The Function of LAT in T Cell Activation 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 (linker for activation of T cells) is an important transmembrane adaptor protein in TCR-mediated signaling. Upon TCR engagement, LAT associates with multiple proteins which allows for the activation of downstream signaling pathways. The interaction between LAT with phospholipase C (PLC-γ1) is especially critical for T cell receptor (TCR)-mediated Ca^{2+} signaling and MAPK activation. Knock-in mice harboring a mutation at the PLC-γ1 binding site (Y136) of LAT develop a severe lymphoproliferative syndrome. These mice have defective thymic development and selection and lack natural regulatory T cells, implicating a breakdown of both central and peripheral tolerance. The phenotype observed in $LAT^{-/-}$ mice is even more severe.T cells are absent in the periphery of these mice due to a complete block in thymocyte development at the DN3 stage thereby making it difficult to study the physiological role of LAT in the activation and function of mature T cells. In order to bypass the developmental defects exhibited by $LAT^{-/-}$ and LATY136F mice, we developed conditional knock-in lines in which only a nonfunctional ($ERCreLAT^{f/-}$) or a LATY136F-mutated allele ($ERCreLAT^{f/m}$) of LAT is expressed in mature T cells after deletion of the wildtype LAT allele.

Analysis of $ERCreLAT^{f/m}$ T cells after LAT deletion indicated that the interaction between LAT and PLC-γ1 plays an important role in TCR-mediated signaling,

proliferation, and IL-2 production. Furthermore, the deletion of LAT induced the development of the LATY136F lymphoproliferative syndrome in these mice. Although $Foxp3^+$ natural Treg cells were present in these mice after deletion, they were unable to suppress the proliferation of conventional T cells. Our data indicated that the binding of LAT to PLC-γ1 is essential for the suppressive function of $CD4^+CD25^+$ regulatory T cells.

We have also performed studies using $ERCreLAT^{f/-}$ T cells to demonstrate that total LAT deficiency reduced the expression of Foxp3, CTLA4, and CD25 in peripheral Treg cells. Interestingly, mice with LAT deleted in peripheral T cells developed a lymphoproliferative syndrome similar to that observed in LATY136F mice although the disease caused by the LATY136F mutation was more severe. These data implicate LAT in both the positive and the negative regulation of mature T cells. Moreover, our findings indicate that LAT is essential in the maintenance of the regulatory T cell profile in the periphery, thereby aiding in the prevention of lymphoproliferative autoimmune disease.

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1. Introduction

1.1 Thymocyte development

The ability of a cell to respond to foreign antigen is only acquired after development within a primary lymphoid organ. The bone marrow and the thymus serve as primary lymphoid organs- they are defined by their provision of environments that are conducive to lymphocyte development. While the bone marrow is the site of hematopoeisis and the development of B cells, the thymus represents the site of T cell development. Immigrant precursors, or early T cell progenitors (ETPs), immigrate from the bone marrow to the thymus. There, these ETPs develop into double negative (DN) thymocytes ($CD4^-CD8^-$) which go through multiple sequential stages of differentiation (outlined in Figure 1.1) and eventually die or emerge from the thymus as mature T cells.

Double negative thymocytes have been divided into 4 subsets based on surface marker expression and consecutive stages of differentiation (Godfrey et al., 1994; Godfrey et al., 1993; Pearse et al., 1989). DN1 thymocytes ($CD44^+CD25^-$) represent the first subset; these cells are still capable of differentiating into multiple lineages, including natural killer (NK), dendritic, myeloid, B, and T cell lineages (Ardavin et al., 1993; Matsuzaki et al., 1993; Moore and Zlotnik, 1995). Upon the expression of CD25, these cells become DN2 thymocytes. β, γ, and δ rearrangement begins as recombination activating gene 1 (RAG-1) and 2 (RAG-2) gene transcription is turned on (Godfrey et al., 1994; Wilson et al., 1994). However, in addition to having T cell lineage potential, DN2

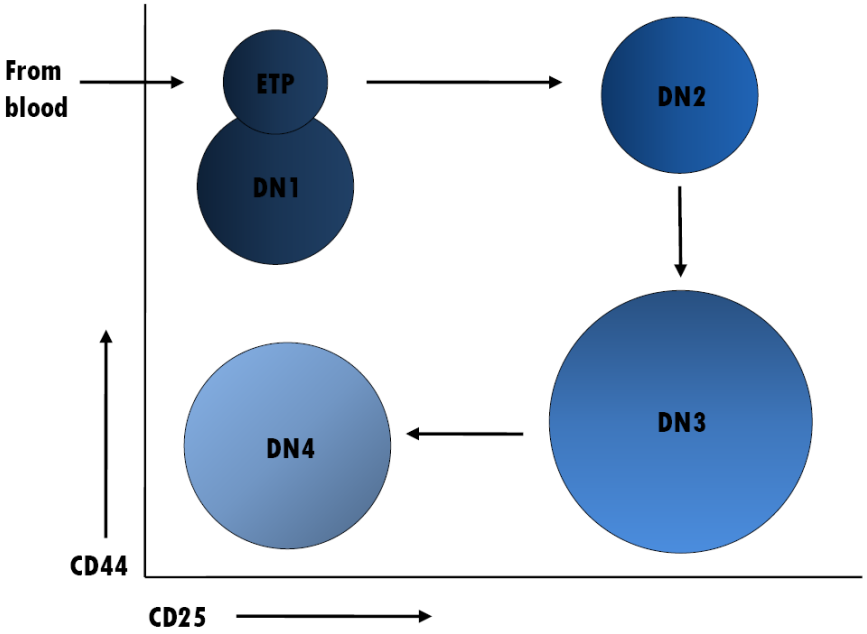


Figure 1.1 E**arly thymocyte differentiation**
Immigrant precursors leave the bone marrow and travel through the blood to the thymus. Early T cell progenitors (ETPs) ($KIT^{hi}CD25^{-}CD44^{hi}$) are developmentallu equivalent to double negative 1 (DN1) thymocytes ($CD25^{-}CD44^{+}$): DN2 cells ($CD25^{+}CD44^{+}$) can originate from either of these populations of cells. The next stage of development leads to a population of DN3 cells ($CD25^{+}CD44^{-}$); at this point, thymocytes are now restricted to the T cell lineage. DN3 cells undergo β-selection and differentiate into DN4 cells ($CD25^{-}CD44^{-}$), also known as pre-double positive (pre-DP) cells due to their expression of *Cd4* and *Cd8* mRNA. (Adapted from (Bhandoola and Sambandam, 2006))

thymocytes still retain the ability to differentiate into NK cell and DC lineages (Schmitt et al., 2004; Wu et al., 1996). Commitment to the T cell lineage is only completed during the DN3 ($CD44^{-}CD25^{+}$) stage (Bhandoola and Sambandam, 2006).

T cells require multiple survival and differentiation signals during thymocyte development. Prior to the DN3 stage, signals for differentiation stem from cells in the surrounding microenvironment such as cortical thymic epithelial cells (cTECs): Until this stage, development has occurred in a T cell receptor (TCR)-independent manner (Petrie and Zuniga-Pflucker, 2007; Rothenberg and Taghon, 2005). As DN3 thymocytes enter the subcapsular zone of the thymus, a small percentage of cells rearrange γ and δ chains, emerging as γδ T cells (MacDonald et al., 2001). However, the majority of DN3 cells undergo gene rearrangement of the β chain followed by the assembly of the pre-TCR complex. This complex consists of CD3, a TCR β chain, and an invariant pre-Tα (pTα) chain (Groettrup et al., 1993; Saint-Ruf et al., 1994). β selection, the selection of cells that have successfully rearranged the TCR β chain, leads cells into the DN4 stage; CD25 surface expression decreases and *cd4* and *cd8* mRNA expression can be detected. pre-TCR signal transduction results in allelic exclusion, continued cell survival, extensive proliferation, and the initiation of α chain rearrangement (O'Shea et al., 1997). These signals induce the transition of cells from the DN4 to the DP ($CD4^{+}CD8^{+}$) stage.

DP thymocytes represent the first subset of cells in the thymus to express a functional αβ TCR as α chain rearrangement occurs at this stage (Hernandez-Munain et

al., 1999). Expression of the TCR during this stage is vital; without survival signals sent via this receptor, cells undergo programmed cell death, called "death by neglect." The majority, approximately 90-95 percent, of DP thymocytes will die as a result of this process (Huesmann et al., 1991; Shortman et al., 1991; Surh and Sprent, 1994). The small amount of cells that express TCRs that are capable of binding self peptide-MHC molecules pass through this checkpoint. This process is known as positive selection. The result is a small population of DP cells that bear potentially useful TCRs.

DP cells must now choose a lineage fate. These cells will differentiate into either $CD4^+$ single positive (SP) or $CD8^+$ SP cells. Various hypotheses addressing how differentiation occurs have been proposed. Popular classical models have been based on the postulation that positive selection signals lead to the irreversible silencing of the gene expression of *cd4* (for $CD8^+$ SP cells) or *cd8* (for $CD4^+$ SP cells). Importantly, these models were all founded on the belief that the TCR signal that leads to CD4/CD8 specification is the same as the TCR signal transduced during positive selection; in other words, positive selection and CD4/CD8 lineage choice occur simultaneously (Singer et al., 2008). These models differ on whether this termination of gene expression is random (the stochastic selection model) or instructed (the strength-of-signal instructional model and the duration-of-signal instructional model). However, recent findings have departed from classical models. It has been shown that the TCR signals that induce positive selection and CD4/CD8 lineage choice are not simultaneous, but rather, are sequential

(Keefe et al., 1999). Furthermore, irreversible termination of either *cd4* or *cd8* does not occur during positive selection. Based on these recent findings, a new nonclassical model, the kinetic signaling model, has been proposed (Figure 1.2) (Singer, 2002; Singer et al., 2008).

Following positive selection, DP thymocytes cease transcription of *cd8* (Brugnera et al., 2000). The resulting cells are phenotypically $CD4^{+}CD8^{low}$cells; these cells have long been identified and are classified as intermediate thymocytes(Lundberg et al., 1995; Suzuki et al., 1995). The kinetic signaling model postulates that if TCR signaling is disrupted, cells differentiate into $CD8^{+}$ SP cells. Alternatively, a sustained TCR signal leads to the induction of $CD4^{+}$ SP cells (Singer et al., 2008). Vast progress has been made in elucidating the complex mechanisms that regulate these two distinct outcomes. Importantly, it has been shown that the TCR signal is not the only signal that plays a role in directing CD4/CD8 lineage choice (Brugnera et al., 2000; Park et al., 2010). Whether the cell differentiates into $CD4^{+}$ SP or $CD8^{+}$ SP depends on additional TCR signals as well as common γ chain signals, specifically, signals emanating from the IL-7R.

The kinetic signaling model has been supported by both old and recent findings. Positive selection dictates that survival signals are to be received only via the TCR;

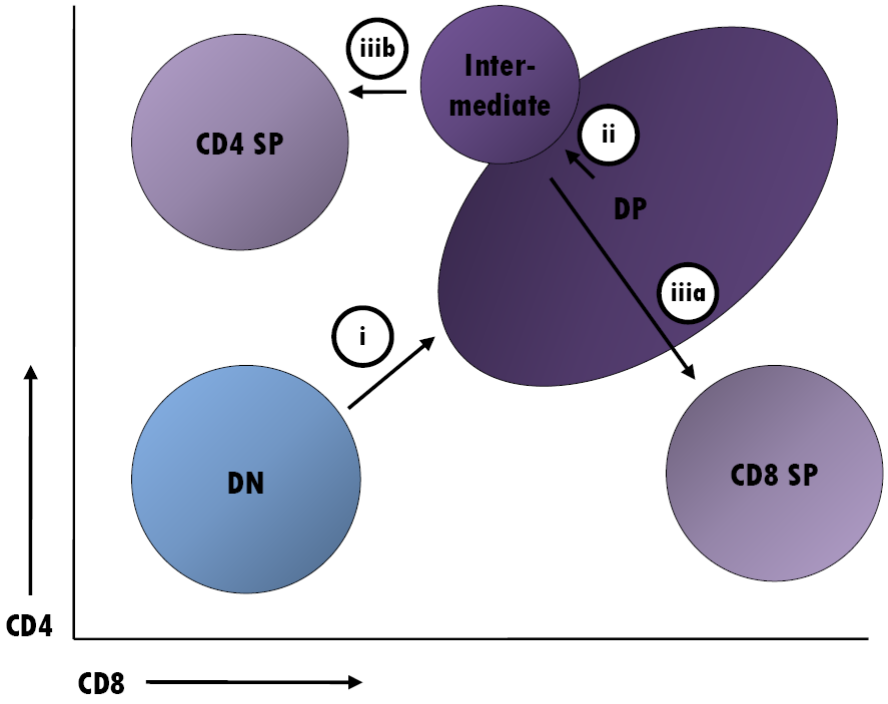


Figure 1.2 Diagram illustrating the kinetic signaling model
(i) Double negative (DN) ($CD4^{-}CD8^{-}$) thymocytes differentiate into double positive (DP) ($CD4^{+}CD8^{+}$) cells. (ii) After receiving a positive selection signal, DP cells terminate the transcription of *Cd8*, appearing phenotypically as $CD4^{+}CD8^{lo}$ intermediate cells. (iiia) If the TCR signal ceases, co-receptor reversal occurs including the upregulation of IL-7 signals and the re-initiation of *Cd8* gene transcription; these cells become $CD8^{+}$ single positive (SP) cells. (iiib) Alternatively, if the TCR signal persists, IL-7 signals are blocked and thymocytes differentiate into $CD4^{+}$ SP cells. (Adapted from(Singer et al., 2008))

therefore, unlike other stages of thymocyte development, early DP thymocytes do not express IL7Rα but express high levels of suppressor of cytokine signaling (SOCS-1) (Chong et al., 2003; Sudo et al., 1993; Yu et al., 2006). After positive selection, there is a progressive reduction of *cd8* expression that occurs during differentiation to the intermediate thymocyte stage. In accordance with the kinetic signaling model, this process may thwart CD8-dependent signaling via MHC class I-restricted TCRs. This disruption of the TCR signal induces a high level of IL-7Rα expression. In this circumstance, cells become $CD8^+$ SP cells in an IL-7 dependent and TCR-independent manner (Park et al., 2010). As a result of the upregulation of IL7Rα, and subsequent STAT-mediated γ_c signaling, these cells also increase their expression level of Runx3 (Park et al., 2010). This transcription factor is central to and essential for CD8 lineage specification (Sato et al., 2005; Setoguchi et al., 2008; Taniuchi et al., 2002). Moreover, Runx3 mediates coreceptor reversal, a concept that is central to the kinetic signaling model (Singer et al., 2008). Coreceptor reversal is the silencing of *cd4* gene transcription and the reactivation of *cd8* expression in intermediate thymocytes (Brugnera et al., 2000). Accordingly, Runx3 negatively regulates Th-POK, a transcription factor whose expression is necessary for CD4 lineage commitment (He et al., 2005; He et al., 2008; Setoguchi et al., 2008; Sun et al., 2005).

The kinetic signaling model also proposes that $CD4^+$ SP cells originate from DP cells that undergo positive selection, differentiate into intermediate thymocytes, and

receive a sustained signal from MHC class II-restricted TCRs. This is contrary to $CD8^+$ SP cells that undergo a disruption of the TCR signal at the intermediate thymocyte stage. Furthermore, unlike CD8 SP cells, CD4 SP thymocytes differentiate from the DP stage in a TCR-dependent but IL-7-independent manner (Singer et al., 2008). A sustained TCR signal in intermediate thymocytes results in the continued silencing of *cd8* transcription and the further upregulation of *cd4* expression (He et al., 2008). This process is mediated by Th-POK which is initially expressed by intermediate thymocytes. While the expression of Th-POK is too low at this stage to limit differentiation into CD8 SP cells, persistent TCR signal transduction induces its upregulation (He et al., 2008). As a result, *cd4* gene silencing by Runx3 does not occur. Together, these processes complete commitment to the CD4 lineage. $CD4^+$ and $CD8^+$ SP cells must now pass a final checkpoint before exiting the thymus.

After CD4/CD8 lineage commitment, SP thymocytes migrate to the medulla of the thymus. There, medulla thymic epithelial cells (mTECs) and thymic DCs present self antigen to SP cells (Klein et al., 2009). Cells that bind self ligand-MHC complexes with high affinity are deleted; this process is known as clonal deletion, the main mechanism of negative selection (Hogquist et al., 2005). This stringent checkpoint prevents the development of autoreactive T cells. After successfully passing this checkpoint, SP cells exit the thymus to enter the periphery as mature T cells.

1.2 TCR-mediated signaling

1.2.1 Initiation of the TCR signal

All immune cells express surface molecules that enable the recognition of antigens. The adaptive branch of the immune system is able to recognize antigens with precise specificity due to the expression of unique molecules (antigen receptors) on the surface of lymphocytes. Antigen recognition by these surface receptors propagate signals that are transmitted to the cytosol and nucleus of the cell. In T cells, the transmission of the signal from the extracellular environment is enabled by the expression and ligation of the TCR. The signaling cascades emanating from TCR engagement thus cause cellular responses specific to the recognized antigen.

TCR-mediated signal transduction is initiated by the interaction of peptides displayed on MHC molecules by APCs with the TCR:CD3 complex located on the surface of the T cell. The TCR:CD3 complex on the surface of an $\alpha\beta$ T cell consists of the α, β, and ζ chains of the TCR as well as the ε, δ, and γ chains of CD3. These CD3 subunits exist as $\varepsilon\gamma$ and $\varepsilon\delta$ dimers while the ζ chain forms a homodimer ($\zeta\zeta$). Together, these molecules are critical for TCR signaling as they are necessary for membrane expression of the TCR. The cytoplasmic tails of the CD3 chains all contain immunoreceptor tyrosine-based activation motifs (ITAMs) which have a consensus sequence of YXXL/IX6-8YXXL/I . There are a total of ten ITAMs on the subunits of the TCR:CD3 complex, each of the CD3 ε, δ, and γ chains contain one ITAM whereas each

TCR ζ chain contains three (Samelson, 2002). Studies have shown that mutating one or more of these motifs leads to impaired proximal T cell signaling and function, demonstrating that these ITAMs are essential for effective propagation and amplification of the T cell signal (Smith-Garvin et al., 2009).

The requirement for ITAMs on CD3 subunits in the initiation of TCR-mediated signaling has been recognized for more than fifteen years. However, the exact mechanism by which recognition of peptide/MHC by the TCR is translated into ITAM phosphorylation and kinase activation at the CD3 complex has not been resolved. Currently, there are several models that can be used to describe this event. These models are based on: the ability of the TCR to aggregrate; the exclusion of inhibitory molecules from the pMHC/TCR contact zone; and conformational changes that occur in the TCR:CD3 complex as a result of TCR ligation (Smith-Garvin et al., 2009). These models are not mutually exclusive and further studies are being done to elucidate the precise mechanism involved. Regardless, crosslinking of the TCR leads to activation of the Src family of protein tyrosine kinases (PTKs), specifically Lck and Fyn. These kinases are constitutively located at the plasma membrane. Upon activation, Lck phosphorylates the ITAMs on the TCR ζ chain homodimer, creating paired docking sites for the ζ chain-associated protein of 70 kDa (ZAP-70) (Iwashima et al., 1994; van Oers et al., 1996). As a result, ZAP-70 undergoes a conformational change from an autoinhibited to an activated conformation that is recruited to the CD3 complex. The Src homology 2 (SH2) domains

of ZAP-70 bind to the phosphorylated ITAMs causing activation of ZAP-70 through the kinase activity of Lck and eventually autophosphorylation (Au-Yeung et al., 2009). Adaptor molecules, such as linker for activation of T cells (LAT) and SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) are phosphorylated by ZAP70, leading to further propagation of the TCR signal (Bubeck Wardenburg et al., 1996; Zhang et al., 1998).

1.2.2 Assembly of the signalosome

The ligation of the TCR induces immediate changes within the T cell, particularly at the cell membrane. The resultant signaling cascade is mediated by molecules with different activities and functions, including phosphatases, kinases, and adaptor molecules. Adaptor proteins do not have enzymatic properties but are able to transduce signals by mediating interactions between proteins and proteins or proteins and lipids; adaptor proteins contain multiple binding domains and sequence motifs which enable such interactions. These domains include pleckstrin homology (PH), Src homology 2 (SH2), SH3, phosphor-tyrosine binding (PTB), and WW domains. There are two major forms of adaptor proteins: transmembrane adaptor proteins (TRAPs) and cytoplasmic adaptor proteins (CAPs). TRAPs contain as many as ten tyrosine-based signaling motifs (TBSMs), which are central to their role in organizing the assembly of multiprotein complexes. Examples of TRAPs that have been shown to play a role in T cell development, activation, and/or function include LAT, LAB/NTAL (linker of activation

for B cells/non-T cell activation linker), LAX (linker for activation of X cells), SIT [SH2 domain-containing phosphatase (SHP2)-interacting TRAP], and TRIM (TCR-interacting molecule)(Simeoni et al., 2008). CAPs, such as SLP-76, growth factor receptor-bound protein 2 (Grb2), and Grb2-related adaptor downstream of Shc (Gads) also play an important role in downstream signal transduction following TCR stimulation.

The formation of a membrane-tethered signalosome is necessary for effective transduction of the TCR; disruption of the involved binding sites can have deleterious effects on critical downstream biochemical events. The assembly of the signalosome is believed to begin with the phosphorylation of scaffolding proteinswhich collectively act as a backbone for the binding of other molecules (illustrated in Figure 1.3). Following TCR ligation, the activation and localization of ZAP-70 to the TCR:CD3 complex leads to the phosphorylation of LAT and SLP-76 (Bubeck Wardenburg et al., 1996; Zhang et al., 1998). LAT is phosphorylated at multiple tyrosine residues, of which the distal four tyrosines are most important for LAT function; the tyrosines serve as binding sites for Gads, Grb2, and phospholipase C γ1 (PLC-γ1) (Lin and Weiss, 2001; Sommers et al., 2001; Zhang et al., 2000; Zhu et al., 2003). The recruitment of Gads to LAT also brings SLP-76, which constitutively binds to Gads, within proximity to LAT (Liu et al., 1999). In addition, SLP-76 constitutively binds to PLC-γ1 and once phosphorylated, can also interact with Vav1, noncatalytic tyrosine kinase (Nck), and IL-2 induced tyrosine kinase (Itk)(Koretzky et al., 2006). The importance of LAT and SLP-76 in the formation of this

Figure 1.3 Formation of the LAT-mediated signalosome
Binding of a self-peptide-MHC complex to the TCR leads to the phosphorylation of ZAP70 and the subsequent activation of LAT and SLP-76. The phosphorylated tyrosines on LAT act as binding sites for the Grb2-Sos complex, PLC-γ1, and the Gads-SLP-76 complex. The binding of Grb2-Sos to LAT leads to activation of the Ras/Erk pathway. Upon its activation via the phosphorylation of ZAP70, SLP-76 interacts with Itk, Vav, and Nck. Additionally, SLP-76 binds constitutively to both Gads and PLC-γ1; the association of the Gads-SLP-76-PLC-γ1 complex with phosphorylated LAT leads to activation of the Ca^{2+} pathway.

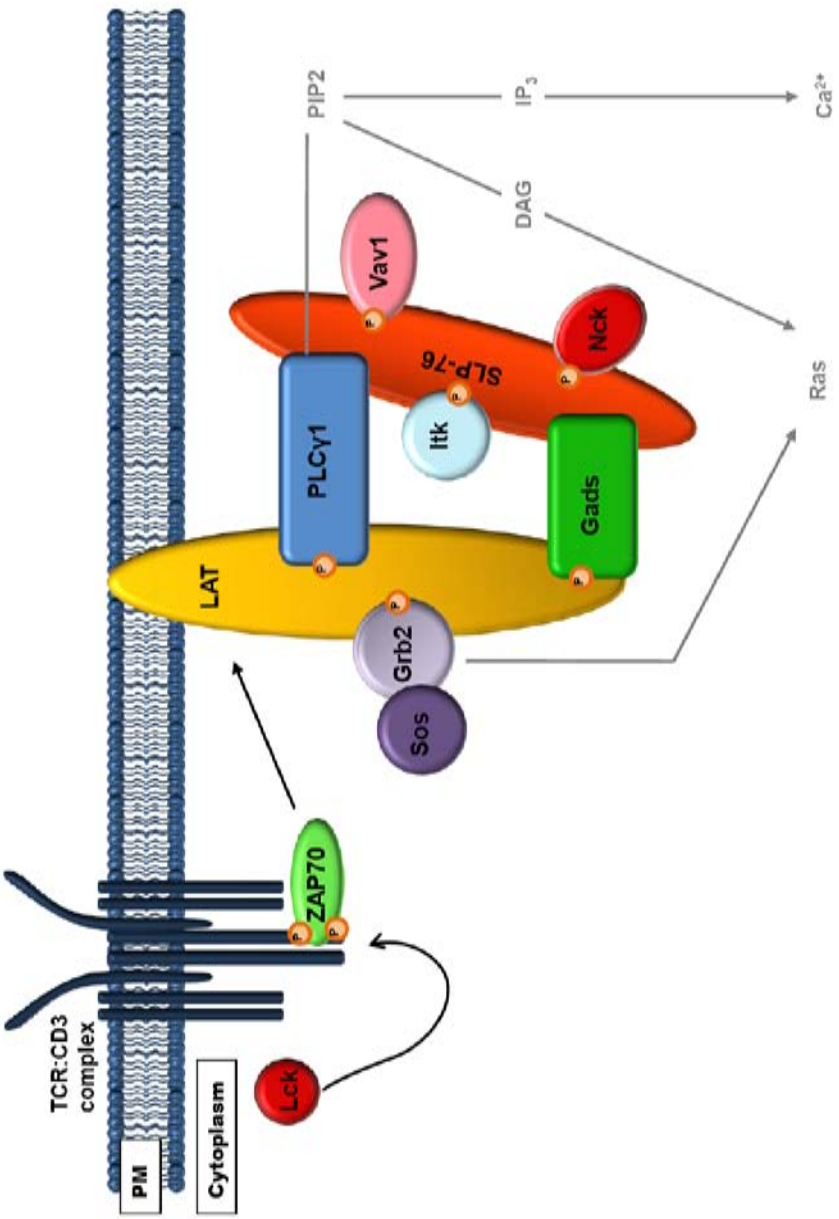

PIP2
IP3
Ca2+
DAG
Ras
Vav1
SLP-76
Nck
Itk
PLCγ1
Gads
LAT
Grb2
Sos
ZAP70
Lck
TCR:CD3
complex
PM
Cytoplasm

Figure 1.3

multiprotein complex was highlighted by the analysis of mice that are deficient in either of these proteins. Both SLP-76$^{-/-}$ and LAT$^{-/-}$ T cells exhibit a near complete loss of pre-TCR signal transduction that is necessary for thymocyte development (Clements et al., 1998; Pivniouk et al., 1998; Zhang et al., 1999b). While the importance of these proteins in the formation of the signalosome is indisputable, the stability of the complex is maintained by several of the other proteins assembled on the scaffolds. Itk is required for the recruitment of Vav1 to the LAT-mediated signalosome. In the absence of Vav1, the phosphorylation of SLP-76 and its recruitment to LAT is defective; in addition, Itk activity, which is necessary for PLC-γ1 phosphorylation, is reduced (Dombroski et al., 2005; Reynolds et al., 2004; Reynolds et al., 2002).

The assembly and stabilization of the LAT-mediated signaling complex are necessary for the ability of T cells to be effectively activated. Specifically, this complex enables the activation of the PLC-γ1-Ca^{2+} and mitogen-activation protein kinase (MAPK) pathways (Finco et al., 1998; Zhang et al., 1999a). Furthermore, this multimolecular complex is essential for the activation of various transcription factors, such as NFAT, AP-1, and NFκB, and the subsequent transcription of numerous genes necessary for T cell proliferation, differentiation, and function.

1.2.3 Linker for activation of T cells

As described earlier, LAT plays a critical role in the formation of the TCR signaling complex, acting as a bridge for multiple molecules in the transduction of the

membrane-potentiated signal to the nucleus. LAT is a 36 to 38 kDa adaptor protein that is expressed in the membranes of T cells, NK cells, mast cells, platelets/megakaryocytes and pre-B cells (Facchetti et al., 1999; Su and Jumaa, 2003; Weber et al., 1998; Zhang et al., 1998). The structure of LAT consists of a short extracellular domain, a transmembrane domain, and a cytoplasmic tail. There are nine conserved tyrosine residues within the cytoplasmic amino acid sequence that, once phosphorylated, represent potential binding sites for other adaptor proteins or effector molecules (Samelson, 2002).

The importance of LAT in TCR signaling was first discovered using LAT-deficient mutants of the Jurkat cell line (J.CaM2). While TCR proximal signaling is unaffected, these cells demonstrate a severe defect in the downstream signaling cascade; specifically, calcium flux, Ras-Erk activation, CD69 upregulation, and NFAT gene transcription are completely abolished (Finco et al., 1998; Zhang et al., 1999a). Moreover, normal TCR signaling and activation of these cells can be rescued with the re-expression of LAT. Further studies using this cell line were able to determine the importance of the tyrosine residues for specific signaling pathways, as well as LAT binding partners and their corresponding interaction sites. One or more tyrosine residues were mutated to phenylalanine on the cytoplasmic tail of LAT and transfected into J.CaM2 cells. Mutations of the four distal tyrosines (human Y132, Y171, Y191, and Y226) led to a complete loss of TCR-mediated activation; this finding demonstrated that these residues

are primarily responsible for the function of LAT. Mutating sites Y110 and Y226 led to defective activation of the Ras-MAPK pathway (Lin and Weiss, 2001); Y132, Y171, and Y191 are required for the Ca^{2+} pathway (Zhang et al., 2000). These studies also revealed the specific molecules that bind to the phosphotyrosines on the cytoplasmic tail of LAT. Specifically, Grb2 binds to phosphorylated Tyr 171, 191, and 226, while Gads binds to Y171 and Y191. PLC-γ1 binds to Y132, though binding was also abrogated in the Y171, Y191, and Y226 triple mutant, demonstrating that the binding of Gads to LAT is also important for recruiting PLC-γ1 to the signalosome (Zhang et al., 2000).

The analysis of LAT-deficient mice illustrated the physiological importance of this adaptor protein in TCR-signaling. In these mice, there is a drastic decrease in the size of the thymus and a corresponding lack of DP and SP cells therein (Zhang et al., 1999b). This phenotype is a direct effect of a complete block in thymocyte development at the DN3 stage; this block also results in a periphery devoid of mature T cells. Additionally, mice harboring mutations at the four distal tyrosines (mouse Y136, Y175, Y195, and Y235) of LAT have a similar phenotype, underscoring the importance of these binding sites in TCR-mediated signaling (Sommers et al., 2001). Collectively, these findings demonstrated that LAT is essential for signals transduced via the pre-TCR. Studies done by Shen et al enabled the analysis of the function of LAT beyond the DN3 stage of thymocyte development. Using conditional knock-in mice crossed onto a CD4Cre background in which deletion occurs at the late DN3 stage, these studies

showed that DP cells are not able to respond to TCR stimulation, indicating that LAT-mediated signaling is also required for the development of thymocytes from DP cells to SP cells (Shen et al., 2009).

Evidence for a negative role of LAT in TCR-mediated signaling has accumulated over the past decade. Studies done in Jurkat cells have found that, via its binding with Grb2/Gads, LAT recruits a complex comprised of Grb2 associated binding protein (Gab2), phosphoinositol 3-kinase (PI3K), and SH2 domain-containing tyrosine phosphatase 2 (SHP2) (Yamasaki et al., 2001; Yamasaki et al., 2003). The activation of SHP2 leads to dephosphorylation of signaling molecules such as the CD3ζ chain, while the binding of Gab2 competes with SLP-76 for the interaction site on Gads. These events lead to inhibition of TCR-mediated signaling. LAT has also been shown to associate with the downstream of kinase 2 (Dok2)/SH2 domain-containing inositol phosphatase (SHIP) complex (Dong et al., 2006). The binding of Grb2 to SHIP enables the recruitment of this Dok2-SHIP complex to the LAT signalosome, resulting in decreased activation of ZAP-70 and Akt, thereby leading to decreased TCR-mediated IL-2 production. In addition to the LAT-mediated recruitment of inhibitory complexes to the signalosome, another negative role for LAT has been identified in human cell lines. The phosphorylation of Thr155 on the cytoplasmic tail of LAT is mediated by extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). The phosphorylation of this site results

in the inability of LAT to effectively bind PLC-γ1. As a result, the mobilization of Ca^{2+} and the activation of the MAPK pathway are defective (Matsuda et al., 2004).

The role of LAT as a positive regulator in TCR-mediated signaling had long been established when physiological evidence of a negative role for LAT was revealed. In 2002, two independent groups generated knock-in mice containing a phenylalanine mutation at the Y136 site of LAT (equivalent to Y132 in the human sequence) (Aguado et al., 2002; Sommers et al., 2002). There is a severe block at the DN3 stage of thymocyte development in these LATY136F mice. At two weeks of age, these mice have a drastic decrease in the number and percentage of $CD4^+$ cells in the periphery; however, starting at four weeks of age, the number of $CD4^+$ cells exponentially increases, resulting in a skewed CD4 to CD8 ratio (Sommers et al., 2002). The expansion of $CD4^+$ conventional T cells results in splenomegaly and lymphadenopathy. These mice eventually die by six months of age from multi-organ infiltration which is mediated by $CD4^+$ T cells, eosinophils, B cells, and macrophages (Aguado et al., 2002; Sommers et al., 2002).

Based on surface marker expression, peripheral $CD4^+$ T cells in LATY136F mice resemble effector/memory T cells with a surface phenotype of $CD62L^{lo}CD44^+CD69^+$, while expressing very low levels of the TCR. In response to TCR stimulation, these cells have defects in calcium mobilization, IL-2 production, and proliferation; they also produce high levels of Th2 cytokines such as IL-4. As a result of the presence of these cytokines and highly activated $CD4^+$ T cells in the periphery, B cells also exhibit a

hyperactive phenotype- a high frequency of B cells upregulate MHC Class II cell surface expression. Furthermore, a large percentage of B cells show decreased B220, IgD, and IgM surface expression, reminiscent of antibody producing cells. Moreoever, analysis of blood serum shows high levels of IgE and IgG_1, but not IgM, suggesting increased isotype switching, likely driven by the predominantly Th2 environment (Aguado et al., 2002). Altogether, the LATY136F phenotype reveals a role for LAT as a negative regulator of immune homeostasis.

Studies by Sommers et al demonstrated that negative selection is defective in LATY136F mice, suggesting that the escape of autoreactive CD4 T cells into the periphery is a major cause of the observed lymphoproliferative phenotype (Sommers et al., 2005). In addition to having a defect in central tolerance, it has also been shown that LATY136F mice are devoid of natural regulatory T cells (Treg cells) in the periphery (Koonpaew et al., 2006). This finding illustrates a role for LAT in the development of Treg cells. These cells are known for their ability to suppress the expansion of conventional T cells; therefore, these findings also implicate a lack of peripheral tolerance as a factor leading to the development of the disease. In fact, reconstitution of LATY136F neonates with wildtype $CD4^+CD25^+$ Treg cells or ectopic expression of Foxp3 in LATY136F conventional T cells results in rescue from the lymphoproliferative phenotype (Koonpaew et al., 2006). Together, these data support a role for defects in both central and peripheral tolerance in the development of the observed disease.

Another strain of LAT mutant mice, termed LAT3YF, contains mutations at the Y175, 195, and Y235 sites, thereby preventing binding of Gads and Grb2 (Nunez-Cruz et al., 2003). Similar to LATY136F mice, there is defective thymocyte development and the periphery is dominated by hyperproliferative, phenotypically activated T cells that produce high levels of Th2 cytokines. However, in LAT3YF mice, the T cells that populate the periphery and cause the lymphoproliferative syndrome are γδ T cells. While γδ T cells progress through thymocyte development and successfully emigrate from the thymus, αβ thymocyte development is completely blocked at the DN3 stage (Nunez-Cruz et al., 2003). After 20 weeks of age, LAT3YF mice develop a Th2-type disorder characterized by hyperproliferative γδ T cells and IgE/ IgG_1 hypergammaglobulinemia. The studies done in LAT3YF mice show that: (1) LAT plays a role in negatively regulating TCR-mediated signals in γδ T cells and (2) there are disparate requirements for LAT in the development of αβ versus γδ T cells. Altogether, studies done on LAT using Jurkat cell lines and different LAT mutant mouse strains have established the necessity of LAT in TCR-mediated signaling and have also illuminated a negative role for LAT in immune homeostasis.

1.3.4 PLC-γ1 activation and Ca^{2+} mobilization

The phenotype observed in LATY136F mice highlights the importance of LAT-mediated PLC-γ1 activation. The phosphorylation of LAT by ZAP-70 leads to the recruitment of PLC-γ1 to the signalosome where it binds to LAT, SLP-76, and Vav. In

turn, Itk, which interacts with SLP-76 following TCR engagement, phosphorylates PLC-γ1. Activated PLC-γ1 hydrolyzes the membrane phospholipid PIP_2 (phospholipid phosphatidylinositol 4,5 biphoshpate) to IP_3 (inositol trisphosphate) and DAG (diacyglycerol) (Rhee, 2001). The production of DAG leads to activation of the Ras-MAPK pathway, which will be discussed in detail later. IP_3 binds to IP_3 receptors (IP3R), located on the endoplasmic reticulum (ER) membrane, which triggers the release of Ca^{2+} from the ER Ca^{2+} store (illustrated in Figure 1.4). Stromal interaction molecules (STIM), transmembrane proteins on the ER, sense the depletion of Ca^{2+} from the ER lumen, inducing the aggregation of these molecules into small clusters (punctae) in the ER membrane (Liou et al., 2005; Roos et al., 2005). The subsequent entry of extracellular Ca^{2+} through CRAC channels into the cytoplasm is known as store-operated Ca^{2+} entry (SOCE). STIM1 is responsible for the initial spike in the SOCE while STIM2 maintains the basal Ca^{2+} level and sustains the late stage of SOCE (Brandman et al., 2007; Oh-Hora et al., 2008). The oligomerization of STIM1 occurs within 10-25nm from the plasma membrane of the cell forming punctae (Liou et al., 2007; Liou et al., 2005; Wu et al., 2006). This allows for binding of Orai1 to the punctae, inducing the formation of an Orai1 tetramer, a Ca^{2+} release-activated Ca^{2+} (CRAC) channel (Feske et al., 2006; Luik et al., 2008; Luik et al., 2006; Penna et al., 2008; Prakriya et al., 2006). The subsequent elevation of intracellular Ca^{2+} concentration leads to the activation of calcineurin and

Figure 1.4 Illustration of TCR-mediated Ca^{2+} mobilization

Upon ligation of the TCR-CD3 complex and the formation of the LAT-mediated signalosome, PLC-γ1 is activated; phosphatidylinositol 3, 4- biphosphate (PIP_2) is cleaved into diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3). IP_3 binds to the IP_3 receptors (IP_3R) located on the membrane of the endoplasmic reticulum (ER). This induces the efflux of Ca^{2+} from the ER into the cytoplasm and the subsequent aggregation of stromal interaction molecule 1 (STIM1). In turn, ORAI1 is activated and Ca^{+} enters the cytosplasm of the cell through the extracellular environment. As a result, the calcineurin-NFAT pathway is activated leading to the translocation of NFAT into the nucleus. In collaboration with other transcription factors (TFs), NFAT leads to the transcription of various proteins necessary for T cell development, differentiation, and function. (Adapted from(Oh-hora, 2009))

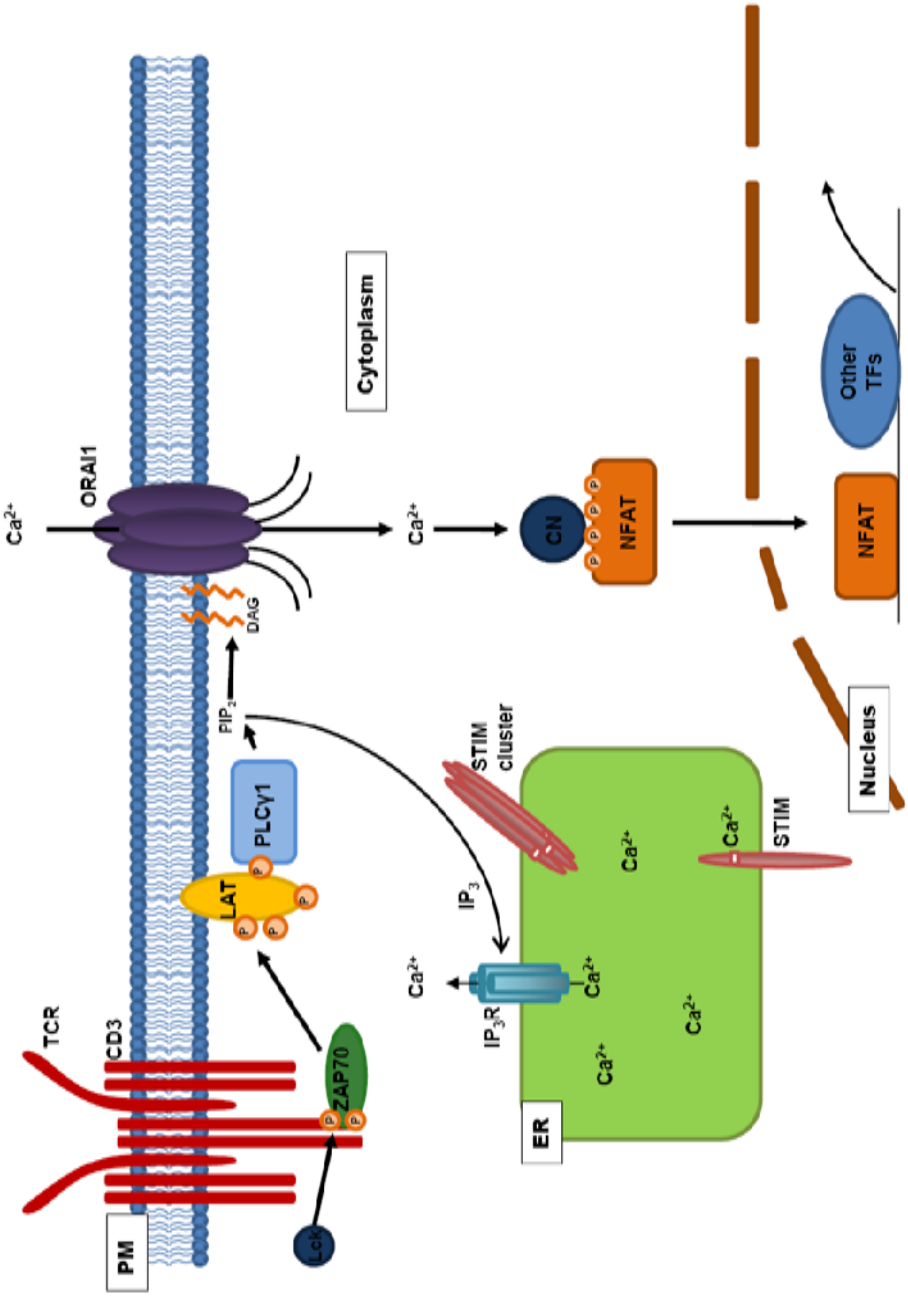

Ca2+
ORAI1
Cytoplasm
Ca2+
CN
NFAT
Other TFs
NFAT
DAG
PIP2
PLCγ1
LAT
STIM cluster
IP3
Nucleus
TCR
CD3
ZAP70
Lck
PM
Ca2+
IP3R
ER
STIM

Figure 1.4

Ca^{2+}-calmodulin-dependent kinase (CaMK). This results in the transcription of genes essential for T cell function and cytokine production.

The increase in intracellular Ca^{2+} resulting from SOCE leads to the binding of Ca^{2+}-calmodulin to calcineurin, displacing the autoinhibitory subunit from the enzyme active site, thereby enabling the phosphatase activity of calcineurin; the activation of calcineurin leads to the dephosphorylation of nuclear factor of activated T cells (NFAT) (Hogan et al., 2003). This modification exposes the nuclear localization sequence of NFAT, inducing its translocation to the nucleus. In conjunction with other transcription factors activated by this or other signaling pathways, NFAT is able to mediate the transcription of genes necessary for T cell development, differentiation, and function. For example, NFAT complexes with STAT proteins, which are activated by various cytokine signaling pathways; these interactions lead to the expression of T-bet and GATA3 which are both essential for T helper cell differentiation (Savignac et al., 2007). Therefore, the activation of the PLC-γ1-Ca^{2+} pathway leads to downstream events, such as NFAT translocation to the nucleus, that are critical for T lymphocyte gene expression and function. Indeed, a number of patients with defects in SOCE and CRAC channel function develop severe combined immunodeficiency (SCID) characterized by impaired lymphocyte function and cytokine expression (Feske et al., 1996; Le Deist et al., 1995; Partiseti et al., 1994).

The importance of PLC-γ1 in T cell activation has recently been highlighted in studies done by Fu et al. As the deletion of PLC-$\gamma1^{-/-}$ is embryonic lethal, this group generated PLC-γ1 conditional knockout mice crossed onto a CD4Cre background, essentially limiting deletion of the protein to the T cell lineage (Fu et al., 2010). As expected, T cells from these mice are unable to flux Ca^{2+}, activate the MAPK pathway, produce IL-2, and proliferate efficiently in response to TCR engagement. As a result of these defects, thymocyte development is impaired. Additionally, these mice develop autoimmune disease, possibly caused by inadequate Treg cell function. These findings further enhance/complement the conglomeration of previous findings demonstrating that the enzymatic activity of PLC-γ1 is essential for TCR-mediated signaling and T cell activation.

1.2.5 The Ras-MAPK pathway

The downstream effects of LAT phosphorylation include the activation of the MAPK pathway via the binding of the Grb2-Sos complex and the production of DAG. Grb2 constitutively binds to Sos, a guanine nucleotide-exchange factor (GEF) for Ras proteins; upon TCR engagement, Grb2 binds to LAT, thereby recruiting Sos to the signalosome (Finco et al., 1998; Zhang et al., 1999a). As a GEF, Sos mediates the conversion of Ras from the inactive to the active state by inducing the release of GDP bound to Ras, thus allowing for the binding of Ras to GTP. The association of GTP with

Ras results in a conformational change, enabling its interaction with various molecules including proteins important for MAPK signaling (Vetter and Wittinghofer, 2001).

Ras guanine nucleotide-releasing protein 1 (RasGRP1), represents another GEF that plays an important role in TCR-mediated signaling. In response to TCR ligation, PLCγ1 is activated, leading to the production of DAG, as well as increased intracellular Ca^{2+}. As a result of high levels of DAG and cytosolic Ca^{2+}, RasGRP1 is activated; this induces its binding to DAG and its subsequent phosphorylation by novel PKC kinases (Ebinu et al., 1998; Roose et al., 2005). The importance of RasGRP1 in TCR-mediated Ras activation was demonstrated in RasGRP1-deficient Jurkat cells which harbor defects in Ras-Erk signaling (Roose et al., 2005). Furthermore, RasGRP1-deficient mice exhibit impaired thymocyte development due to abrogated TCR-mediated signaling, particularly Erk phosphorylation (Dower et al., 2000).

Erk is one of three major groups of MAP kinases that are important in T lymphocyte activation. The activation of Ras in T cells, which is mediated by Sos and RasGRP1, induces the activation of Raf-1, a serine-threonine kinase. Raf-1 stimulates MAPK/Erk kinase (MEK) activity, which leads to the phosphorylation of Erk1 and Erk2 (Izquierdo et al., 1993). The dual phosphorylation of Erk on threonine and tyrosine residues induces kinase activity. Phosphorylated Erk dimerizes and translocates to the nucleus; this results in the activation of Elk1, a transcription factor that contributes to the assembly of the activator protein-1 (AP-1) (Jun/Fos) transcription complex. Accumulated

data have indicated that Erk is involved in thymocyte development and the differentiation, survival, growth, and activation of mature T cells (Dong et al., 2002).

p38 and Jnk are the other two major groups of MAP kinases that are activated upon TCR-mediated signaling. Unlike Erk, which is activated by Ras via the Raf MAPK kinase kinase (MAPKKK) family, p38 and Jnk are activated by the Rho family of GTPases. These small GTPases, such as Rac and Cdc42, activate MKK3, MKK4, and MKK6, as well as MKK4 and MKK7, which lead to p38 and Jnk activation, respectively (Dong et al., 2002). p38 is important for the early stages of T cell development, although a constitutively active form of p38 leads to a defect in the progression of thymocytes from the DN to the DP stage due to a p38-induced cell cycle arrest (Diehl et al., 2000; Sugawara et al., 1998). This indicates a role for p38 in regulating development and proliferation in the thymus. Furthermore, *in vitro* experiments using SB203580, a p38 inhibitor, have suggested a role for p38 in negative selection (Sugawara et al., 1998). In addition, p38 has been implicated in IFN-γ production associated with Th1 differentiation (Rincon et al., 1998). Interestingly, there is also evidence that p38 is important for the suppressive function of Treg cells: As a result of p38 activation, p27Kipl, an inhibitor of cell cycle promoter cdk, induces and maintains anergy in Treg cells (Adler et al., 2007).

Jnk, the third MAP kinase that plays a role in TCR-mediated signaling has also been shown to be important in T cell activation, proliferation, and differentiation. Jnk is

encoded by two different genes in T cells - Jnk1 and Jnk2. T cells deficient in either of these genes exhibit defects in T cell-mediated immune responses (Dong et al., 1998; Yang et al., 1998). Though the sequences of Jnk1 and Jnk2 are similar, there are significant differences in the activity and outcomes from signals transduced via each of these proteins. Jnk1 deficiency leads to the inhibition of the development of Th1 cells while increasing Th2 cytokine production and having no effect on Th1 cytokine production (Dong et al., 1998). On the other hand, the ablation of Jnk2 blocks the generation of Th1 cells due to ineffective IFN-γ production (Yang et al., 1998). Jnk may also play a role in negative selection in the thymus through c-Jun phosphorylation (Sabapathy et al., 1999; Sabapathy et al., 2001). However, it has been shown that while Jnk1 induces c-Jun phosphorylation and its subsequent transcriptional activity, Jnk2 targets c-Jun for ubiquitination resulting in its degradation (Derijard et al., 1994; Fuchs et al., 1996; Fuchs et al., 1997). In conclusion, the differential signaling of MAP kinases and their separate isoforms leads to a potential of numerous disparate immunological outcomes.

1.3 T helper cell differentiation

1.3.1 Polarizing signals

More than two decades ago, studies revealed two phenotypically distinct subsets within the peripheral CD4 T cell population. These subsets were called Th1 and Th2 cells, characterized by the different cytokines produced by each subset (Mosmann et al., 1986). The discovery of these two separate subclasses led to a plethora of studies

aiming to further characterize these cells. While it was clear that these cells did not differentiate until after exiting the thymus, many challenging questions remained. What conditions and signals lead to this differentiation? What are the specific effector functions of each helper subset? What transcription factors play a role in the differentiation and effector functions of Th1 versus Th2 cells? Are there more than just two subsets of T helper cells? This latter question was answered with definitive proof in 2005 when the Th17 subset was unveiled (Harrington et al., 2005; Park et al., 2005); this discovery proved that there were in fact more than two T helper subsets. At about this time, inducible regulatory T cells (iTregs) were defined (Chen et al., 2003; Fantini et al., 2004). Recent work has even suggested that a fifth subset exists, called T follicular helper cells (Tfh) (King et al., 2008). However, more studies are needed to clarify whether these Tfh cells actually differentiate from uncommitted naïve T cells or exist as different states of each of the Th1, Th2 and, Th17 subsets.

The signals necessary for T helper cell differentiation are provided by antigen presenting cells (APCs). Dendritic cells (DCs) are the most effective APCs; they constitutively express costimulatory molecules and MHC Class II molecules on their cell surfaces. Immature dendritic cells (DCs) reside in areas of the body prone to pathogenic invasion. In response to the detection of a "non-self" antigen, these DCs undergo maturation (De Smedt et al., 1996). This process is initiated by the binding of pathogen-associated molecular patterns (PAMPs) on pathogens to pattern recognition receptors

(PRRs) on DCs. TLRs represent the most common PRRs involved in DC maturation (Akira et al., 2001). Depending on the PAMPs that are present in the surrounding environment, DCs are capable of differential maturation (Kapsenberg, 2003) which is mediated by the ability of TLRs to discriminate between different types of pathogens. Multiple DC subsets have been defined, including $CD8^+$ DCs, $B220^+$ DCs, and $CD11b^+$ DCs. Some models have proposed that each DC subset signals to a T cell in a manner that is different from the other DC subsets and that this plays a role in the differentiation of T cells into different subsets (Maldonado-Lopez et al., 1999; Pulendran et al., 1999). However, other studies suggest that more than one DC subset can give rise to the same T cell subset (Huang et al., 2001). This argues that different DC subsets may play a role in differential Th subset skewing, but there are other more important driving forces that determine T helper lineage.

Upon maturation, DCs migrate from the periphery to lymphoid organs where they present antigen on MHC class II molecules to naïve T cells ($CD44^{lo}$ and $CD62L^{hi}$). Other important signals are also provided by DCs to T cells including signals from coreceptors, costimulatory molecules, and cytokines (Kapsenberg, 2003). Upon contact between the DC and the T cell, an immunological synapse is formed. The cytoskeletal structure of both the T cell and the APC rearranges, allowing for the movement of multiple molecules to the synapse (Dustin and Cooper, 2000). Foremost, MHC Class II molecules presenting antigen on the DC cell surface move to the center of the synapse.

The binding of the TCR to the MHC-antigen complex initiates a signaling cascade that was described in the previous section of this chapter. Some studies have suggested that antigen dose as well as the duration and strength of the initial TCR signal plays a role in the development of different T effector phenotypes (Constant et al., 1995; Hosken et al., 1995; Rogers and Croft, 1999). A high level of stimulation leads to the differentiation of Th1 cells whereas a moderate level of stimulation leads to differentiation of Th2 cells; a low level of stimulation leads to the development of uncommitted cells. This differential signaling can cause a skewing of the signaling cascade downstream of the TCR. Accordingly, low PKC levels and high Ca^{2+} mobilization favor differentiation into the Th1 subset while the converse induces cells to differentiate into Th2 cells (Noble et al., 2000). In addition to the TCR signal, DCs provide the T cell with other means to induce T cell differentiation.

The binding of the TCR to the MHC-antigen complex is followed by the movement of several costimulatory molecules to surround the center of the immunological synapse. These molecules include CD40, CD80, and CD86, which bind to CD40L and CTLA4/CD28, respectively, on the T cell. The costimulation signals that result from these interactions are important for preventing the development of anergic T cells. DCs and other APCs also provide signals to the T cells in the form of cytokines. These signals are the most important polarizing signals for T cell differentiation (Zhu et al., 2010). Upon cytokines binding to their respective receptors on the surface of the T

Figure 1.5 T helper cell differentiation
Upon activation of a naïve T cell by an antigen presenting cell, commonly a dendritic cell (DC), lineage fate is dependent on the presence, or absence, of specific cytokines in the surrounding environment. Cytokine signaling induces the expression of different transcription factors which define the subset to which they belong.

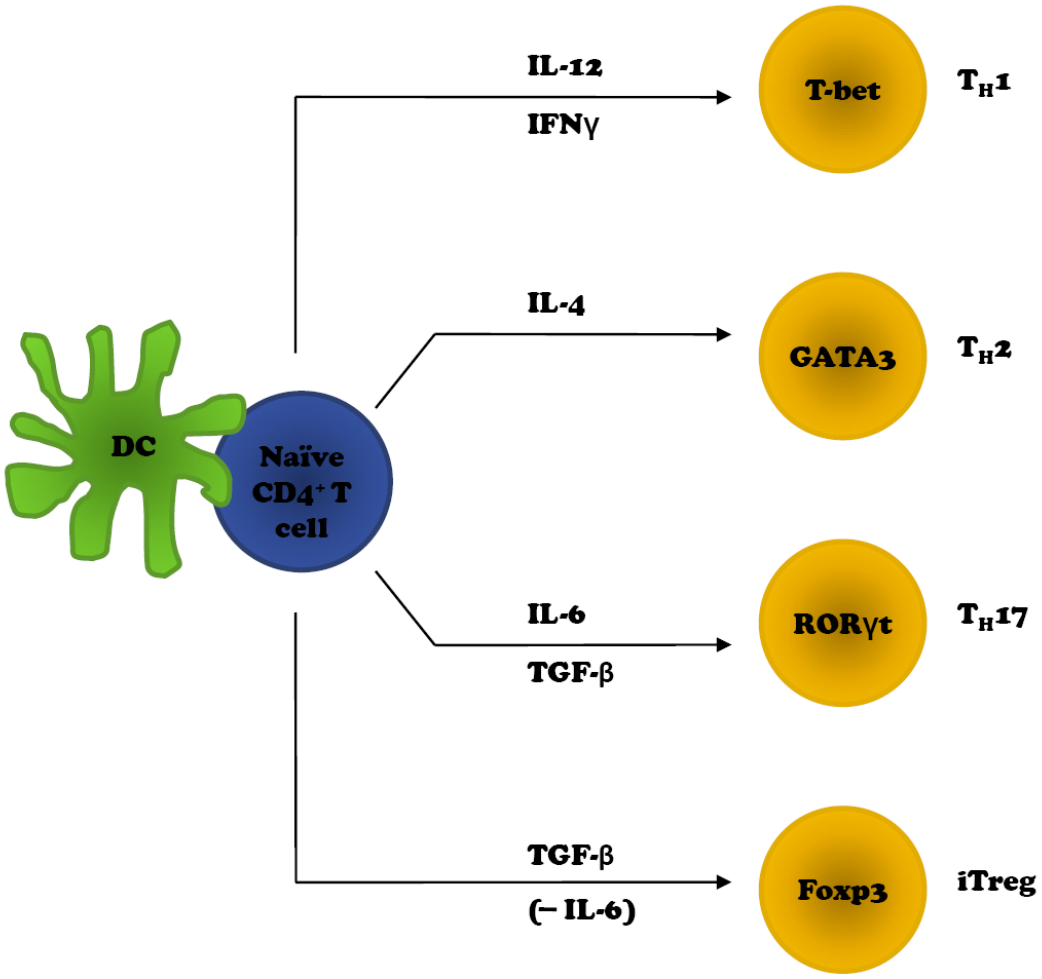

IL-12
IFNγ
T-bet
T_H1
IL-4
GATA3
T_H2
DC
Naïve
$CD4^+$ T
cell
IL-6
TGF-β
RORγt
T_H17
TGF-β
(– IL-6)
Foxp3
iTreg

Figure 1.5

cell, these receptors dimerize and activate the Janus kinase and signal transducers and activators of transcription (JAK-STAT) pathways. The transduction of these signaling pathways and the subsequent translocation of STAT proteins to the nucleus leads to the activation of various gene transcription programs. In response to the activation of a particular cytokine gene profile, cells commit to a certain T helper subset (Figure 1.5). IFN-γ and IL-12 production by APCs leads to the differentiation of uncommitted T cells to Th1 cells. Alternatively, IL-4 and IL-2 lead to the emergence of the Th2 subset. More recently, Th17 cells have been defined as a bonafide subset; differentiation into this subset is induced by IL-6 and TGF-β. In the absence of IL-6, but in the presence of TGF-β along with IL-2, naïve T cells can differentiate into inducible regulatory T cells (iTregs). Simultaneously, opposing cytokine genes are suppressed and the T helper cell subset phenotype stabilizes. After multiple rounds of cell division and exiting the lymphoid organs, these T cells arrive at the site of insult where they carry out their programmed effector function.

1.3.2 Th1 cells

Th1 effector cells are important for the control of intracellular infections by viruses, bacteria, and other micro-organisms. This effector role is orchestrated by Th1 cell production of interferon (IFN)-γ as well as IL-2 and TNF-α, cytokines known for their deleterious effects on intracellular pathogens. IFN-γ, the "signature" cytokine of

the Th1 subset, activates macrophages; this induces an increase in phagocytosis, MHC Class I and II surface expression, and superoxide and nitric oxide production.

IL-12 and IFNγ are necessary for the differentiation of uncommitted CD4 T cells to Th1 cells. IL-12 is produced by APCs, namely DCs, macrophages, and monocytes. However, TCR stimulation is necessary for CD4 T cells to be responsive to IL-12 as IL-12R expression on T cells is TCR-dependent (Presky et al., 1996). The binding of IL-12 to the IL-12R initiates the JAK-STAT signaling pathway specifically, Jak2 and Tyk2 kinases (Gately et al., 1998). This leads to the activation of STAT4 and an increase in the expression of T-box expressed in T cells (T-bet); this also results in the further upregulation of IL-12R on the T cell surface. T-bet is a transcription factor that is essential for Th1 cell differentiation (Szabo et al., 2000; Szabo et al., 2002). STAT4 and T-bet play a role in remodeling the *Ifng* gene, which induces IFN-γ production (Mullen et al., 2001; Mullen et al., 2002). Both of these transcription factors bind to the promoter of *Ifng*, whereby the presence of one factor stabilizes the chromatin binding of the other (Zhu et al., 2010). IFNγ production also leads to the further activation of T-bet. Upon the interaction of IFNγ to its ligand on the T cell, STAT1 is activated via the Jak1 and Jak2 kinases, leading to the activation of T-bet (Afkarian et al., 2002). This positive feedback loop is an important characteristic of T helper cell differentiation. It is also important to note that during the process of Th1 differentiation, there is evidence that genes required for delineation of other Th subsets are suppressed. For example, it has been shown that

T-bet negatively regulates the expression of GATA3, the master regulator of the Th2 cell lineage (Usui et al., 2006).

1.3.3 Th2 cells

Th2 cells are notorious for their role in asthma and other respiratory and allergic diseases. However, their beneficial roles are not to be disregarded, including the activation of B cells as well as the stimulation of mast cell proliferation and degranulation. These activities defend the host against an array of parasites including worms. Th2 cells mainly produce IL-4, IL-5, and IL-13 but can also produce IL-3, IL-9, and IL-10.

Early studies demonstrated the need for IL-4 and IL-2 for Th2 differentiation. It has since become apparent that IL-4 is in fact the most important cytokine in this process *in vivo*. The binding of IL-4 to the surface IL-4R/γc dimer leads to the phosphorylation of STAT6 via Jak1 and Jak3 activation (Hou et al., 1994; Quelle et al., 1995). The activation of this signaling cascade leads to enhanced GATA3 expression; GATA3 is necessary for Th2 differentiation (Zheng and Flavell, 1997). This transcription factor binds to the *il4*, *il5* and *il13* genes; the binding of GATA3 to these genes results in chromatin remodeling which enables the binding of other transcription factors (Ho et al., 2009). GATA3 therefore instructs Th2 commitment, but it also stimulates the expansion of Th2 cells (Zhu et al., 2006). Furthermore, GATA3 suppresses Th1 differentiation through its mutual antagonism with T-bet.

In addition to STAT6, studies have also implicated STAT5 in the indirect upregulation of GATA3 levels. In fact, in the absence of STAT5a, one of the two isoforms of STAT5, Th2 differentiation is abrogated (Kagami et al., 2001). IL-2 activates STAT5, resulting in increased expression of IL4-Rα on the T cell surface, thereby further enhancing IL-4 signaling (Liao et al., 2008). IL-4 signaling then leads to the increased activity of GATA3 and the production of more IL-4. As seen with Th1 differentiation, this positive feedback loop is necessary for the commitment and maintenance of T cells to the Th2 lineage.

1.3.4 Th17 cells and iTregs

Initially it was believed that Th1 and Th2 subsets were the only distinct CD4 subsets that existed in the periphery. However, there still remained a gap in the capabilities of the host defense that could not be explained by the function of either the Th1 or the Th2 subset. For instance, certain fungi and pathogens are not adequately cleared by Th1 or Th2 immunity; however, these infections are controlled by the host immune response. Eventually, a new T helper subset was defined which was able to fill the gap in our understanding of T cell immunity. The major cytokines produced by the Th17 subset include IL-17, IL-17F, IL-21, and IL-22 (reviewed in (Korn et al., 2009)). These cells, and the related soluble factors, are important for the control of extracellular bacteria and some fungi within the host. Specifically, Th17 cells defend against the invasion of bacteria at barrier surfaces and mediate the inflammatory reaction both

locally and systemically. However, due to their propensity to engineer inflammatory conditions, Th17 cells also play a role in the development of autoimmunity, such as inflammatory bowel disease, arthritis, and multiple sclerosis, as well as certain types of cancer.

IL-6 and TGF-β are responsible for the differentiation of naïve T cells into Th17 cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006); although it has also been found that in the absence of IL-6, IL-21 can also work in conjunction with TGF-β towards Th17 differentiation (Korn et al., 2007). These signaling pathways, IL-6 and TGF-β or IL-21 and TGF-β, result in the STAT3-dependent increase in RORγt expression (Korn et al., 2009). This transcription factor has recently been identified as the definitive transcription factor for the Th17 subset (Ivanov et al., 2006). RORγt is important for continued IL-17 production by these cells and it also mediates the expression of cell surface IL-23R. This is necessary for the stabilization of the Th17 cells as IL-23 signaling has been shown to play a role in maintaining commitment to this subset (Korn et al., 2009). While RORγt is necessary for specifying IL-17 induction and the subsequent positive feedback loop, STAT3 has also been shown to bind to the IL-17 and IL-21 promoters (Chen et al., 2006; Wei et al., 2007).

Intriguingly, TGF-β, by way of STAT3 activation, can also lead to the differentiation of uncommitted T cells into a completely different subset of cells, termed inducible Tregs (iTregs) (Chen et al., 2003; Kretschmer et al., 2005); differentiation of

iTregs occurs in the absence of IL-6. This demonstrates the pleiotropic nature of TGF-β whereby it plays a dominant role in the induction of Th17 cells, a subset of cells that initiates a high level of inflammatory conditions, but can also produce iTregs, cells that are renowned for their ability to diminish immune responses. The remarkable features of these anti-inflammatory iTregs, specifically their thymus-derived counterparts, natural Tregs (nTregs), will be discussed in the next section.

1.4 Immunological tolerance

1.4.1 Central tolerance

The adaptive branch of the immune system is fine-tuned to operate in a manner that supports the balance between defense against pathogens and protection against self-attack. If this balance scale is tipped to either extreme, immunodeficiency (in the case of defective pathogen recognition) or autoimmunity (excessive/inappropriate response to self) is likely to occur.

Within the thymus, more than 90% of developing T cells will undergo apoptosis due to the stringent checkpoints that have to be passed before being allowed to exit as a mature T cell. Most of the cell death that occurs in the thymus is a result of positive selection (Surh and Sprent, 1994). This process selects for thymocytes that bind to self peptide presented by MHC molecules with intermediate to high affinity. As a result, invading pathogens are more likely to be recognized in the periphery by one of these T cells due to the enormous diversity of the T cell receptors. Conversely, this selection

process enriches TCRs that bind with high affinity to self peptide-MHC complexes. Thus, positive selection increases the potential for the development of autoimmunity. One way in which the immune system prevents the development of autoimmune disease is through central tolerance.

Central tolerance, or recessive tolerance, is defined as the lack of responsiveness to self that occurs during lymphocyte development in primary lymphoid organs. Clonal deletion, a hallmark of negative selection, is the predominant mechanism of central tolerance (Palmer, 2003). During this process, cells that bind to self antigens with a high affinity undergo apoptosis. Although clonal deletion eliminates the majority of high-affinity binding thymocytes, a few subsets of cells are paradoxically selected for; this mechanism results in the immune system's ability to restrain immune responses triggered by antigens recognized as self. These subsets include $CD4^{+}Foxp3^{+}$ regulatory T cells (Tregs), $CD8\alpha\alpha^{+}$ intestinal epithelial lymphocytes, and natural killer T (NKT) cells (Hogquist et al., 2005).

The recognition of self peptides presented by MHC molecules during negative selection mostly occurs within the medulla of the thymus. This process is enabled by antigen presenting cells such as thymic dendritic cells, thymic macrophages, thymic B cells, and medullary thymic epithelial cells (mTECs). mTECs are arguably the most prominent thymic APCs involved in clonal deletion (Kyewski and Klein, 2006). These cells express a remarkably vast amount of tissue-restricted self-antigens (TRAs),

representing virtually every parenchymal organ thereby efficiently screening and allowing for the deletion of potentially autoreactive thymocytes. The expression of TRAs by mTECs is termed promiscuous gene expression (Derbinski et al., 2001). The autoimmune regulator (Aire) is a molecule that has proven to be indispensible in the function of mTECs during negative selection. This gene controls the promiscuous gene expression that is so vital to mTEC function. Indeed, Aire deficient mTECs lose the expression of TRAs; T cells from these mice escape negative selection, leading to the development of autoreactive T cells (Anderson et al., 2002). As a result, autoimmunity develops in multiple organs of $Aire^{-/-}$ mice (Liston et al., 2003). In addition to mTECs, thymic DCs also play a role in negative selection. In contrast to mTECs, DCs do not reside in the thymus, but have been shown to immigrate from peripheral tissues to present their antigens to the thymocytes (Bonasio et al., 2006).

Receptor editing also contributes to central tolerance; this process plays a role in preventing the development of autoreactive lymphocytes. During their 3 to 4 day lifespan, DP thymocytes undergo sequential rounds of recombination at the TCRα locus. This mechanism promotes secondary rearrangement of the TCRα chain following the failure of the TCR to successfully bind to an MHC-peptide complex, which increases the potential for the TCR to be matched with an MHC molecule (Petrie et al., 1993). This mechanism can also replace TCRα chains that would otherwise render the T cell autoreactive by binding too strongly to self antigen. However, receptor editing does not

seem to play a major role in the central tolerance of T cells, but is the major mechanism used to induce tolerance during B cell development (Hogquist et al., 2005).

1.4.2 Peripheral tolerance

Despite the efficiency of central tolerance in deleting most autoreactive T cells during thymic development, some of these T cells still manage to escape negative selection. Accumulating evidence suggests that these T cells are able to pass the negative selection checkpoint because the avidity of their TCRs to self-pMHC is low enough to evade apoptosis (Liu et al., 1995; Zehn and Bevan, 2006). It is also possible that autoreactive peripheral T cells originate from thymocytes that bind with a strong affinity to TRAs that are not sufficiently expressed in the medulla. These T cells enter the periphery and pose a potential threat to immunological homeostasis as they are capable of triggering an immune response upon recognition of self-peptide. However, there are mechanisms in place that prevent this from occurring in healthy individuals, thereby ensuring peripheral tolerance. Active or dominant tolerance is mediated by the suppressive activities of $CD4^{+}Foxp3^{+}$ Treg cells. These cells are capable of dampening the activation and proliferation of autoreactive T cells by a variety of mechanisms. This aspect of tolerance will be discussed in detail later in this chapter.

In addition to Treg cells, there are other mechanisms that exist which aid in the promotion of peripheral tolerance, including the physical sequestration of potentially autoreactive T cells from cells that express TRAs and the induction of anergy to prevent

activation of these autoreactive T cells. Due to the expression of CCR7 and CD62L on their cell surface membranes, naïve T cells are restricted to a seemingly stringent trafficking pattern. These cells circulate in the blood, enter secondary lymphoid organs, such as the spleen and lymph nodes, migrate into the efferent lymphatic vessels, and then return to the blood (Lammermann and Sixt, 2008). If T cells do not recognize pathogen-derived pMHCs presented by DCs in the secondary lymphoid organs, they eventually re-enter the blood stream. This cycle prevents naïve autoreactive T cells from entering nonlymphoid organs where TRAs are highly expressed, thereby decreasing the chances that these T cells will ever encounter pMHC that would elicit an autoimmune response.

Another way to attain peripheral tolerance is through anergy, in which autoreactive T cells are made unresponsive to antigen or immunogenic stimulus. Tolerogenic APCs, particularly DCs, have been implicated as a major cause of anergy induction. In the absence of inflammation, these DCs exist in the secondary lymphoid organs in a semi-mature state, inducing anergy in naïve T cells that express TCRs with a strong binding affinity to self MHC (Mueller, 2010). Studies have also suggested that autoreactive T cells can recognize TRA on mature tolerogenic DCs in lymph nodes and spleens, resulting in anergy and potentially the elimination of these T cells. It must be noted, however, that DCs do not work alone in eliciting anergy but require the presence of Tregs for optimal tolerance induction (Mueller, 2010).

In addition to the influence of DCs, T cell anergy can also be mediated by co-stimulatory molecules. TCR stimulation in the absence of costimulation, such as the signal propagated by CD28, leads to an anergic state. However, CD28 competes with CTLA-4, its structural homolog, for binding to its ligands CD80 and CD86; furthermore, CTLA-4 binds with higher affinity to these ligands compared to CD28. In response to antigen stimulation, CTLA-4-deficient CD4 T cells and wildtype T cells incubated with CTLA-4 blocking antibodies are resistant to anergy induction (Greenwald et al., 2001; Kearney et al., 1995; Perez et al., 1997). The importance of CTLA-4 in anergy induction can also be partially attributed to its role in regulatory T cell function, but evidence for its role in inducing unresponsiveness in CD4 T cell in the absence of Treg cells has been demonstrated (Eggena et al., 2004). While CTLA-4 is important for anergy induction, another costimulatory molecule, PD-1, is critical to maintain an anergic state. For this reason, mice deficient in PD-1 or its ligands, PD-L1 and PDL-2, develop systemic autoimmunity (Freeman et al., 2000; Nishimura et al., 1999). Studies have suggested that PD-1 is required for thwarting efficient TCR engagements by inhibiting tissue migration "stop signals", thus promoting the maintenance of anergy (Fife et al., 2009).

While physically sequestering autoreactive T cells from TRAs in parenchymal tissue and inducing anergy in these cells are essential mechanisms in establishing peripheral tolerance, the function of regulatory T cells is undoubtedly of the utmost importance in achieving this goal. Scurfy mice, in which the *foxp3* gene is nonfunctional,

lack Treg cells, resulting in uncontrolled T cell expansion, development of autoimmunity, multi-organ failure, and early death (Blair et al., 1994; Fontenot et al., 2003; Lyon et al., 1990) (Brunkow et al., 2001). This discovery inspired an interest in Treg cell signaling and function that has led to expansive studies which have collectively demonstrated that these cells are in fact essential for the establishment of peripheral tolerance.

1.4.3 The discovery of Tregs

Immune responses elicited by autoreactive T cells are normally dampened by Treg cells. However, the role of Treg cells extends farther than controlling the development of autoimmune diseases; these cells are also important in thwarting inappropriate immune responses and have been implicated in abrogating the anti-tumor response. Cellular targets of Treg suppression include the following: CD4 and CD8 T cells, B cells, DCs, NK cells, NKT cells, macrophages and mast cells. In the past decade, the literature describing the many characteristics and roles of Treg cells has grown drastically. However, this field has not always been popular.

It was observed by Nishizuka and Sakakura in 1969 that neonatal thymectomy of wild-type mice led to the development of autoimmune disease, but this disease could be rescued with the transfer of $CD4^+$ T cells (Nishizuka and Sakakura, 1969). Studies done in sublethally irradiated adult rats also found that the transfer of $CD4^+$ T cells could rescue the animals from autoimmune disease (Penhale et al., 1973). Accordingly, it was

postulated that a portion of T cells had the ability to suppress immune responses; this hypothesis was also supported by other studies (Gershon and Kondo, 1970, 1971). However, studies done within that decade could not identify the factors that would allow for such a function in T lymphocytes. As a result, investigations based on this theory ceased during the 1980s and the idea of suppressor T cells had been all but discarded (Green and Webb, 1993; Moller, 1988). In fact, most immunologists had begun to believe that these cells did not exist- they were just artifacts of scanty data.

Fortunately, this field of study was revitalized when definitive evidence emerged that supported the existence of such a population. In 1995, Sakaguchi et al. identified the suppressive capability of $CD4^+CD25^+$ T cells in maintaining immunological tolerance (Sakaguchi et al., 1995). These cells, which represent 10% of the total $CD4^+$ population, were shown to prevent the development of autoimmune disease in BALB/C athymic nude (nu/nu) mice that had been reconstituted with $CD4^+CD25^-$ cells; this rescue occurred in a dose dependent manner (Sakaguchi et al., 1995). Since the identification of CD25 (the IL-2Rα chain) on CD4 cells as a marker for suppressor cells, great advances have been made in further characterizing these cells.

Around the same time that Sakaguchi et al made their groundbreaking discovery, Scurfy mice were being analyzed by others. These mice exhibit severe lymphoproliferative disease with multi-organ lymphocytic infiltration and wasting disease (Godfrey et al., 1991). An early study demonstrated that transferring $CD4^+$ T cells

from Scurfy mice to nu/nu or SCID mice leads to the development of a similar phenotype, implicating a role for these cells in mediating the disease (Blair et al., 1994). Further analysis of Scurfy mice identified a mutation in a novel member of the forkhead/winged helix family, Foxp3. Indeed, the mutation of this gene is responsible for the development of the observed autoimmune disease (Brunkow et al., 2001). Similarly, the human homolog of Foxp3 is mutated in patients with a severe autoimmune disease known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) (Bennett et al., 2001; Chatila et al., 2000; Wildin et al., 2001).

Soon after these initial studies on Foxp3, the link between this transcription factor and Treg cells was made. It was demonstrated that Foxp3 expression was largely limited to naturally occurring thymic and peripheral $CD4^+CD25^+$ Treg cells; Foxp3 was not expressed by TCR-activated conventional T cells that had upregulated CD25 expression (Fontenot et al., 2003). These studies also showed that Foxp3 is required for the development of $CD4^+CD25^+$ Treg cells in the thymus. Furthermore, it was proven that the phenotype observed in Foxp3-deficient mice and Foxp3-mutated Scurfy mice was a direct result of a lack of $CD4^+CD25^+$ Treg cells, rather than an intrinsic defect in $CD4^+CD25^-$ conventional T cells (Fontenot et al., 2003). Indeed, when Foxp3 was ectopically expressed in $CD4^+CD25^-$ T cells, these cells gained suppressor function. Together, these data established that Foxp3 is the gene responsible for $CD4^+CD25^+$ Treg cell development and function.

Following the discovery of the importance of Foxp3 in Treg cell function, studies revealed another subset of Foxp3-expressing Treg cells, called adaptive Tregs or inducible Tregs (iTregs). In contrast to natural Tregs (nTregs) that develop in the thymus, iTregs develop in the periphery in response to different stimuli, such as the recognition of foreign antigen in certain conditions (Curotto de Lafaille and Lafaille, 2009). This subset of cells can be induced *in vitro* by culturing TCR-stimulated $CD4^+CD25^-$ cells with TGF-β and IL-2. nTregs and iTregs behave differently due to the differences in their thymic development and TCR repertoires. Their functions in regulating immune responses are likely to be complementary but more studies are needed to understand this relationship. As most of the studies presented in this thesis address natural regulatory T cells, as opposed to iTregs, the term "Treg cell" will refer to thymus-derived $CD4^+CD25^+$ Treg cells unless otherwise noted.

1.4.4 Mechanisms of Treg suppression

Treg cells are characterized by their ability to attenuate immune responses. Since their discovery, how they are able to carry out this function has been an intriguing question. It is known that Treg cells become functional upon TCR-mediated activation. They are able to suppress the activation and proliferation of conventional T cells by interacting with the responder T cells and influencing them directly, secreting molecules such as suppressive cytokines, and inducing changes in dendritic cells that inhibit responder cell function.

Treg cells produce several cytokines, including IL-10 and TGF-β, which are known for their anti-inflammatory properties (Figure 1.6a). Early *in vitro* studies suggested that these cytokines may not be important in Treg function as cell-cell contact was shown to be necessary for the suppression of responder proliferation (Takahashi et al., 1998; Thornton and Shevach, 1998). Moreover, the addition of IL-10 and TGF-β blocking antibodies to *in vitro* suppression assays, or the use of Treg cells unable to produce or respond to either of these cytokines, does not prevent responder T cell function (Dieckmann et al., 2001; Piccirillo et al., 2002; Piccirillo and Shevach, 2001; Takahashi et al., 1998; Thornton and Shevach, 1998). However, the role of these cytokines in mediating Treg suppression *in vivo* has been well-documented. IL-10 plays an essential role in controlling immune responses at environmental interfaces such as in the colon and lungs where foreign antigens are likely to elicit inflammatory responses (Rubtsov et al., 2008). Interestingly, this study also showed that the deletion of IL-10 specifically in Treg cells did not induce systemic autoimmune disease, downplaying the role of this cytokine in the suppression of autoreactive T cells by Treg cells. TGF-β is also produced by Treg cells and is important in suppressing allergic responses, preventing colitis in IBD, and controlling inflammation during *M. tuberculosis* infection (Joetham et al., 2007; Kursar et al., 2007; Li et al., 2007). In contrast, there have been datasuggesting that Treg-derived TGF-β is not essential for the efficient function of Treg cells. These studies have shown that TGF-β can also be produced by other cell types (Shevach, 2009).

The differences in these findings may be attributed to different models that were used but further studies are needed to resolve this issue.

One of the hallmarks of Treg cells is their constitutively high surface expression of IL-2Rα (CD25). Published data have suggested that this characteristic is utilized in suppressing immune responses that are mediated by effector $CD4^+$ T cells. One such study shows that, when cultured with conventional T cells, Treg cells are able to sop up IL-2 from the surrounding environment, essentially starving the effector T cells of this cytokine which is so important for effector function (illustrated in Figure 1.6b) (Pandiyan et al., 2007). As a result, the effector T cells do not proliferate and eventually undergo apoptosis. This process is referred to as "IL-2 sink". However, other studies have suggested that this process is not sufficient to disturb the function of effector T cells (Shevach, 2009). Therefore, more work needs to be done to confirm the physiological importance of this process in Treg cell suppression.

$Foxp3^+$ Treg cells are the only known subset of lymphocytes that constitutively express CTLA-4. As previously mentioned, this inhibitory molecule competes with CD28 for binding of CD80 and CD86 on APCs. CTLA-4 expressed on Treg cells binds to these ligands on the surface of DCs, preventing costimulation of naïve T cells via CD28 and thereby resulting in immunosuppression. Indeed, CD80 and CD86 expression is downregulated on DCs cultured with CTLA-4 deficient Treg cells (Wing et al., 2008). Mice with a selective deletion of CTLA-4 in $Foxp3^+$ cells develop severe autoimmune

Figure 1.6 Major mechanisms of suppression used by Treg cells

(a) Upon activation, Treg cells produce inhibitory cytokines, such as IL-10 and TGF-β, which can dampen an effector T cell-mediated immune response. (b) Treg cells express a high level of surface CD25 which absorbs IL-2 from the surrounding environment, thereby depriving conventional $CD4^+$ T cells of this cytokine and inducing apoptosis. (c) The association of CTLA-4 on Treg cells with CD80 and CD86 molecules on dendritic cells (DCs) can cause the abrogation or retardation of DC maturation; this mechanism may be mediated by indoleamine 2, 3- dioxygenase (IDO), an immunosuppressor. The result is decreased DC-initiated activation of conventional T cells. (d) Treg cells may also kill conventional T cells in a granzyme- or perforin-dependent manner. (Adapted from (Vignali et al., 2008))

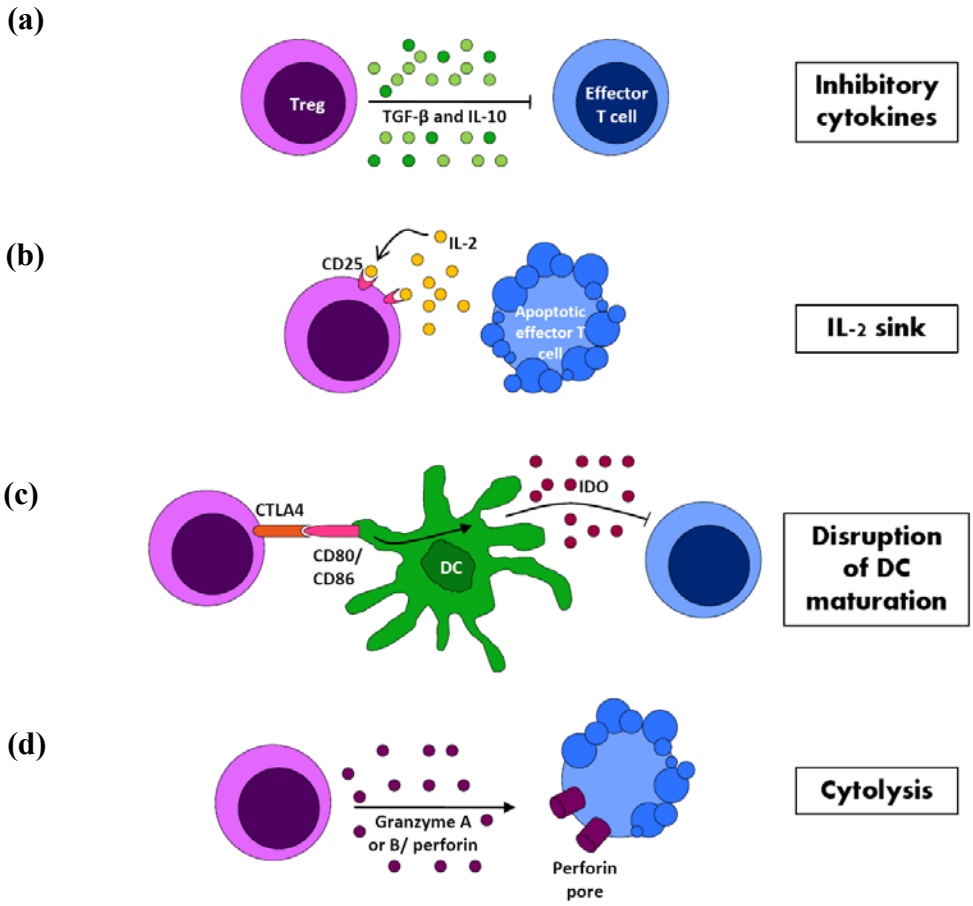

(a)
Treg
TGF-β and IL-10
Effector T cell
Inhibitory cytokines
(b)
CD25
IL-2
Apoptotic effector T cell
IL-2 sink
(c)
CTLA4
CD80/ CD86
DC
IDO
Disruption of DC maturation
(d)
Granzyme A or B/ perforin
Perforin pore
Cytolysis

Figure 1.6

disease characterized by T cell-mediated lymphoproliferation. These cells do not have defects in Treg cell development or homeostasis but exhibit impaired suppressive function *in vivo* and *in vitro*, highlighting the importance of CTLA-4 in Treg-mediated suppression (Wing et al., 2008). In addition, the association of Tregs with DCs leads to the increased activity of indoleamine 2,3-dioxygenase (IDO) in a manner dependent on CTLA-4-CD80/CD86 binding (Grohmann et al., 2002). IDO is a potent immunosuppressive enzyme whose activity leads to increased production of pro-apoptotic metabolites, resulting in reduced effector T cell function (von Boehmer, 2005). However, the physiolocigal importance of this process has not yet been defined.

Another mode of inducing apoptosis in effector T cells is dependent on the cytolytic properties of granzyme B and/or perforin (Figure 1.6d). Upon activation, Treg cells upregulate granzyme B expression; Treg cells deficient in granzyme B have defective suppressive function *in vitro* (Gondek et al., 2005). Additionally, one study has suggested that Treg cells may be able to kill responder cells in a granzyme B- and perforin-dependent manner (Cao et al., 2007). Presently, there is not much evidence to support this notion of responder cell cytolysis. However, other studies have found that activated Treg cells are capable of killing APCs *in vitro*; this killing is mediated, at least in part, by granzyme B (Zhao et al., 2006). Perforin, a molecule with cytolytic properties similar to granzyme B, can be detected in Treg cells and has also been implicated in mediating effector cell death (Zhao et al., 2006).

This field of study is constantly changing. There seem to be an extensive number of mechanisms utilized by Treg cells to suppress immune responses. Studies are ongoing and it is inevitable that the details for already proposed and entirely new mechanisms will be revealed.

While the importance of Foxp3 as a master regulator of gene transcription in Treg cells has been revealed, not much is known about what regulates Foxp3. Here, we show data that suggests that LAT plays a role in regulating Foxp3 expression in Treg cells. Furthermore, while TCR-mediated signaling is important for Treg cell activation, the importance of TCR signals in Treg cell function and survival has not been demonstrated. Therefore, we sought to assess the importance of LAT and the LAT-PLCγ1 interaction in the function and survival of Treg cells.

Past studies on LAT and its interaction with PLC-γ1 have been done in Jurkat cell lines or in primary T cells from mice with pre-existing autoimmune disorders, defects in thymocyte selection and development, and/or environments involving elevated levels of cytokines. In addition to our interest in the role of LAT in Treg cells, we sought to study the role of LAT and the LAT-PLC-γ1 interaction in mature T cells after normal thymocyte development and without pre-existing conditions that likely affect TCR-mediated signaling. In order to do this, we generated ERCreLAT$^{f/-}$ and ERCreLAT$^{f/m}$ mice (where m is the Y136F-mutated allele of LAT). In these mice, treatment with tamoxifen-induced the deletion of LAT in T cells following normal thymocyte

development and before the development of autoimmune disorders. This allowed for the study of these cells in a more physiological setting compared to studies done in cell lines. In the data presented here, we show that LAT and the LAT-PLC-γ1 interaction are essential for effective TCR-mediated signaling. The deletion of LAT in ERCreLAT$^{f/-}$ or ERCreLAT$^{f/m}$ T cells in vivo leads to the development of a lymphoproliferative disorder characterized by CD4^{+} T cells. These data demonstrate that while LAT is a positive regulator of TCR-mediated signaling, LAT also plays a role in negatively regulating homeostasis.

2. Materials and Methods

2.1 Mouse models

LAT$^{f/f}$ mice were generated as previously described (Shen et al., 2009). These mice were crossed with ERCre transgenic mice (kindly provided by Dr. Thomas Ludwig, Columbia University, NY) to produce ERCre^{+}LAT$^{f/f}$ mice, which were then bred with LAT$^{m/+}$ mice (m=Y136F) to generate ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice. All mice were used in accordance with the National Institutes of Health guidelines. The procedures described in this study were reviewed and approved by the Duke University Institutional Animal Care and Use Committee. Mice were housed in specific pathogen-free conditions at the Duke University Animal Care facility.

2.2 Antibodies

2.2.1 Antibodies for flow cytometry

Texas Red conjugated to anti-IgM was purchased from Southern Biotechnology Associates (Birmingham, AL). Biotinylated CD69 and TCRβ were from BD Biosciences (San Jose, CA). The cell viability marker, 7AAD, was purchased from Invitrogen (Carlsbad, CA). Thy1.1 Pacific Blue was purchased from Bio-Legend (San Diego, CA). All other antibodies used to stain cell markers for FACS analyses were obtained from eBioscience (San Diego, CA). These include anti-CD3ε, CD4, CD8α, B220, CD11c, CD25, IAb, IgD, CD44, CD62L, CD40, CD80, CD86, Mac-1, Gr-1, IL-2, IL-4, IFN-γ, Foxp3, and CTLA-4.

2.2.2 Antibodies for biochemical studies

Monoclonal anti-phospho-Erk1/2 (Thr202/Tyr204) antibody and polyclonal anti-phospho-PLCγ1 (Tyr783) antibody were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal anti-PLCγ1 antibody and anti-phosphotyrosine (4G10) were bought from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-Erk2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-HSP70 was bought from BD Transduction Laboratories (San Jose, CA). For secondary antibodies, goat anti-mouse and anti-rabbit Ig conjugated with Alexa Fluor® 680 were purchased from Molecular Probes (Eugene, OR). IRDye ™800 anti-rabbit-IgG was purchased from Rockland Immunochemicals (Gilbertsville, PA).

2.3 Cell culture, isolation, and purification

2.3.1 Isolation of lymphocytes from thymus, spleen, and lymph nodes

Thymuses, spleens, and lymph nodes were extracted from sacrificed mice. Single cell suspensions were created by homogenizing organs with frosted glass slides. Spleens were subjected to ACK lysis buffer (0.15M NH4Cl, 10mM KHCO3, 0.1mM EDTA pH7.4) for 1 minute at room temperature in order to lyse red blood cells. These cells were then washed with PBS. Cell suspensions were filtered through mesh and resuspended to the desired concentration after being counted using a hemocytometer.

2.3.2 Purification of T cells and regulatory T cells

For positive selection of $CD4^+$ T cells, MACS® beads were used with LS columns as recommended by the manufacturer (Miltenyi Biotec, Auburn, CA). For negative selection of $CD4^+$ T cells, the EasySep® Mouse $CD4^+$ T cell Enrichment Kit was purchased from Stem Cell Technologies. For purifying $CD4^+CD25^+$ T cells, the EasySep® Mouse Regulatory T cell Positive Selection Kit was used to negatively select for $CD4^+$ T cells followed by positive selection of $CD25^+$ cells as recommended by the manufacturer. For purification of $CD4^+CD25^+GFP^+$ T cells, $CD4^+$ cells were enriched by negative selection using biotin-conjugated antibodies for B220, Gr1, Mac-1, CD11c, CD8, TER119, and CD49b (all from eBioscience) on ice for 30 minutes followed by incubation with streptavidin-Dynabeads for 1-2 hours. Cells were then incubated with cell surface antibodies and sorted by flow cytometry using a MoFlo sorter (Beckman Coulter, Brea, CA).

2.3.3 Generation of activated T cells

Splenocytes were cultured in αCD3 antibody (2C11)-coated 6-well plates in the presence of murine IL-2 (10 ng/ml) for 2 days. Cells were then moved into T75 flasks to expand for 3 more days in the presence of IL-2, without αCD3. T cells were then purified by negative selection.

2.4 Flow cytometry analysis

2.4.1 Surface staining

For staining of cell surface markers, cells were incubated with anti-CD32/16 (2.4G2) supernatant for 15 minutes on ice. Cells were washed with FACS buffer (PBS with 2% FBS) and incubated with different combinations of fluorochrome-conjugated antibodies for approximately 20 minutes on ice and washed in FACS buffer before FACS analysis. If biotin conjugated antibodies were used, cells were washed with FACS buffer followed by incubation with fluorochrome-conjugated streptavidin (eBioScience) on ice for 20 minutes. Samples were washed with FACS buffer and analyzed on FACSDiva or FACSCanto II (BD Biosciences). The FACS plots shown were analyzed using Flowjo software.

2.4.2 Intracellular staining

For intracellular staining of cytokines, splenocytes were stimulated with 40 ng/ml of PMA and 500 ng/ml of ionomycin for 1 hour. After addition of Golgi-Stop (BD Bioscience), cells were stimulated for an additional 4 hours. Cells were washed and

Figure 2.1 Outline of adoptive transfer of T cells into LAT$^{-/-}$ mice and the induction of LAT deletion after reconstitution

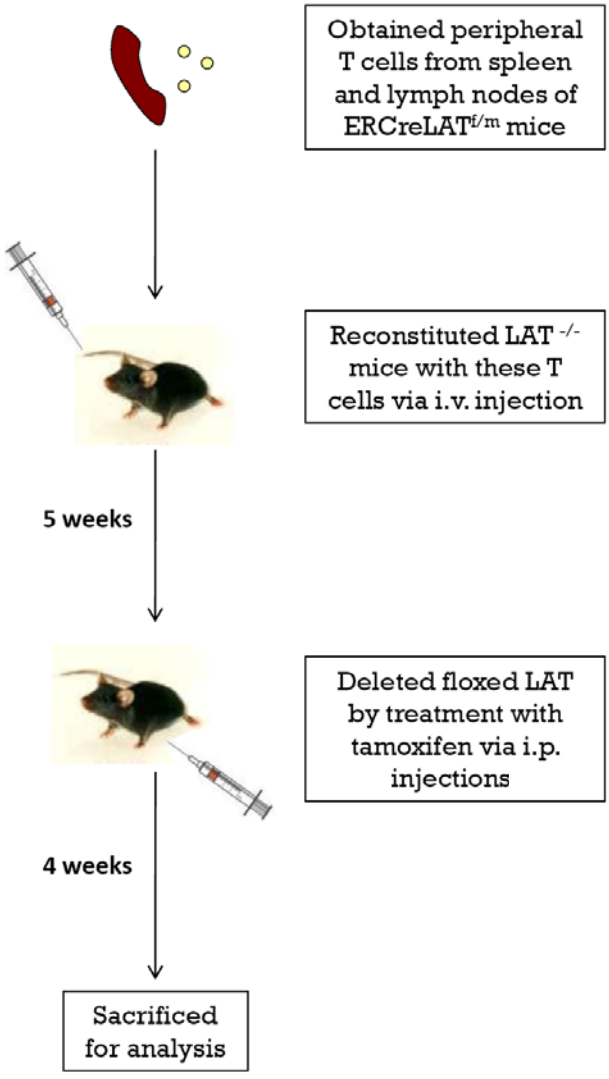

Obtained peripheral T cells from spleen and lymph nodes of ERCreLAT f/m mice
Reconstituted LAT -/- mice with these T cells via i.v. injection
5 weeks
Deleted floxed LAT by treatment with tamoxifen via i.p. injections
4 weeks
Sacrificed for analysis

Figure 2.1

stained with cell surface antibodies as described in 2.4.1. Cells were then washed, fixed, and permeabilized on ice using the Foxp3 staining buffer set (eBioscience) according to the manufacturer's guidelines. For intracellular staining using anti-Foxp3 and anti-CTLA-4 antibodies, cells were surface stained, fixed, and permeabilized as above. Samples were analyzed on FACSDiva or FACSCanto II (BD Biosciences). FACS plots shown were analyzed on Flowjo software.

2.4.3 CFSE staining

T cells were isolated as described above. They were then labeled with 5μM CFDA-SE for 10 minutes at 37°C in the dark in 5%FBS/PBS. These cells were washed three times with 5%FBS/PBS. The cells were then left at 37°C for 10 minutes, washed, and cultured for regulatory cell suppression assays for 60-72hrs. At this time, cells were washed and stained with cell surface antibodies as described earlier. Samples were collected on FACSDiva (BD Bioscience) and analyzed using Flowjo software.

2.5 Adoptive transfer of T cells

Single-cell suspensions were prepared from the lymph nodes and spleens of ERCreLAT$^{f/m}$, ERCreLAT$^{f/+}$, ERCreLAT$^{f/-}$, or LAT$^{f/-}$ mice. T cells were enriched by negative selection using biotinylated αB220, αGr1, αMac-1, αCD11c, and αNK1.1 (all from eBioscience) followed by incubation with streptavidin-conjugated Dynabeads® (Invitrogen). 20x10^6 cells were injected into each 6 week-old LAT$^{-/-}$ recipient via tail vein

injections. After 5 weeks, blood was collected from the recipients and FACS analysis was done to ensure successful reconstitution.

For reconstitution of $LAT^{-/-}$ mice with cells from both $ERCreLAT^{f/+}$ mixed with $ERCreLAT^{f/m}$ T cells, T cells were enriched from the spleens and lymph nodes from each of these mice using Dynabeads® as described above. Cells were then analysed by FACS to assess the percentage of $TCR\beta^{+}$ cells. After calculation, $TCR\beta^{+}$ cells from both mouse genotypes were mixed at a one to one ratio. Cells were again analysed by FACS to ensure a 1:1 ratio. Once this ratio was achieved, approximately 8 million total cells (4 million of each genotype) were injected via tail vein injection into each $LAT^{-/-}$ recipient.

2.5 Deletion of LAT in T cells from conditional knock-in mice

To delete the floxed *lat in vivo*, 1.5mg of tamoxifen (Sigma, St. Louis, MO) dissolved in corn oil was injected intraperitoneally into mice for two consecutive days. For long-term deletion, the same dose of tamoxifen was administered once a week thereafter. For LAT deletion *in vitro*, cells were incubated with 50nm 4OHT (Sigma) for 96 hours following activation with anti-CD3 as described above.

2.6 ELISA

2.6.1 TCR-mediated IL-2 production

$2x10^5$ positively-selected T cells were cultured in a 96-well plate coated with αCD3 (5μg/ml) with or without soluble αCD28 (0.5μg/ml). PMA (40ng/ml) plus ionomycin (500ng/ml) stimulation was used as a control to bypass TCR-mediated

signaling. Cells were cultured in triplicate for 36 to 48 hours at 37°C and the supernatants from these cultures were harvested and used for ELISAs using IL-2 capture and detection antibodies according to the manufacturer's protocol (eBioscience).

2.6.2 Serum antibody levels

For serum antibody detection, blood was harvested via cardiac puncture and allowed to clot at room temperature for one hour. After centrifugation, serum was obtained and serially diluted. ELISAs for IgG_1 and IgM were done using 96 well plates coated with unlabelled anti-mouse (H+L) antibody. Supernatants were incubated with IgG_1-AP or IgM-AP at room temperature. After one hour, PNPP substrate was added (all reagents from Southern Biotech, Birmingham, AL). Absorbance was read at 405nm. ELISAs for IgE were done using 96 well plates coated with purified IgE (eBioscience). Supernatants were incubated with IgE biotin (BD Pharmingen) and Avidin-HRP (eBioscience) at room temperature. The reaction was stopped with H_2SO_4. Absorbance was read at 450nm.

2.7 TCR-mediated calcium flux

$CD4^+$ T cells were purified from splenocytes and lymph node cells using positive selection. Indo-1 acetoxymethyl ester dissolved in DMSO was added to cells in loading buffer (1% FBS-HBSS with 10mM HEPES) for a final concentration of 1.5μM for 30 minutes at 30°C. Cells were then washed with loading buffer and incubated in a 37°C water-bath for 5 mins. TCR-stimulation was initiated by incubation with biotinylated

CD4 (1μg/ml) and CD3 (5μg/ml) followed by crosslinking with streptavidin (25μg/ml, Sigma-Aldrich). Calcium flux on GFP^+ cells was recorded using FACStar (BD Biosciences). To induce TCR-independent calcium flux, ionomycin (500ng/ml) was added after 5 mins and cell events were collected for an additional 3 mins. Calcium flux was measured by the fluorescence emission ratio at 405/495nm.

2.8 TCR-mediated cell proliferation assay

$2x10^5$ purified T cells were cultured in a flat-bottomed 96-well plate coated with αCD3 (5μg/ml) with or without soluble αCD28 (0.5μg/ml). PMA (40ng/ml) plus ionomycin (500ng/ml) stimulation was used as a control to bypass TCR-mediated signaling. Cells were cultured in triplicate for 36 to 48 hours at 37°C and were then pulsed with 1μCi of [^{3}H]thymidine (GE Healthcare) for an additional 6 hours. Cells were harvested and [^{3}H]thymidine incorporation was measured by a liquid scintillation luminescence counter (Perkin Elmer).

2.9 Western blotting

$CD4^+$ T cells were purified from activated T cells by negative selection and were rested in medium without IL-2 for 6 hr before being incubated with biotinylated αCD3, αCD4 and αCD8, washed, and treated with streptavidin for the indicated time points at 37°C. Cells were lysed with RIPA buffer (25mMTris·HCl (pH 7.5), 150mM NaCl, 5mM EDTA, 1% Triton-100, 0.1% SDS, 0.5% deoxycholic acid) containing a cocktail of protease inhibitors (1mM AEBSF, 1mM aprotinin, 1mM leupeptin hemisulfate, and 1mM sodium

vanadate) on ice for 5 mins. The lysate were centrifuged and the insoluble pellet was discarded. The supernatant was boiled with an equal portion of 2x SDS (100mM Tris·HCL (pH 6.8), 4% SDS, 20% glycerol, 5% β-mercaptoethanol and 0.02% bromphenol blue) for 5 mins at 95°C.

Samples were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Membranes were incubated in blocking buffer (1% fish gelatin in PBS) at room temperature for one hour. Membranes were then blotted with primary antibodies at 4°C overnight, washed with 1xPBST buffer, and then probed with secondary antibody for one hour at room temperature. After membranes were washed, protein-bound antibody was visualized and quantified with an infrared fluorescence imaging system (LI-COR Bioscience).

2.10 RT-PCR and semi-quantitative PCR

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified using magnetic separation as described above or cells were sorted on GFP^+ Treg and conventional T cell populations. In some cases, cells were stimulated with plate-bound αCD3 and soluble αCD28 (2μg/ml) for one hour. Cells were washed and supernatants were discarded. After dislodging of the pellet, Trizol (Invitrogen) was added and RNA was extracted per the manufacturer's suggestions. RNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) and was then used for RNA extraction. The cDNA templates generated were amplified during PCR using Taq polymerase. The following primers

were used for semi-quantitative RT-PCR: 5′-CTATGCTGCCTGCTCTTACTGAC-3′ and 5′-CGGAGAGAGGTACAAACGAGG-3′ for IL-10; 5′-TGCTGCTTTCTCCCTCAACCT-3′ and 5′-CACTGCTTCCCGAATGTCTGA-3′ for TGF-β; 5′-TGTTTGAGACCTTCAACACC-3′ and 5′-TAGGAGCCAGAGCAGTAATC-3′ for β-Actin; 5′GGGAGCCAGCTCTACTCTGCACC-3′ and 5′-GGCCTTGCCTTTCTCATCCAGG -3′ for Foxp3.

2.11 In vitro regulatory T cell suppression assay

$CD4^+CD25^+$ cells (Treg cells) and $Thy1.1^+CD4^+CD25^-$ T cells (responders) were purified using a regulatory T cell isolation kit (StemCell Technologies). $2x10^4$ responders were cultured with 1μg/ml αCD3 (2C11), $4x10^4$ APCs (splenocytes from $LAT^{-/-}$ mice), and Treg cells at different ratios. Cells were cultured for 66 to 72 hours. Proliferation of responder T cells was analyzed by CFSE staining, in which responder cells were labeled with CFSE prior to culture and subsequently analyzed using FACS. Alternatively, proliferation was measured by [^{3}H] thymidine incorporation; cells were pulsed with 1μCi of [^{3}H]thymidine after 66 to 72 hour culture and incubated for an additional 6 hours. Cells were harvested and [^{3}H]thymidine incorporation was measured by a liquid scintillation luminescence counter (Perkin Elmer).

3. The role of the LAT-PLCγ1 interaction in the function of conventional and regulatory T cells

3.1 Introduction

LAT is a transmembrane adaptor protein that plays a central role in linking TCR engagement to the activation of downstream signaling events, such as Ras-Erk activation and calcium mobilization. Upon T cell activation, LAT is phosphorylated by ZAP-70 and binds to Grb2, Gads, PLC-γ1, and other signaling proteins (Finco et al., 1998; Weber et al., 1998; Zhang et al., 1999a; Zhang et al., 1998; Zhang et al., 2000). Two of the highly conserved tyrosines in the cytoplasmic tail of LAT, Y171 and Y191, bind to Gads, which constitutively interacts with SLP-76. One critical tyrosine, Y132, associates with PLC-γ1; the interaction of LAT with PLC-γ1 and the Gads-SLP-76 complex is important for the activation of PLC-γ1. Upon activation, PLC-γ1 hydrolyzes phosphatidylinositol 4,5 biphosphate (PIP_2) to generate diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). While DAG activates PKC and RasGRP1, IP_3 binds to IP_3 receptors on the endoplasmic reticulum (ER), inducing the flux of Ca^{2+} from the ER into the cytoplasm (Jayaraman et al., 1996; Putney and Bird, 1993). This influx eventually leads to the dephosphorylation and translocation of NFAT proteins to the nucleus (Vig and Kinet, 2009).

Studies using LAT-deficient Jurkat cells also show that the interaction of LAT with PLC-γ1 through Y132 is important in TCR-mediated calcium flux and Ras activation (Zhang et al., 2000). This interaction is also critical for thymocyte development

and the control of T cell homeostasis and autoimmunity (Sommers et al., 2002; Von Boehmer, 1995). In LAT knock-in mice with a mutation at the PLC-γ1 binding site (Y136 in murine LAT), thymocyte development is partially blocked at the DN3 ($CD25^+CD44^-$) stage, likely due to impaired pre-TCR signaling, a necessity for the transition from the DN3 stage to the DN4 ($CD25^-CD44^-$) stage. Consequently, very few DP ($CD4^+CD8^+$) cells are present in the thymus. Despite the severe developmental defect, mature $CD4^+$ T cells are present in these mice; however, they undergo uncontrolled proliferation in the periphery which results in the development of splenomegaly and lymphadenopathy (Aguado et al., 2002; Sommers et al., 2002). The majority of these $CD4^+$ T cells exhibits an effector/memory ($CD44^+CD62L^{lo}$) phenotype and produces high levels of Th2 cytokines, specifically IL-4 and IL-5. As a result of the dominance of Th2 cytokines, B cells are highly activated and secrete large amounts of IgE and IgG_1 (Sommers et al., 2002). Recent studies indicate that the dominance of Th2 cells in these mice is dependent on STAT6. Interestingly, in the absence of STAT6, LATY136F mice still develop a lymphoproliferative disease; however, the T cell compartment is dominated by Th1 and $CD8^+$ T cells (Archambaud et al., 2009).

Several studies have investigated the cause of the autoimmune disease observed in LATY136F mice. One early study suggests that autoimmunity in these mice is likely caused by defective thymic selection. In this study, by using an HY transgenic TCR model, it was shown that both positive selection and negative selection are impaired in

LATY136F mice (Sommers et al., 2005). A defect in negative selection may allow autoreactive T cells to survive and exit the thymus, contributing to the development of autoimmune disease. In addition to impaired negative selection, previous studies from our lab show that the LATY136F mice lack naturally arising T regulatory cells ($CD4^+CD25^+$ cells)(Koonpaew et al., 2006). Correspondingly, the level of Foxp3 RNA in thymocytes and peripheral $CD4^+$ T cells is dramatically reduced. In addition, Foxp3 protein is hardly detectable in $CD4^+$ T cells. However, another study using *Foxp3EGFP* knock-in mice clearly shows that $Foxp3^+$ cells, as indicated by GFP expression, are present in these mice (Wang et al., 2008). The GFP intensity in $CD4^+$ LATY136F T cells appears to be lower than that in normal Treg cells, suggesting that LAT-mediated signaling is important for maintaining Foxp3 expression during thymocyte development or in the periphery. Additionally, data from our lab show that adoptive transfer of normal Treg cells into these mice can prevent the development of lymphoproliferative disease (Koonpaew et al., 2006). These data suggest that the unchecked expansion of T cells observed in LATY136F mice may also be due to a lack of peripheral tolerance.

The defect in thymocyte selection processes in LATY136F mice is an obstacle in studying the role of the LAT-PLC-γ1 interaction in mature T cells. Here, we used LAT conditional knock-in mice to examine the role of the LAT-PLC-γ1 interaction in the regulation of TCR-mediated signaling, Treg cell function, and T cell homeostasis. We utilized the ERCre transgenic system in which a floxed gene can be deleted upon

tamoxifen treatment. This allowed for the evaluation of mature T cells containing the Y136F-mutated form of LAT that had undergone normal development in the thymus. This chapter reports that the LAT-PLC-γ1 interaction is required for optimal signaling and function in mature T cells as well as the *in vitro* and *in vivo* function of Treg cells.

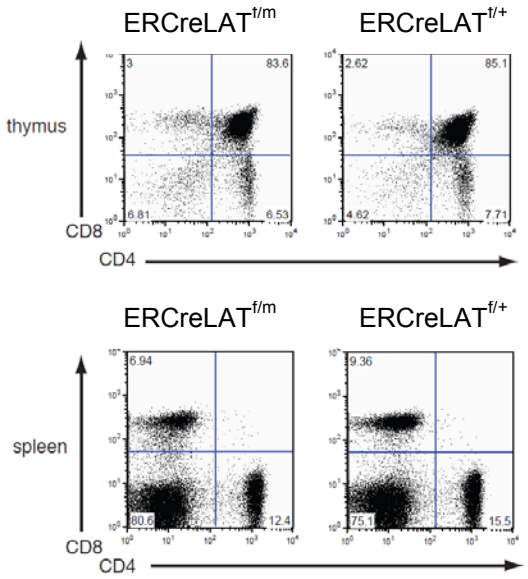
3.2 Results

3.2.1 ERCreLAT$^{f/m}$ T cells in the thymus and periphery

Mice with a mutation at the LAT-PLCγ1 interaction site, LATY136F mice, exhibit a partial block at the DN3 stage during thymocyte development (Aguado et al., 2002; Sommers et al., 2002). Positive and negative selection are also impaired in these mice (Sommers et al., 2005). In order to study the function of the LAT-PLC-γ1 interaction in TCR-mediated signaling and the regulation of T cell homeostasis and autoimmunity, we needed to bypass this developmental block. This was achieved by utilizing LAT knock-in mice previously generated by others in the lab (Shen et al., 2009). In the *lat* gene of these mice, exons 7-11 are flanked by two Loxp sites. Deletion of these exons by Cre leads to the production of non-functional LAT. In addition, cells with the *lat* gene deleted are marked by the expression of GFP. To be able to induce deletion of LAT, we crossed ERCre mice, in which Cre recombinase is fused to a mutated estrogen receptor (ER) ligand-binding domain, with LAT knock-in mice (LAT$^{f/f}$) and LATY136F mice (LAT$^{m/+}$) to generate ERCreLAT$^{f/+}$ and ERCreLAT$^{f/m}$ mice. In these mice, T cells should develop normally before injection of tamoxifen due to the presence of the WT allele.

Figure 3.1 T cell development in untreated ERCreLAT$^{f/m}$ mice
(a) CD4 and CD8 expression on thymocytes (upper panel) and splenocytes (lower panel) from six-week old ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ littermates. (b) TCRβ expression on ERCreLAT$^{f/m}$ (dotted line) and ERCreLAT$^{f/+}$ (solid line) CD4^{+} splenocytes. The shaded area represents TCRβ expression on B cells. Data shown are representative of three mice for each genotype.

(a)


ERCreLAT f/m
ERCreLAT f/+
thymus
3
83.6
6.81
6.53
2.62
85.1
4.62
7.71
CD8
CD4
ERCreLAT f/m
ERCreLAT f/+
spleen
6.94
80.6
12.4
9.36
75.1
15.5
CD8
CD4

(b)

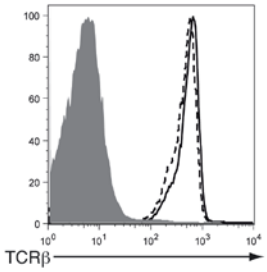

100
80
60
40
20
0
TCRβ

Figure 3.1

Upon treatment with tamoxifen, the floxed *lat* allele is deleted in cells expressing ERCre. As a result, all T cells should express either Y136F (in the case of ERCreLAT$^{f/m}$ mice) or wild-type LAT (in the case of ERCreLAT$^{f/+}$ mice). As shown in the top panel of Figure 3.1a, prior to tamoxifen treatment, both ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice showed normal percentages of DN, DP, and SP T cells in the thymus, indicating that the floxed LAT allele is able to support normal thymocyte development. In addition, the percentages of CD4$^+$ and CD8$^+$ T cells in the spleens of these mice were normal (Figure 3.1a, bottom panel). In LATY136F mice, expression of the TCR on peripheral T cells is very low (Sommers et al., 2002) while in untreated ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice peripheral CD4$^+$ T cells expressed normal surface levels of TCRβ (Figure 3.1b).

In order to delete the floxed *lat* allele, mice were treated with tamoxifen for two consecutive days. At day 4 after tamoxifen injections, the floxed LAT allele was efficiently deleted as indicated by the expression of GFP on over 95% of splenic T cells (Figure 3.2a). Tamoxifen-induced deletion affected thymocyte development in ERCreLAT$^{f/m}$ mice. There was a reduced percentage of DP thymocytes accompanied by an increase in the frequency of DN thymocytes (Figure 3.2b, top panel). The percentages of peripheral CD4$^+$ and CD8$^+$ T cells were similar to those in ERCreLAT$^{f/+}$ mice (Figure 3.2b, bottom panel). Previously, studies from our lab have shown that LATY136F mice lack CD4$^+$CD25$^+$ Treg cells (Koonpaew et al., 2006). At day 4 after tamoxifen injection, the percentage of CD4$^+$Foxp3$^+$ cells in ERCreLAT$^{f/m}$ mice was similar to that in

Figure 3.2 T cell development in tamoxifen-treated ERCreLAT$^{f/m}$ mice
Mice were treated with tamoxifen via intraperitoneal injections on two consecutive days. Four to five days after the first treatment, mice were sacrificed and analyzed. (a) Efficient deletion of LAT after tamoxifen treatment as demonstrated by GFP expression. CD4$^+$ splenocytes were analyzed; the shaded area represents CD4$^+$ T cells from a C57BL/6 mouse. (b) CD4 and CD8 expression on thymocytes (upper panel) and splenocytes (lower panel) from tamoxifen-treated ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ littermates. (c) Expression of CD4 and Foxp3 on GFP$^+$ cells from the lymph nodes of tamoxifen-treated ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ littermates. Data shown are representative of three mice for each genotype.

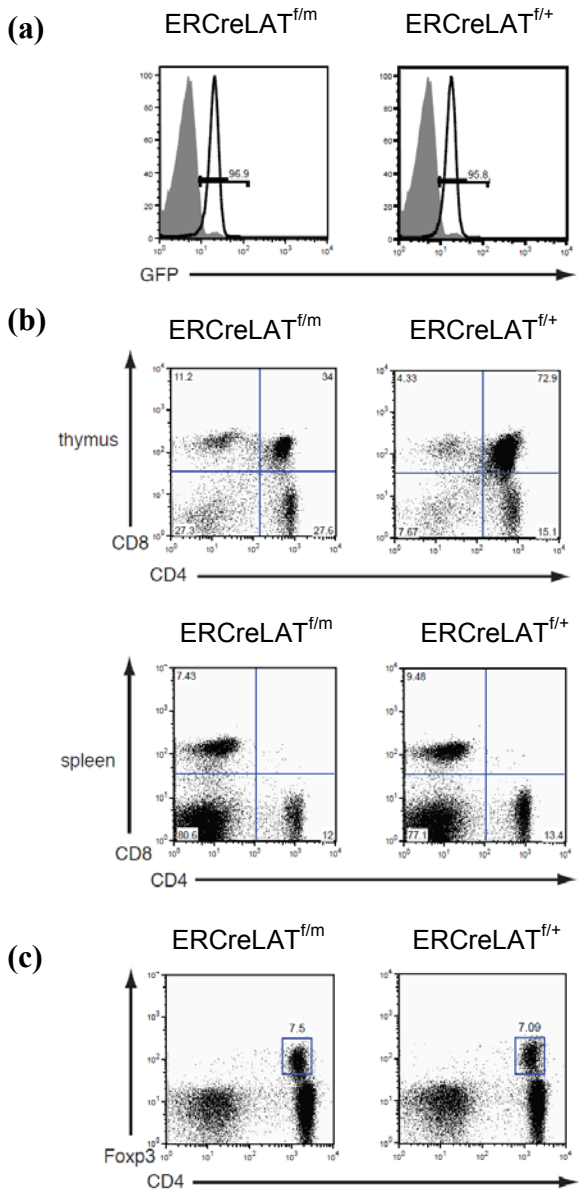

(a)
ERCreLAT f/m
ERCreLAT f/+
96.9
95.8
GFP
(b)
ERCreLAT f/m
ERCreLAT f/+
thymus
11.2
34
27.3
27.6
4.33
72.9
7.67
15.1
CD8
CD4
ERCreLAT f/m
ERCreLAT f/+
spleen
7.43
80.5
12
9.48
77.1
13.4
CD8
CD4
(c)
ERCreLAT f/m
ERCreLAT f/+
7.5
7.09
Foxp3
CD4

Figure 3.2

ERCreLAT$^{f/+}$ mice (Figure 3.2c). Together, these data showed that we have successfully established an experimental system which allows us to delete functional LAT in mature T cells and bypass the developmental block observed in LATY136F mice. These mice will be used to analyze the importance of the LAT-PLC-γ1 interaction in the control of T cell activation and autoimmunity.

3.2.2 Impaired signaling in ERCreLAT$^{f/m}$ T cells

T cells from LATY136F mice exhibit defects in TCR-mediated phosphorylation of LAT and PLCγ1. Consequently, these mutant T cells fail to flux calcium upon TCR engagement (Sommers et al., 2002). These results are similar to previously published data using Jurkat cells (Zhang et al., 2000). However, TCR-mediated Erk activation is surprisingly normal in LATY136F CD4$^+$ T cells. In addition, these T cells express low cell surface levels of TCR (Aguado et al., 2002; Sommers et al., 2002). Consequently, T cells from LATY136F mice are not suitable to study the role of the LAT-PLC-γ1 interaction in primary T cells. Our experimental model allows us to bypass this obstacle.

In order to prepare a large number of cells for biochemical analysis, we deleted the floxed allele *in vitro*. To this end, splenocytes from ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice were activated with anti-CD3 for 2 days and then cultured in IL-2 medium in the presence of hydroxyltamoxifen for 3-4 days. These T cells were rested for 6 hours in medium without IL-2 and used for analysis of TCR-mediated signaling. As shown in Figure 3.3a, surface expression of the TCR on ERCreLAT$^{f/m}$ T cells was comparable to

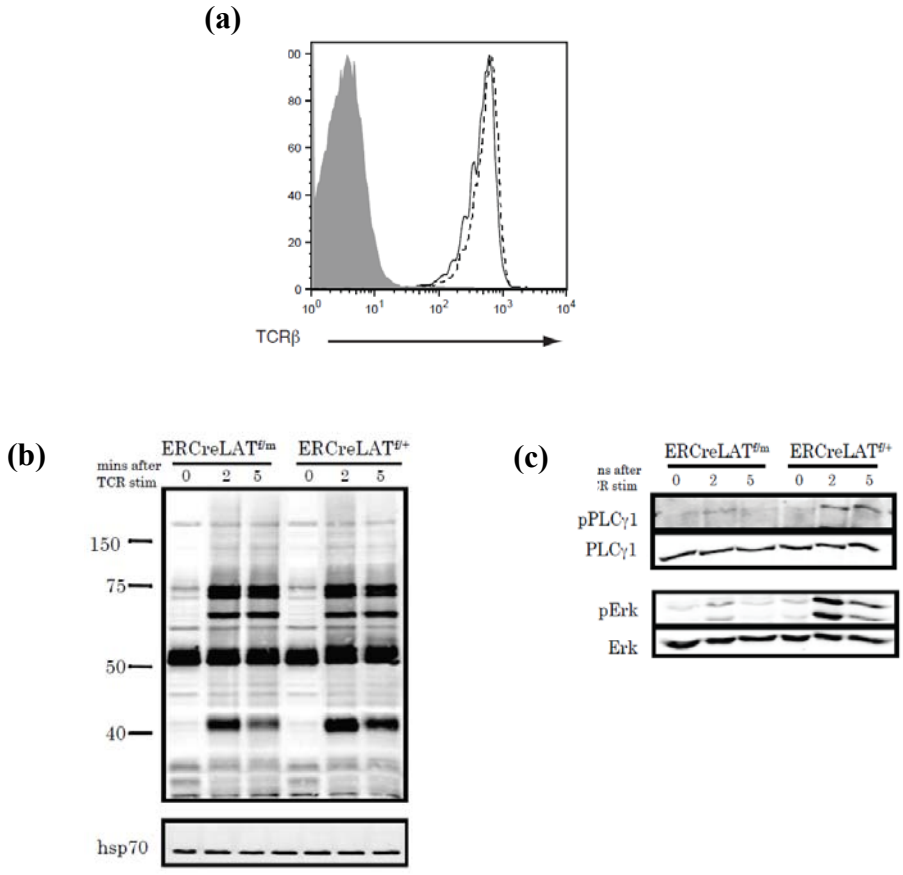


Figure 3.3 Decreased TCR-mediated phosphorylation of proteins in mature ERCreLAT$^{f/m}$ T cells

(a) TCRβ expression on ERCreLAT$^{f/m}$ (solid line) and ERCreLAT$^{f/+}$ (dotted line) in GFP^{+}CD4^{+} lymph node cells four to five days after tamoxifen injections. The shaded area represents TCRβ expression on B cells. Data shown are representative of 3 experiments using 2 mice of each genotype for each experiment. (b) Western blot analysis showing total protein tyrosine phosphorylation in ERCreLAT$^{f/m}$ and ERCreLAT$^{f/m}$ activated T cells after 4-OHT treatment and anti-CD3 stimulation. (c) Western blots showing phosphorylation of PLC-γ1 and Erk1/2. Data shown are representative of three independent experiments.

that on T cells from ERCreLAT$^{f/+}$ mice. While overall tyrosine phosphorylation of proteins was seemingly unaffected by the Y136F mutation (Figure 3.3b), TCR-mediated PLC-γ1 phosphorylation and Erk activation were significantly decreased in ERCreLAT$^{f/m}$ T cells when compared to the ERCreLAT$^{f/+}$ control (Figure 3.3c). We next analyzed TCR-mediated calcium mobilization in these cells. In contrast to published data showing that LATY136F T cells fail to mobilize calcium (Sommers et al., 2002), ERCreLAT$^{f/m}$ T cells were able to flux some calcium, albeit reduced when compared with ERCreLAT$^{f/+}$ T cells (Figure 3.4). Stimulation with ionomycin, a Ca^{2+} ionophore that bypasses TCR proximal signaling, induced similar levels of calcium flux between the two experimental groups. We next investigated the ability of ERCreLAT$^{f/m}$ CD4$^+$ T cells to produce IL-2. TCR-mediated IL-2 production was impaired in ERCreLAT$^{f/m}$ CD4$^+$ T cells as shown in Figure 3.5a. In addition, TCR-mediated proliferation, assayed by [^{3}H]thymidine incorporation, was impaired in ERCreLAT$^{f/m}$ CD4$^+$ T cells while PMA and ionomycin-induced proliferation was similar (Figure 3.5b). Together, these results suggested that the LAT-PLC-γ1 interaction plays an important role in TCR-mediated PLC-γ1 activation, calcium mobilization, Erk phosphorylation, IL-2 production, and cell proliferation.

3.2.3 Development of the LATY136F phenotype in ERCreLAT$^{f/m}$ mice

LATY136F mice develop an autoimmune syndrome characterized by splenomegaly, lymphadenopathy, eosinophilia, and infiltration of lymphocytes into various tissues (Aguado et al., 2002; Sommers et al., 2002). CD4$^+$ T cells from these mice

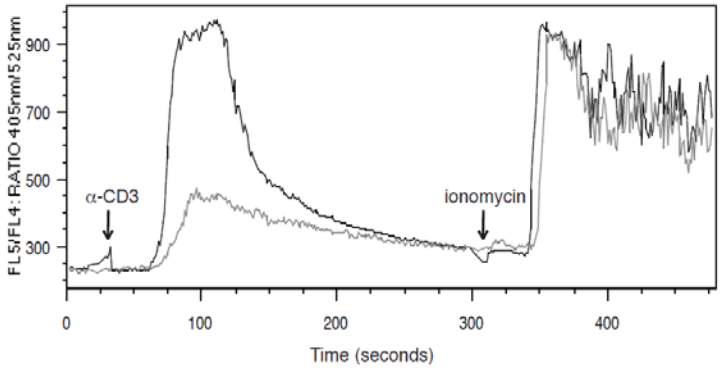


Figure 3.4 Impaired calcium flux in mature ERCreLAT$^{f/m}$ T cells
Calcium flux in ERCreLAT$^{f/m}$ (gray line) and ERCreLAT$^{f/+}$ (black line) GFP^{+}CD4^{+} cells in response to TCR stimulation four to five days after mice were injected with tamoxifen. α-CD3 antibody was added at 15 seconds while ionomycin was added at 5 minutes. Data shown are representative of four independent experiments.

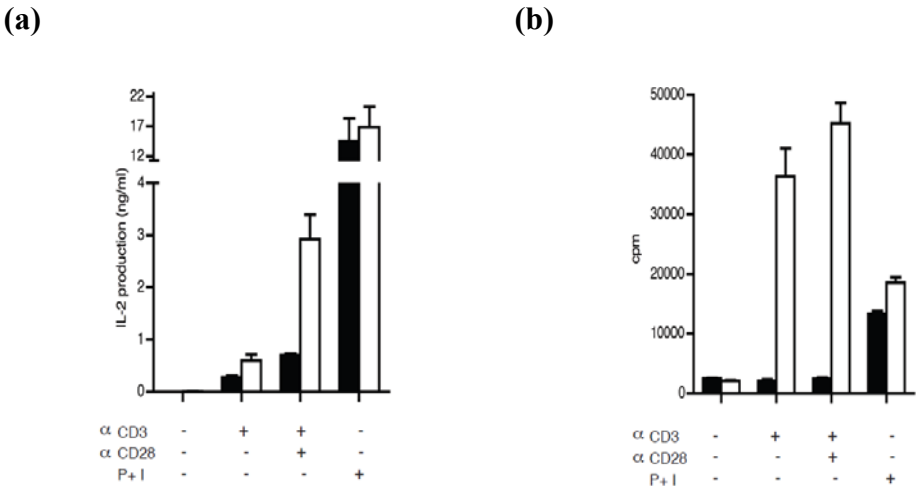


Figure 3.5 Defective function of mature ERCreLAT$^{f/m}$ CD4^{+} T cells
Mice were treated with tamoxifen for four to five days. CD4^{+} T cells were purified and analysed. (a) IL-2 production in ERCreLAT$^{f/m}$ (filled bars) and ERCreLAT$^{f/+}$ (empty bars) CD4^{+} cells before and after stimulation. (b) Proliferation in ERCreLAT$^{f/m}$ (filled bars) and ERCreLAT$^{f/+}$ (empty bars) CD4^{+} cells before and after stimulation. Each figure is representative of 4 independent experiments. Error bars on bar graphs represent the standard error of the mean of triplicates done in an independent experiment.

undergo uncontrolled expansion and produce a copious amount of Th2 cytokines. As a result, B cells are also activated and undergo isotype-switching, producing high levels of IgG_1 and IgE. Since we planned to study the LAT-PLCγ1 interaction in the control of autoimmunity, we first investigated whether ERCreLAT$^{f/m}$ mice could be induced to develop a disease similar to that observed in LATY136F mice. ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice were treated with tamoxifen once a week for four weeks to induce and maintain deletion of the floxed allele. ERCreLAT$^{f/m}$ mice exhibited a similar phenotype to what is observed in LATY136F mice, as evidenced by enlarged lymph nodes and spleens (Figure 3.6a) both of which contained an increased number of B220$^+$ and CD4$^+$ cells in the periphery. The numbers of CD4$^+$ and B220$^+$ cells were increased by approximately two fold while the number of CD8$^+$ cells was significantly decreased (Figure 3.6b).

Long-term treatment with tamoxifen induced an early block in thymocyte development in ERCreLAT$^{f/m}$ mice, as indicated by a drastically reduced percentage of DP thymocytes and an accumulation of DN cells (Figure 3.7a). The percentage of DN3 thymocytes (CD25$^+$CD44$^-$) was also increased in ERCreLAT$^{f/m}$ mice (Figure 3.7b). This result indicated that tamoxifen-induced deletion of LAT could occur in early progenitor cells. This block in thymocyte development is similar to that observed in LATY136F mice. In the periphery, the ratio of CD4$^+$ to CD8$^+$ T cells was dramatically increased in ERCreLAT$^{f/m}$ mice (approximately 15:1) compared to control mice (approximately 0.7:1)

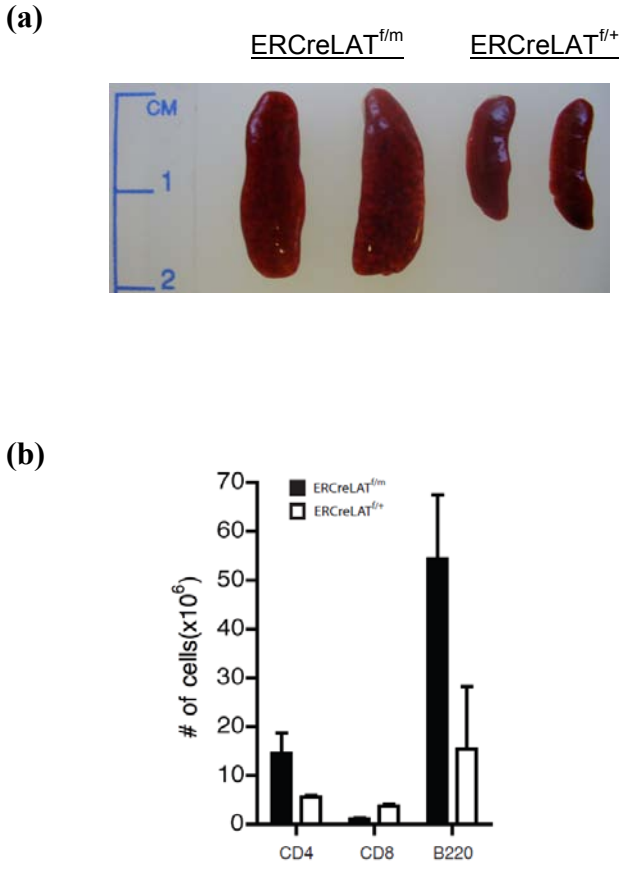


Figure 3.6 Increased lymphocyte numbers in ERCreLAT$^{f/m}$ mice after four weeks of tamoxifen treatment

ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ littermates were treated with tamoxifen on two consecutive days followed by weekly tamoxifen injections for four weeks. (a) Picture of spleens and lymph nodes from ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ after four weeks of tamoxifen treatment. (b) Total numbers of CD4$^+$, CD8$^+$, and B220$^+$ cells in the spleens of ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice. The figure shown is one representative of 5 mice analyzed. Error bars on bar graphs represent the standard error of the mean of three mice analyzed in an independent experiment.

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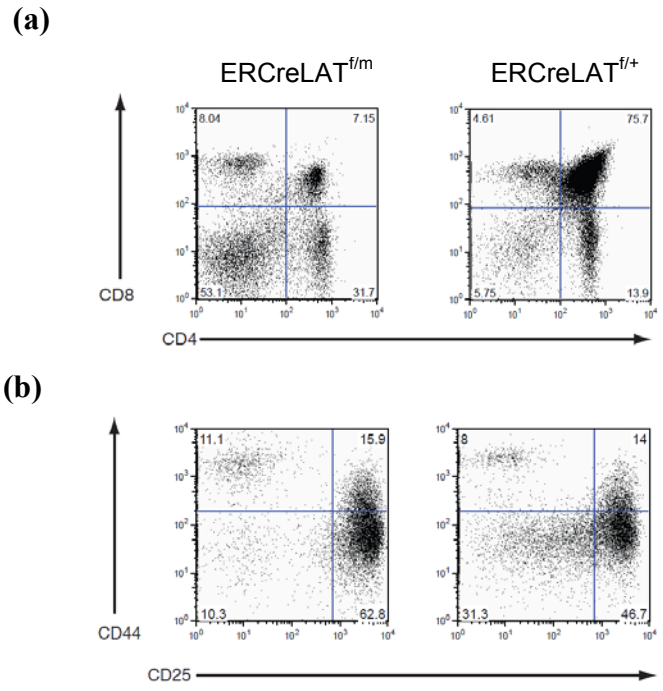


Figure 3.7 Defective thymocyte development in ERCreLAT$^{f/m}$ mice after four weeks of tamoxifen treatment

Mice were treated with tamoxifen for 4 weeks on a weekly basis. (a) CD4 and CD8 expression on GFP^{+} thymocytes from ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice. (b) CD25 and CD44 expression profile on DN thymocytes. Figures shown are representative of 3 mice of each genotype.

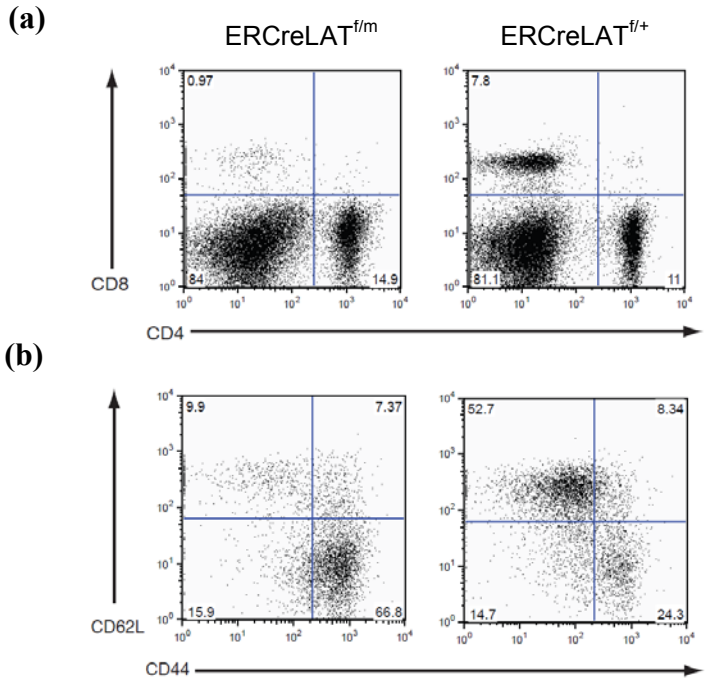


Figure 3.8 Increased CD4⁺ T cell activation in ERCreLAT$^{f/m}$ mice after four weeks of tamoxifen treatment

Tamoxifen was injected into mice via intraperitoneal injections for four weeks. (a) CD4 vs CD8 expression in the spleens of ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice. (b) CD62L vs CD44 surface expression on GFP⁺CD4⁺ splenocytes. Figures shown are representative of five mice from each genotype.

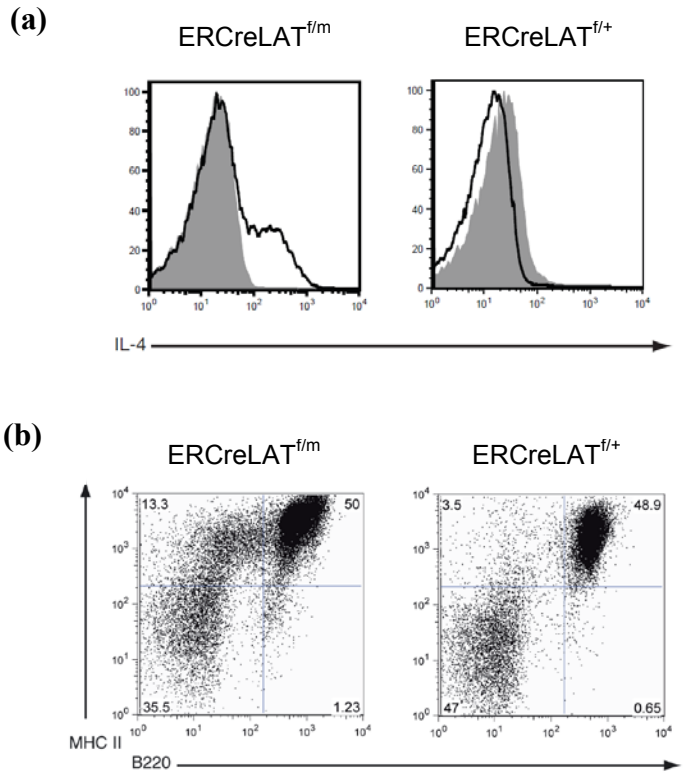


Figure 3.9 Increased activation of B cells in ERCreLAT$^{f/m}$ T cells after four weeks of tamoxifen treatment

Mice were injected with tamoxifen weekly for 4 weeks. (a) Intracellular IL-4 expression in ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ GFP^{+}CD4^{+} splenocytes after P+I stimulation. The shaded area represents unstimulated controls. (b) Expression of B220 vs MHC Class II on splenocytes. Figures shown are representative of five mice from each genotype.

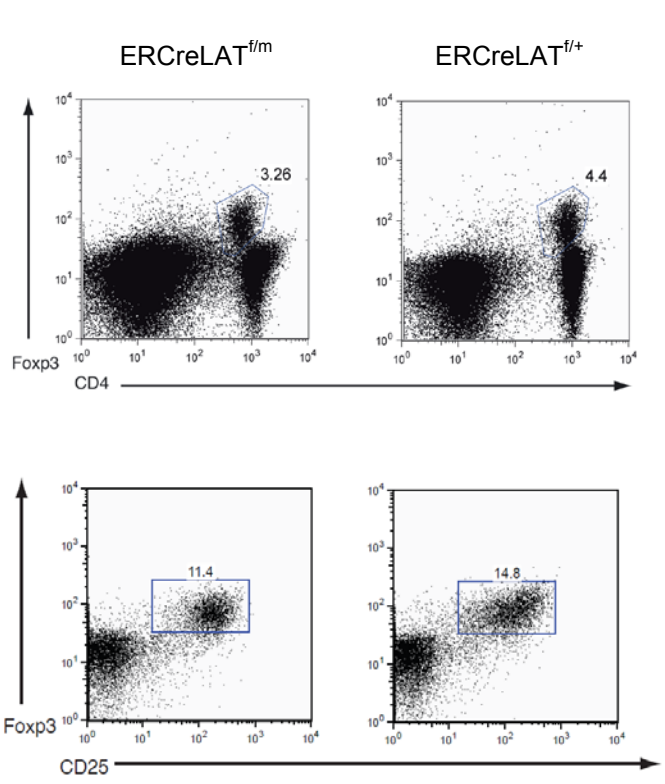


Figure 3.10 Survival of ERCreLAT$^{f/m}$ regulatory T cells after 4 weeks of tamoxifen treatment

Mice were treated with tamoxifen weekly for a total of four weeks. (a) CD4 vs Foxp3 expression in lymph node cells from ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice. (b) Surface CD25 and intracellular Foxp3 expression in GFP^{+}CD4^{+} lymph node cells. FACS plots shown are representative of five mice per genotype.

(Figure 3.8a). Most of those $CD4^+$ T cells were $CD44^+CD62L^{lo/-}$, a phenotype of activated/memory T cells (Figure 3.8b). Additionally, these $CD4^+$ T cells produced a high level of intracellular IL-4 (Figure 3.9a). B cells were hyperactivated, as indicated by a high expression level of MHC Class II (Figure 3.9b).

In LATY136F mice, $CD4^+CD25^+$ Treg cells are absent in the periphery (Koonpaew et al., 2006). Interestingly, in $ERCreLAT^{f/m}$ mice treated with tamoxifen, $CD4^+CD25^+Foxp3^+$ cells were present and the percentage of these cells was similar to that in $ERCreLAT^{f/+}$ mice (Figure 3.10). This result suggested that these regulatory T cells are not able to suppress the expansion of $CD4^+$ effector cells. Collectively, these data indicated that the LATY136F phenotype can be recapitulated in tamoxifen-treated $ERCreLAT^{f/m}$ mice. Moreover, the LAT-PLC-γ1 interaction might not be important in the survival of Treg cells in the periphery.

3.2.4 Increased numbers of dendritic cells in $ERCreLAT^{f/m}$ mice

$ERCreLAT^{f/m}$ mice treated with tamoxifen for 4 weeks developed a lymphoproliferative disease characterized by the expansion of CD4 T cells and the hyperactivation of both T and B lymphocytes. While B cells represent one type of antigen presenting cells, dendritic cells are even more efficient at activating T cells due to their constitutive expression of MHC Class II and costimulatory molecules as well as their ability to activate naïve T cells without first being activated themselves. We next sought to characterize dendritic cells in the periphery of $ERCreLAT^{f/m}$ mice.

Tamoxifen was administered to ERCreLAT$^{f/m}$ and control mice for 4 weeks as described earlier. In the spleen, approximately 1.6% of cells in ERCreLAT$^{f/m}$ mice expressed high levels of CD11c and MHC Class II compared to just 0.4% in ERCreLAT$^{f/+}$ control mice (Figure 3.11a). Furthermore, more than 8% of splenocytes expressed high levels of MHC Class II and intermediate levels of CD11c; this is a drastic increase compared to control mice in which less than 0.5% of splenocytes were CD11c^{int}MHCII^{hi}. Overall, the number of dendritic cells (CD11c^{+}MHCII^{+}) was dramatically increased in ERCreLAT$^{f/m}$ mice compared to control mice by more than 25-fold (Figure 3.11b).

DCs can be divided into multiple subsets based on their surface markers, including CD8^{+} (conventional DCs), CD11b^{+} (myeloid DCs), and B220^{+} (plasmacytoid DCs). We next investigated which of these subsets was increased in ERCreLAT$^{f/m}$ mice. In mutant and control mice, the total number of plasmacytoid DCs was higher than the other two subsets (Figure 3.11c). Moreover, while both conventional and myeloid DCs were approximately five-fold higher in ERCreLAT$^{f/m}$ mice compared to control mice, ERCreLAT$^{f/m}$ plasmacytoid DCs were approximately fifty-fold higher than controls. Additionally, all subsets of DCs that we investigated expressed high levels of CD80, CD86, and CD40, molecules that are upregulated on the surfaces of activated DCs (Figure 3.12). In summary, these data demonstrate that there is an increase in the total number of ERCreLAT$^{f/m}$ DCs compared to controls; furthermore, these cells are highly activated. It is possible that dendritic cells, particularly plasmacytoid DCs may play a

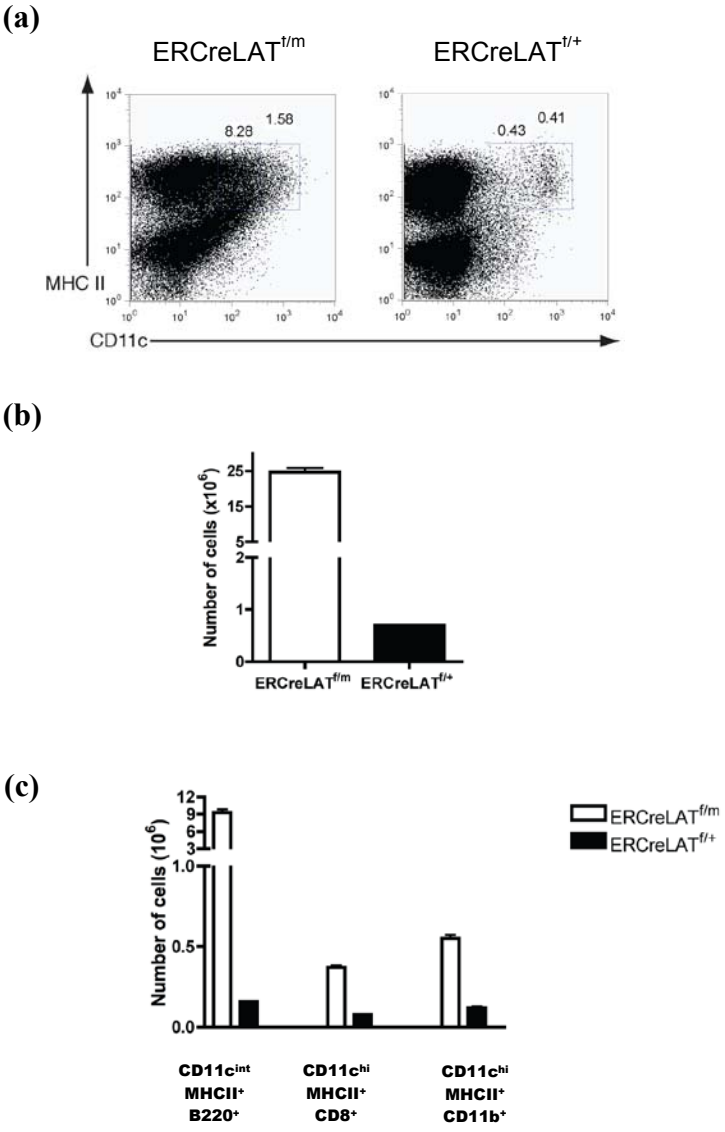


Figure 3.11 Increased number of dendritic cells in ERCreLAT$^{f/m}$ mice
ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice were treated with tamoxifen weekly for 4 weeks. (a) CD11c vs MHC Class II expression in splenocytes. Numbers on plots represent percentages of gated populations. (b) Number of CD11c^{+}MHCII^{+} cells, including both CD11c^{int} and CD11c^{hi} cells. (c) Total number of different subsets of dendritic cells in the spleen. Data shown are representative of three independent experiments. Error bars on bar graphs represent the standard error of the mean of three mice analyzed in an independent experiment.

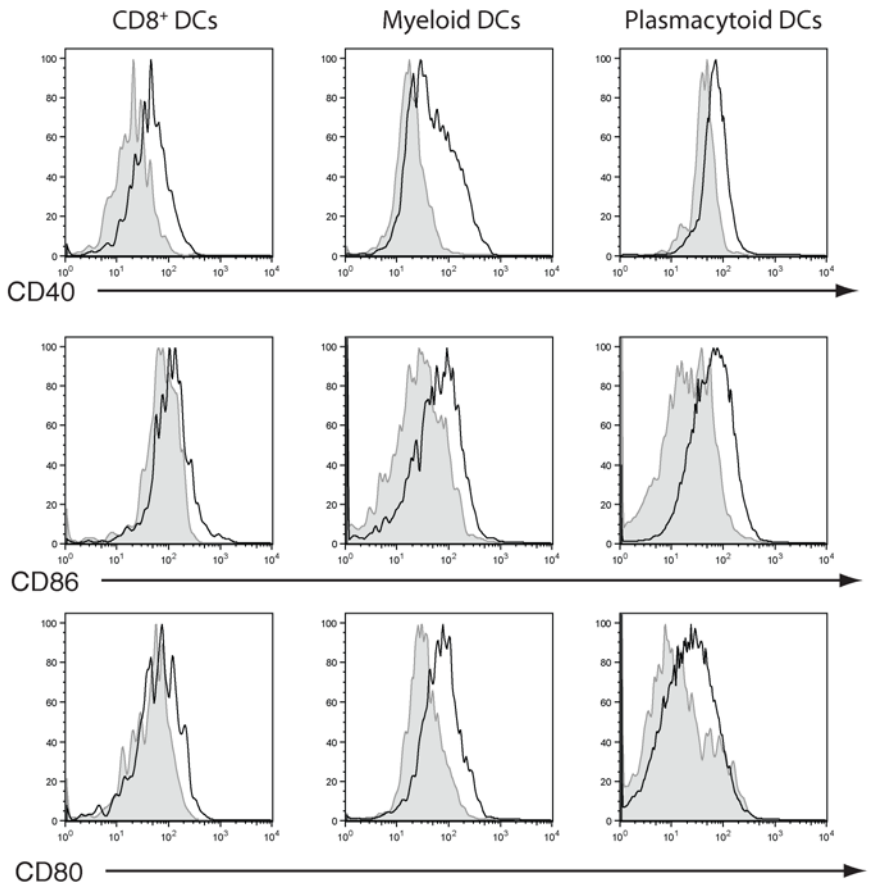


Figure 3.12 Increased expression of activation markers on ERCreLAT$^{f/m}$ dendritic cells

Mice were injected weekly with tamoxifen for 4 weeks. ERCreLAT$^{f/m}$ (black line) and ERCreLAT$^{f/+}$ (gray shaded region) splenocytes were analyzed and gated as follows: CD8^{+} DCs as CD11c^{hi}MHCII^{+}CD8^{+}; myeloid DCs as CD11c^{hi}MHCII^{+}CD11b^{+}; plasmacytoid DCs as CD11c^{int}MHCII^{+}B220^{+}. Data shown are representative of two independent experiments.

role in mediating the lymphoproliferative disease observed in ERCreLAT$^{f/m}$ mice.

3.2.5 Reconstitution of LAT$^{-/-}$ mice with ERCreLAT$^{f/m}$ T cells

The data shown in section 3.2.3 demonstrates that deletion of the floxed allele in ERCreLAT$^{f/m}$ mice led to the development of an autoimmune syndrome similar to that in LATY136F mice. However, thymic selection may still be affected in ERCreLAT$^{f/m}$ mice after 4 weeks of tamoxifen treatment, allowing for the escape of autoreactive T cells into the periphery. To determine whether impaired thymic selection is the cause for autoimmunity, we established a system in which LAT$^{-/-}$ mice were reconstituted with untreated mature ERCreLAT$^{f/m}$ or ERCreLAT$^{f/+}$ T cells. Approximately 20x10^6 T cells were adoptively transferred into LAT$^{-/-}$ mice via tail vein injection. We waited about 5 weeks to allow expansion of these T cells by homeostatic proliferation prior to tamoxifen treatment. To ensure complete deletion of the floxed allele, we injected tamoxifen once a week for four weeks before analysis. As shown in Figure 3.13a, mice reconstituted with ERCreLAT$^{f/m}$ T cells developed splenomegaly after tamoxifen treatment. Lymph nodes were also enlarged in these mice. These enlarged lymphoid organs were partially due to the expansion of CD4$^+$ T cells present in the mice reconstituted with ERCreLAT$^{f/m}$ cells (Figure 3.13b and 3.13c), whereas the total number of CD8$^+$ T cells was not significantly affected by the Y136F mutation. Mice reconstituted with ERCreLAT$^{f/+}$ T cells and treated with tamoxifen had spleens and lymph nodes similar in size to those of B6 mice (Figure 3.13a). Analysis of ERCreLAT$^{f/m}$ CD4$^+$ T cells showed that these cells also downregulated

TCRβ on their cell surfaces like LATY136F T cells (Figure 3.13d), suggesting that LAT-mediated signaling is required for maintaining cell surface expression of the TCR. These data suggested that mature $CD4^+$ T cells expressing the LATY136F mutant are hyperproliferative compared with T cells expressing wildtype LAT.

We next investigated whether these hyperactivated T cells also produce a large amount of Th2 cytokines. Splenocytes from mice reconstituted with $ERCreLAT^{f/m}$ or $ERCreLAT^{f/+}$ T cells were stimulated with PMA and ionomycin for 5 hours and the production of cytokines in these cells was analyzed by intracellular staining. As shown in Figure 3.14, $ERCreLAT^{f/+}$ and $ERCreLAT^{f/m}$ $CD4^+$ T cells produced similar levels of IFNγ and IL-2; however, $ERCreLAT^{f/m}$ $CD4^+$ T cells produced much more IL-4. More than 70% of the $ERCreLAT^{f/m}$ $CD4^+$ T cells produced IL-4 compared to approximately 3% of the control cells (Figure 3.14, bottom panel). B cells were also highly activated in the mice reconstituted with $ERCreLAT^{f/m}$ T cells as many of the B cells had downregulated B220 and upregulated MHC Class II molecules (Figure 3.15, top panel). In addition, almost one third of the $B220^+$ cells expressed low levels of surface IgD and IgM (Figure 3.15a, bottom panel), indicating that many of these B cells had undergone maturation and isotype switching. In accordance with the above results, the serum concentrations of IgE and IgG_1, but not IgM, were elevated in mice reconstituted with $ERCreLAT^{f/m}$ T cells (Figure 3.15b). These results demonstrated that, despite undergoing normal thymic

Figure 3.13 Increased numbers of donor ERCreLAT$^{f/m}$ T cells in LAT$^{-/-}$ recipients after 4 weeks of tamoxifen treatment

LAT$^{-/-}$ mice were reconstituted with ERCreLAT$^{f/m}$ or ERCreLAT$^{f/+}$ T cells and treated with tamoxifen for 4-5 weeks. (a) Pictures of spleens and lymph nodes from LAT$^{-/-}$ mice reconstituted with ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ cells. C57BL/6 mice were also used as controls. (b) Total numbers of CD4^{+}, CD8^{+}, and B220^{+} cells in the spleens of tamoxifen treated LAT$^{-/-}$ recipients reconstituted with ERCreLAT$^{f/m}$ (filled bars) or ERCreLAT$^{f/+}$ T cells (empty bars). Error bars on bar graphs represent the standard error of the mean of three mice analyzed in an independent experiment. (c) CD4 and CD8 expression on splenocytes. (d) TCRβ expression on GFP^{+} CD4^{+} cells in LAT$^{-/-}$ recipients that received cells from either ERCreLAT$^{f/m}$ (solid line) or ERCreLAT$^{f/+}$ (dotted line) mice. The shaded area represents a negative control. 3 mice of each genotype were analyzed. Data shown is representative of 4 such experiments.

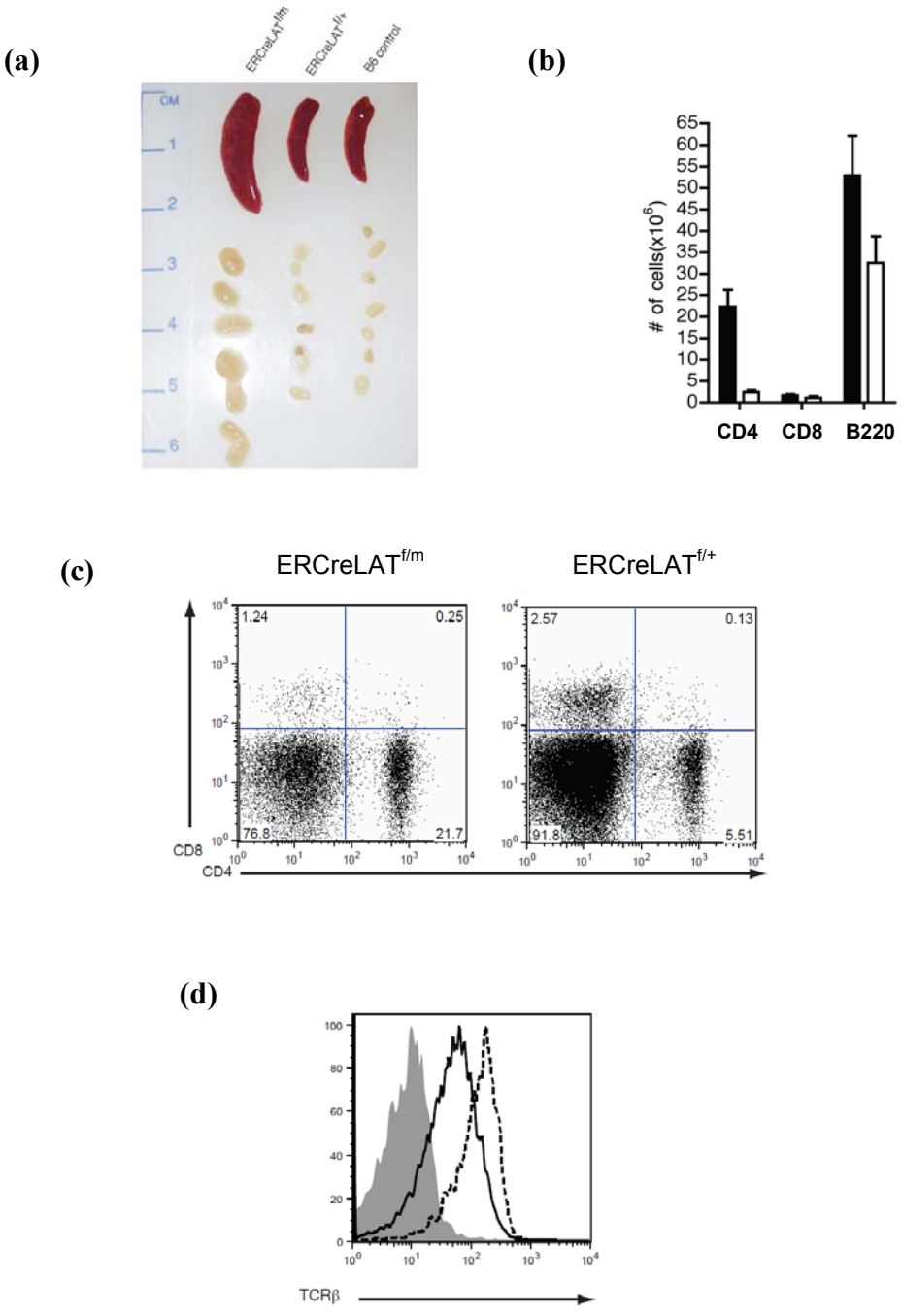

(a)
ERCreLAT f/m
ERCreLAT f/+
B6 control
CM
1
2
3
4
5
6
(b)
# of cells(x10^6)
65
60
55
50
45
40
35
30
25
20
15
10
5
0
CD4
CD8
B220
(c)
ERCreLAT f/m
ERCreLAT f/+
1.24
0.25
76.8
21.7
2.57
0.13
91.8
5.51
CD8
CD4
(d)
TCRβ

Figure 3.13

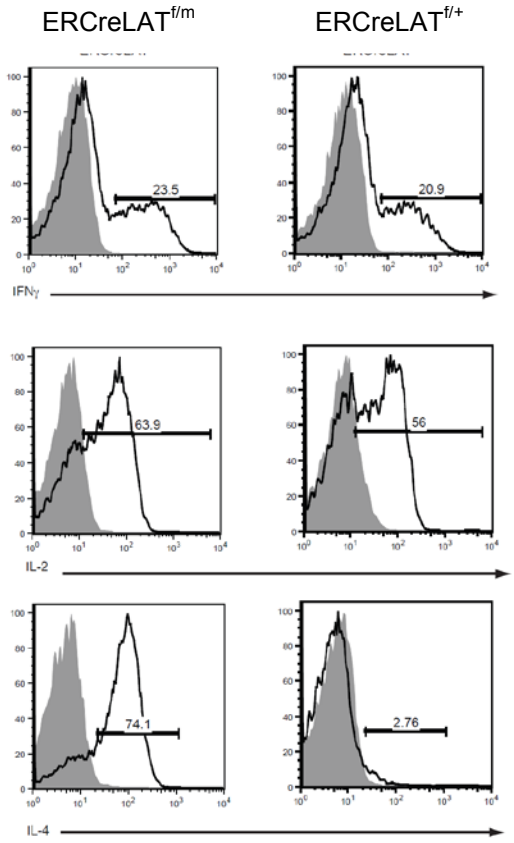


Figure 3.14 Increased IL-4 expression in ERCreLAT$^{f/m}$ donor T cells after 4 weeks of tamoxifen injections

LAT$^{-/-}$ mice were reconstituted with ERCreLAT$^{f.m}$ or ERCreLAT$^{f/+}$ T cells and treated with tamoxifen for 4 weeks. IFN-γ, IL-2 and IL-4 production in GFP^{+}CD4^{+} cells after P+I stimulation. Unstimulated controls (shaded area) and stimulated cells (solid line) are depicted. 3 mice of each genotype were analyzed. Data shown is representative of 4 such experiments.

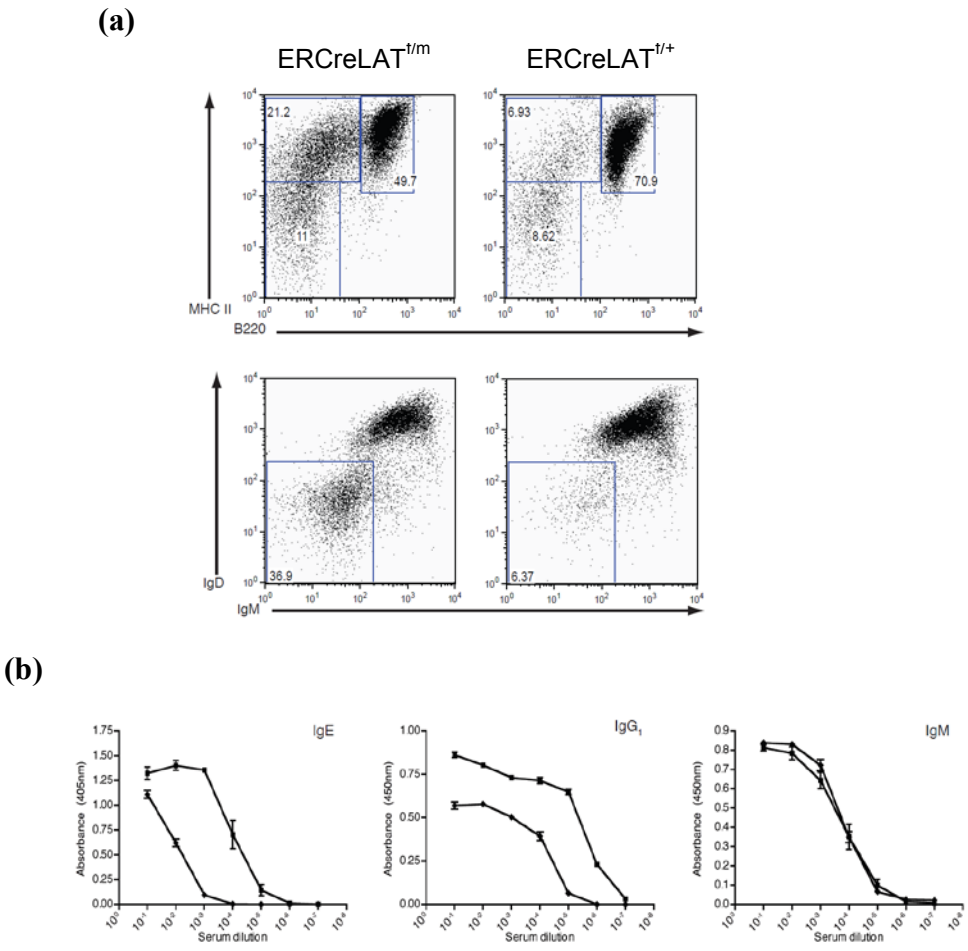


Figure 3.15 Activation of B cells after four weeks of tamoxifen treatment in LAT$^{-/-}$ mice reconstituted with ERCreLAT$^{f/m}$ T cells

(a) B220 and MHC Class II expression on lymph node cells (top panel). IgD and IgM expression on B220^{+} cells (bottom panel). (b) Blood serum levels of IgE, IgG$_1$, and IgM from LAT$^{-/-}$ recipients injected with T cells from ERCreLAT$^{f/m}$ (squares) and ERCreLAT$^{f/+}$ (diamonds) mice. 3 mice of each genotype were analyzed. Data shown is representative of 3 such experiments.

selection, ERCreLAT$^{f/m}$ CD4 T cells still exhibited uncontrolled expansion and produced high levels of IL-4.

3.2.6 Rescue of the autoimmune-like syndrome

The uncontrolled proliferation of conventional (CD4$^+$CD25$^-$) T cells in LATY136F mice occurs independently of signals transduced via MHC Class II binding to the TCR (Wang et al., 2008). This intrinsic defect has raised questions regarding whether these cells are able to be effectively suppressed *in vivo*. While one study has suggested that these cells cannot be suppressed by wildtype regulatory T cells, Koonpaew et al. demonstrated that CD4$^+$CD25$^+$ T cells adoptively transferred into LATY136F neonates are able to rescue these mice from the lymphoproliferative syndrome. The latter study implicates a role for Treg cells in the development of this phenotype. In order to resolve whether conventional T cells with mutated LATY136F can indeed be suppressed, we reconstituted LAT$^{-/-}$ mice with untreated Thy1.1$^+$ERCreLAT$^{f/+}$ and Thy1.1$^-$ERCreLAT$^{f/m}$ T cells mixed at a one to one ratio. We allowed 5 weeks for efficient reconstitution, followed by weekly treatments with tamoxifen for 4 weeks. Spleen and lymph nodes from recipient mice reconstituted with mixed cells were similar in size to the ERCreLAT$^{f/+}$ control; spleens from both of these mice were about half the size of the spleen from the ERCreLAT$^{f/m}$ control (Figure 3.16a). Accordingly, there was no evidence of uncontrolled proliferation of CD4$^+$ T cells as both CD4$^+$ and CD8$^+$ T cell numbers were relatively normal in LAT$^{-/-}$ mice receiving mixed cells (Figure 3.16b). Moreover,

ERCreLAT$^{f/m}$ CD4^{+} T cells from these mice did not produce high levels of IL-4 in response to P+I stimulation. This is in contrast to the recipient mice reconstituted with only ERCreLAT$^{f/m}$ T cells, the majority of which produced elevated levels of IL-4 in response to stimulation (Figure 3.17a). As a result of high levels of this Th2 cytokine, B cells become activated. As demonstrated in Figure 3.17b, B220^{+} cells from mice receiving ERCreLAT$^{f/m}$ T cells expressed high levels of surface MHC Class II. However, when ERCreLAT$^{f/m}$ T cells were administered along with ERCreLAT$^{f/+}$ T cells, B cells remained in the resting state, similar to B cells from mice receiving only ERCreLAT$^{f/+}$ T cells. These data cumulatively indicate that the hyperactivation and uncontrolled expansion of CD4 T cells harboring the Y136F mutation can indeed be suppressed, presumably by wildtype Treg cells that were present within the transferred wildtype T cells.

3.2.7 Defective ERCreLAT$^{f/m}$ regulatory T cell function

Our previous data show that CD4^{+}CD25^{+} Treg cells are largely absent in LATY136F mice. Moreover, adoptive transfer of WT Treg cells into LATY136F mice suppresses the development of the autoimmune syndrome in these mice, suggesting that the absence of these Treg cells is the cause of this disease (Koonpaew et al., 2006). However, LAT$^{-/-}$ mice that received ERCreLAT$^{f/m}$ T cells and underwent tamoxifen treatment still exhibited signs of autoimmunity, as evidenced by hyperactivation of T and B cells, despite the fact that Treg cells are present in ERCreLAT$^{f/m}$ mice. Moreover,

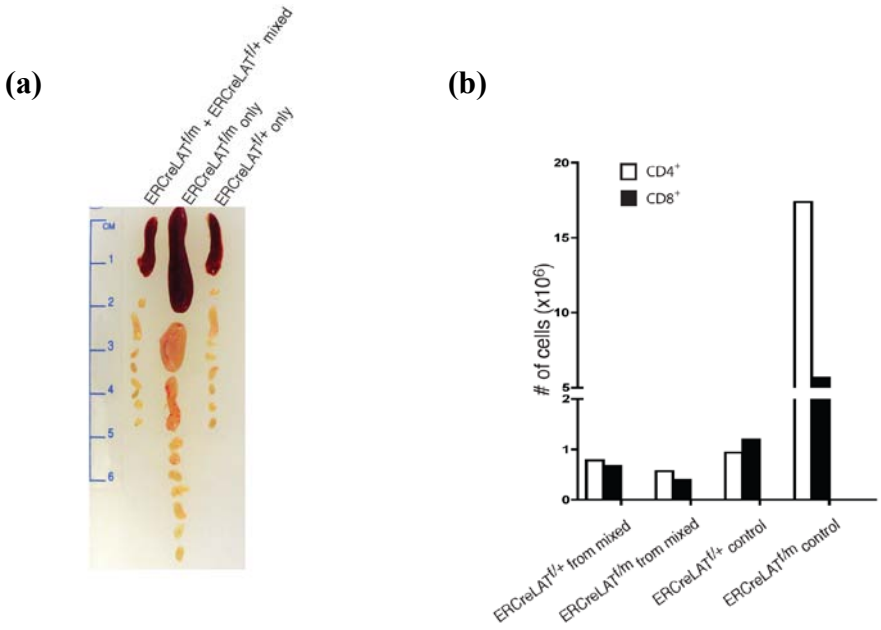
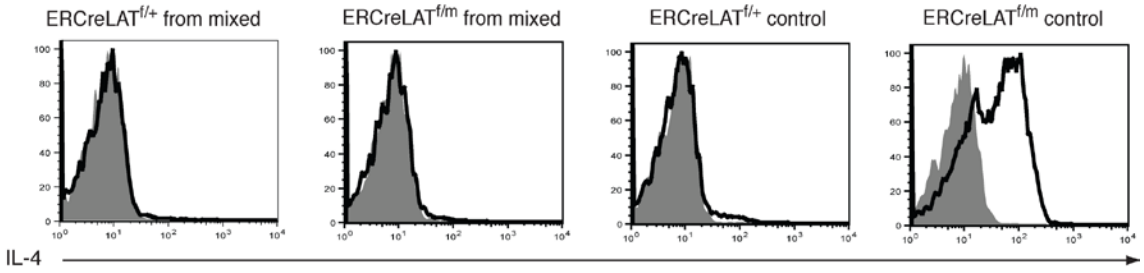


Figure 3.16 The absence of ERCreLAT$^{f/m}$ T cell expansion in the presence of ERCreLAT$^{f/+}$ T cells *in vivo*

Thy1.1^{-} ERCreLAT$^{f/m}$ T cells were mixed with Thy1.1^{+} ERCreLAT$^{f/+}$ T cells at a ratio of 1:1 and reconstituted into LAT$^{-/-}$ mice. After 5 weeks, these mice were treated with tamoxifen for 4 weeks. (**a**) Spleens and lymph nodes from LAT$^{-/-}$ mice receiving ERCreLAT$^{f/m}$ cells, ERCreLAT$^{f/+}$ cells, or both. (**b**) Total numbers of GFP^{+} cells, CD4^{+}, and CD8^{+}. These data are representative of 2 independent experiments using 3 mice per group for each experiment.

(a)



(b)

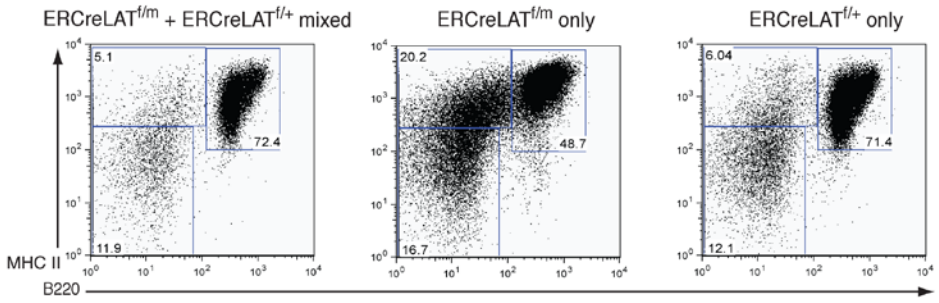


Figure 3.17 A lack of Th2 skewing and B cell activation in the presence of ERCreLAT$^{f/=}$ T cells *in vivo*

LAT$^{-/-}$ mice were reconstituted with Thy1.1^{-} ERCreLAT$^{f/m}$ T cells mixed with Thy1.1^{+} ERCreLAT$^{f/+}$ T cells at a ratio of 1:1. After 5 weeks, these mice were treated with tamoxifen for 4 weeks. (**a**) Expression of IL-4 on CD4^{+}GFP^{+} splenocytes after 5 hour stimulation with P+I. (**b**) MHC Class II and B220 expression on cells from the lymph nodes of reconstituted mice. These data are representative of 2 independent experiments using 3 mice per group for each experiment.

our data in Section 3.2.6 demonstrates that the expansion and hyper-activation of ERCreLAT$^{f/m}$ CD4^{+} T cells can be suppressed by co-transfer with T cells from "wildtype" mice, suggesting that a subset of wildtype T cells is capable of suppressing the LATY136F phenotype. From these data, we hypothesize that regulatory T cells from ERCreLAT$^{f/m}$ mice are nonfunctional.

As shown in Figure 3.18a (upper panel), a similar percentage of Treg cells was present in ERCreLAT$^{f/m}$ mice 4-5 days after tamoxifen treatment. Four weeks after deletion of LAT, about 6% of GFP^{+}CD4^{+} T cells in the LAT$^{-/-}$ mice that received ERCreLAT$^{f/m}$ T cells were CD25^{+}Foxp3^{+} regulatory T cells, as compared to 18% in mice reconstituted with ERCreLAT$^{f/+}$ T cells (Figure 3.18b). However, the total number of regulatory T cells in the lymph nodes of mice reconstituted with ERCreLAT$^{f/m}$ T cells was approximately three fold more than those in mice reconstituted with ERCreLAT$^{f/+}$ T cells (Figure 3.18c). There was also an increase in the number of Treg cells in the spleen. These data suggested that the LAT-PLCγ1 interaction is not required for Treg cell survival; however, it might be important for the function of Treg cells to suppress the proliferation of conventional T cells and to control autoimmunity.

To examine the function of ERCreLAT$^{f/m}$ Treg cells, CD4^{+}CD25^{+} T cells were purified from ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice 5 days after treatment with tamoxifen and used in the assay for Treg cell function. Wildtype Thy1.1^{+}CD4^{+}CD25^{-} conventional T cells were used as responder cells. As shown in Figure 3.19, anti-CD3-induced

Figure 3.18 Survival of ERCreLAT$^{f/m}$ regulatory T cells in LAT$^{-/-}$ recipients after four weeks of tamoxifen treatment

(a-b) CD25 and Foxp3 expression in CD4^{+}GFP^{+} cells in ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice 4 days after tamoxifen treatment (a) and in LAT$^{-/-}$ mice reconstituted with ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ T cells after 4 weeks of maintained tamoxifen treatment (b) 3 mice were analyzed for each genotype. The figure shown is one representative of 4 experiments performed. (c) Total number of cells from the mesenteric, inguinal, brachial and axillary lymph nodes (left panel) and the spleen (right panel) of LAT$^{-/-}$ mice reconstituted with ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ T cells 4 weeks after tamoxifen treatment. Numbers were calculated from four mice from each genotype. Error bars on bar graphs represent the standard error of the mean of the four mice analyzed in an independent experiment.

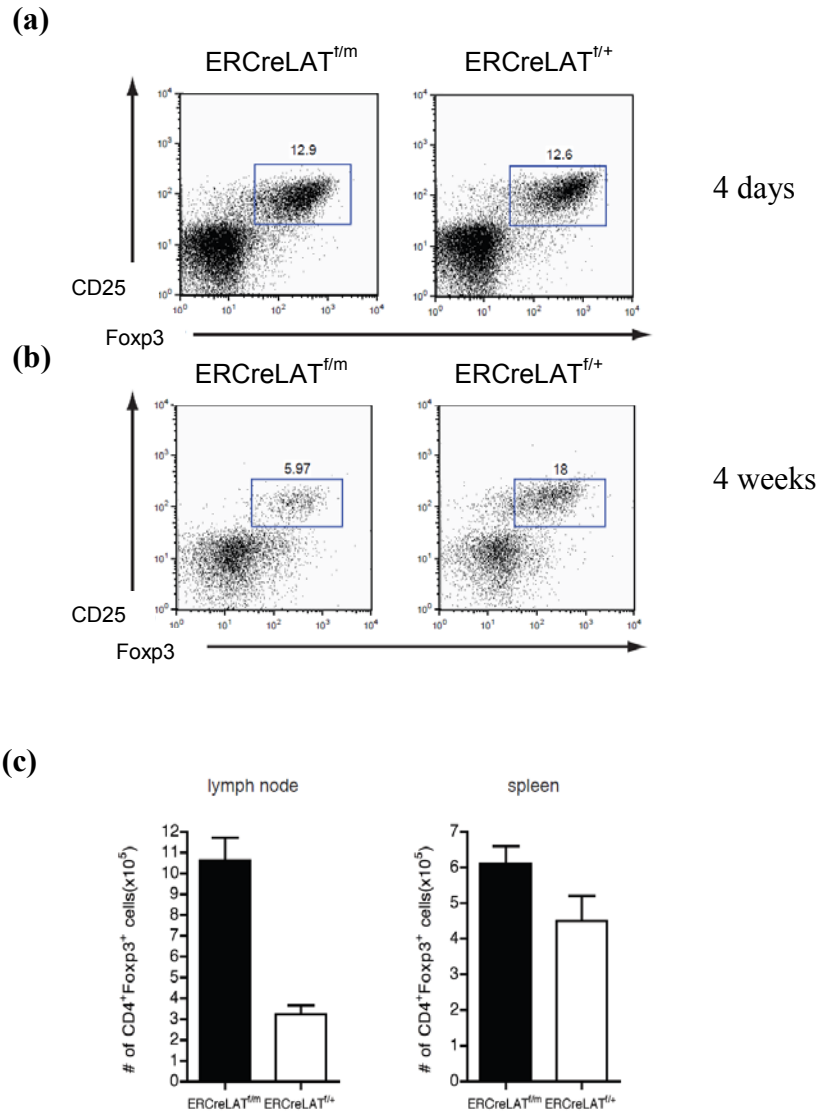

(a)
ERCreLAT f/m
ERCreLAT f/+
12.9
12.6
CD25
Foxp3
4 days
(b)
ERCreLAT f/m
ERCreLAT f/+
5.97
18
CD25
Foxp3
4 weeks
(c)
lymph node
spleen
# of CD4+Foxp3+ cells(x10^5)
ERCreLAT f/m
ERCreLAT f/+

Figure 3.18

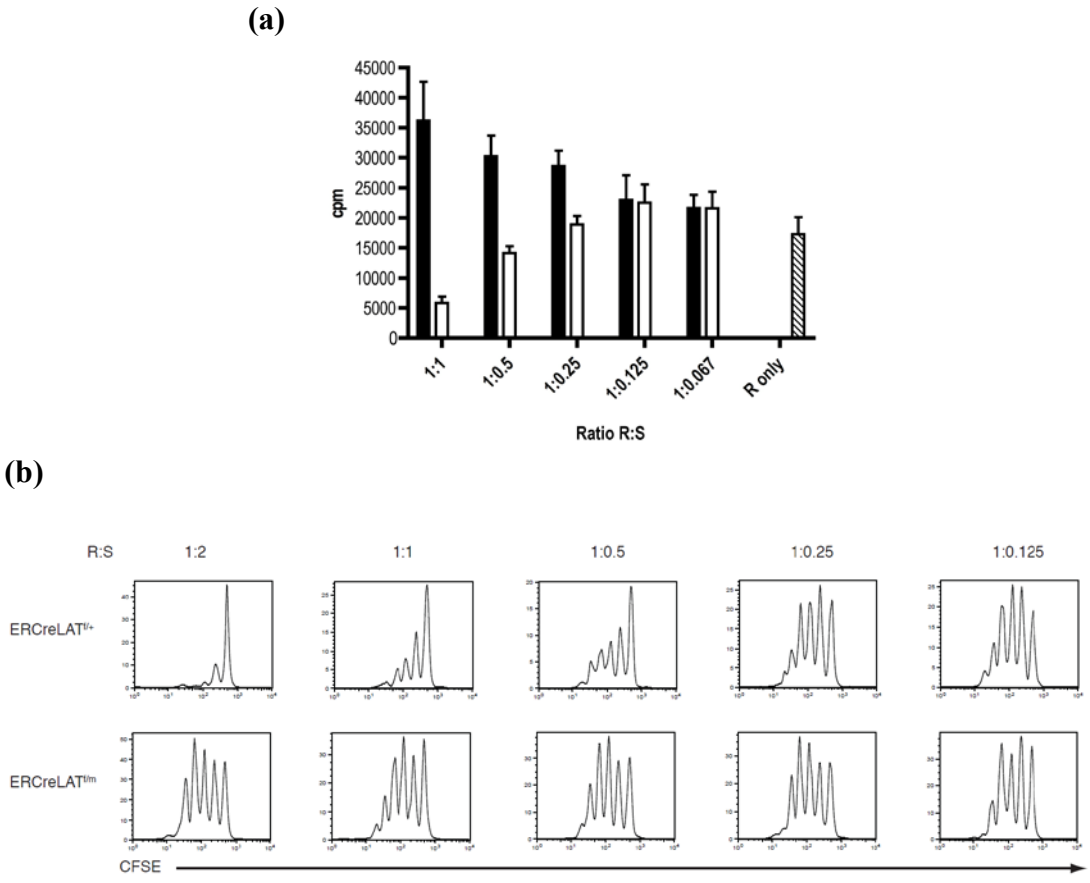


Figure 3.19 Impaired suppressive function of ERCreLAT$^{f/m}$ regulatory T cells
In vitro suppression assay using various ratios of CD4^{+}CD25^{-} responder cells (R) to CD4^{+}CD25^{+} suppressor cells (S) from ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ mice 4-5 days after tamoxifen treatment. (a) Proliferation of wildtype responder cells was measured by [^{3}H] thymidine incorporation. Results shown are combined from two independent experiments. Error bars on bar graphs represent the standard error of the mean of triplicates done in the two independent experiments. (b) Proliferation of wildtype Thy1.1^{+}CD4^{+}CD25^{-} responder cells is shown as indicated by CFSE dilution. The figure shown is one representative of 3 experiments performed.

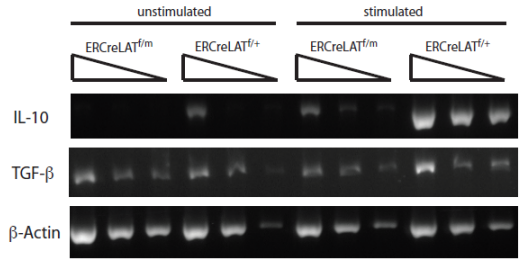
proliferation of responder cells could be suppressed efficiently by ERCreLAT$^{f//+}$ Treg cells in the presence of APCs, but not by ERCreLAT$^{f/m}$ Treg cells. IL-10 and TGF-β are two suppressive cytokines that are produced by Treg cells. Compared with ERCreLAT$^{f/+}$ Treg cells, ERCreLAT$^{f/m}$ Treg cells had reduced expression of IL-10 and TGF-β RNA after stimulation via the TCR (Figure 3.20a). These data suggested that the LAT-PLC-γ1 interaction in regulatory T cells plays an important role in the production of suppressive cytokines and in the suppression of conventional T cell proliferation.

Recent data indicate that the expression of CTLA-4 on Treg cells is essential for their ability to suppress proliferation of conventional T cells (Friedline et al., 2009; Wing et al., 2008). Furthermore, mice with CTLA-4 deleted in Foxp3^{+} cells develop a severe autoimmune disease (Wing et al., 2008). Hence, we next examined whether CTLA-4 expression on ERCreLAT$^{f/m}$ Treg cells is normal. As shown in Figure 3.20b, 4 days after tamoxifen treatment, the mean fluorescence intensity (MFI) of CTLA-4 in Treg cells from ERCreLAT$^{f/m}$ mice was reduced by approximately two-fold when compared with the CTLA-4 expression in ERCreLAT$^{f/+}$ Treg cells. This result suggested that the LAT-PLC-γ1 interaction is required for maintaining CTLA-4 expression in Treg cells.

3.3 Discussion

Our data demonstrate that the LAT-PLC-γ1 interaction is essential for efficient signaling in mature CD4 T cells in response to TCR crosslinking. Specifically, this interaction is important for TCR-mediated Erk activation, Ca^{2+} flux, IL-2 production, and

(a)



(b)

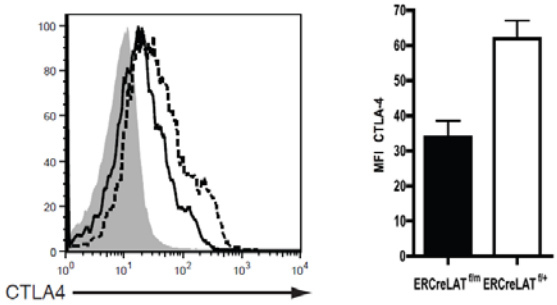


Figure 3.20 Impaired cytokine and CTLA-4 expression in ERCreLAT$^{f/m}$ Treg cells
ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ littermates were treated with tamoxifen; after four days, mice were analyzed. (a) Expression of IL-10 and TGFβ in ERCreLAT$^{f/m}$ and ERCreLAT$^{f/+}$ CD4^{+}CD25^{+} T cells after one hour of anti-CD3 stimulation. Data shown are representative of three independent experiments. (b) CTLA-4 expression on GFP^{+}CD4^{+}Foxp3^{+} cells from the lymph nodes of ERCreLAT$^{f/m}$ mice (solid line) and ERCreLAT$^{f/+}$ mice (dashed line) 4-5 days after tamoxifen treatment. GFP^{+}CD4^{+}Foxp3^{-} cells were used as a control (filled area). Data shown are representative of five mice per genotype. Error bars on bar graphs represent the standard error of the mean of three mice analyzed in an independent experiment.

proliferation. Moreover, when LAT$^{-/-}$ mice were reconstituted with ERCreLAT$^{f/m}$ T cells and treated with tamoxifen, they developed a disease similar to that observed in LATY136F mice. Although regulatory T cells were present in ERCreLAT$^{f/m}$ mice, our *in vitro* data show that they were nonfunctional, demonstrating that the LAT-PLC-γ1 interaction is not necessary for the survival of regulatory T cells in the periphery but is essential for Treg cell function.

LATY136F T cells have a partial block at the DN3 stage of thymocyte development as well as defects in negative and positive selection (Aguado et al., 2002; Sommers et al., 2005; Sommers et al., 2002). Our experimental model allowed for the analysis of T cells mutated at tyrosine 136 of LAT after they had undergone normal development and thymic selection. We were therefore able to assess the effects of this mutation specifically on signaling within mature T cells as well as on the function of regulatory T cells without the complications of any pre-existing developmental defects. Our results showed that phosphorylation of PLC-γ1 was defective in ERCreLAT$^{f/m}$ T cells when stimulated via the TCR. Consequently, calcium flux was impaired in these mature T cells. These results are similar to those seen in studies using LAT-deficient Jurkat cells expressing human LAT mutated at Tyr^{132} (Lin and Weiss, 2001; Zhang et al., 2000). However, in contrast to LATY136F knock-in CD4^{+} T cells, calcium flux was not completely absent in ERCreLAT$^{f/m}$ T cells; this difference can be attributed to the normal TCR expression level on the surfaces of ERCreLAT$^{f/m}$ T cells 5 days after tamoxifen

treatment, whereas LATY136F T cells express very low TCR levels (Aguado et al., 2002; Sommers et al., 2002). The residual calcium flux may be a result of the activation of PLC-γ1 through the association of the Gads-SLP76-PLC-γ1 complex to LAT via the direct binding of Gads to LAT.

In addition to causing defects in TCR-mediated PLC-γ1 activation and Ca^{2+} signaling, the Y136F mutation also affected Erk activation in mature T cells, as phosphorylation of Erk was decreased in ERCreLAT$^{f/m}$ T cells when compared to the ERCreLAT$^{f/+}$ control. This is not surprising as it has been shown that the phosphorylation and activation of PLC-γ1 are important for RasGRP1 and MAPK activation (Roose et al., 2005; Zhang et al., 2000). However, Sommers et al. found that LATY136F peripheral T cells exhibited normal Erk phosphorylation in response to stimulation via the TCR, even though TCR surface expression is very low on these cells (Sommers et al., 2002). This Erk activation occurs despite the lack of LAT and PLC-γ1 phosphorylation and the abrogation of calcium flux in these T cells. The reason for the difference observed between Erk phosphorylation in LATY136F and ERCreLAT$^{f/m}$ T cells could be that LATY136F T cells are sensitized by the presence of excess cytokines in the autoimmune environment, allowing for Erk activation upon TCR engagement. It is therefore likely that Erk activation in LATY136F T cells occurred in a LAT-independent manner, possibly through a cytokine-mediated Jak-Stat signaling pathway (Winston and Hunter, 1996).

Three to four weeks after tamoxifen treatment, ERCreLAT$^{f/m}$ mice developed a similar phenotype to that in six-week-old LATY136F mice. Thymocyte development was impaired in ERCreLAT$^{f/m}$ mice, including a partial block from the DN3 to the DN4 stage and a subsequently low percentage of DP thymocytes. CD4^{+} T cells underwent expansion, existing in a highly activated state and producing excessive IL-4; this phenomenon induced B cell hyper-activation and production of excessive IgE and IgG$_1$. These data demonstrate that after three weeks of tamoxifen injection, the phenotype of ERCreLAT$^{f/m}$ mice closely resembles that of LATY136F mice.

In order to more closely investigate the effect of mutating the LATY136 site in mature T cells on the breakdown of peripheral tolerance, LAT$^{-/-}$ mice were reconstituted with peripheral ERCreLAT$^{f/m}$ T cells. This experimental design eliminated aspects of the LATY136F phenotype related to impaired pre-TCR signaling and the partial block from the DN3 to the DN4 stage of thymocyte development, as well as issues associated with defective positive and negative selection. After tamoxifen treatment, LAT$^{-/-}$ mice that were reconstituted with peripheral ERCreLAT$^{f/m}$ T cells exhibited signs of a lymphoproliferative disorder similar to that in LATY136F mice. The development of this lymphoproliferative syndrome in our experimental model, despite normal thymocyte development and selection processes, suggested that the Y136F mutation in mature T cells alone is sufficient for the development of the lymphoproliferative disorder in LATY136F mice.

Even though negative selection is indeed impaired in LATY136F thymocytes, it might not contribute significantly to the development of the autoimmune disorder in these mice. It is possible that a breakdown in peripheral tolerance is the predominant cause of this disease. Our previous studies show that $CD4^+CD25^+$ T cells are absent in LATY136F mice. Adoptive transfer of wildtype $CD4^+CD25^+$ T cells into LATY136F neonates rescued these mice from the autoimmune syndrome, demonstrating a role for these cells in the control of autoimmunity (Koonpaew et al., 2006). While these data showed a defect in Treg cell development, another study suggested that a small population of $Foxp3^+$ cells is actually present in LATY136F mice (Wang et al., 2008). In our model, $ERCreLAT^{f/m}$ Treg cells developed normally before deletion of the wildtype allele. After deletion, regulatory T cells expressing the LATY136F mutant were able to survive in the periphery. In fact, the total number of $ERCreLAT^{f/m}$ $CD4^+Foxp3^+$ regulatory T cells in lymph nodes was approximately 3 times that of the $ERCreLAT^{f/+}$ control. However,our *in vitro* suppression assays demonstrated that $ERCreLAT^{f/m}$ $CD4^+CD25^+$ regulatory T cells lacked the ability to control the proliferation of wildtype $CD4^+CD25^-$ cells. These data suggested that the LAT-PLC-γ1 interaction is essential for the suppressive function of regulatory T cells.

Our data *in vivo* and *in vitro* indicated that, although they expressed Foxp3, $ERCreLAT^{f/m}$ Treg cells were not functional. However, previous studies from this lab have shown that ectopic expression of Foxp3 in LATY136F T cells restores their

suppressive capability (Koonpaew et al., 2006). While these results appear to be contradictory, this can be easily explained by the differences of Foxp3 expression in these cells. In ERCreLAT$^{f/m}$ Treg cells, Foxp3 expression was similar or slightly lower than that in WT Treg cells (Figure 3.19a). In the retroviral experiments done by Koonpaew et al, the expression of ectopic Foxp3 in LATY136F T cells should be much higher than the endogenous levels. Investigations by Ramsdell's group show that even CD8$^+$ T cells with overexpression of Foxp3 have suppressive function (Khattri et al., 2003). Thus, it is not surprising that overexpression of Foxp3 by retroviral transduction restores LATY136F Treg cell suppressive function. Importantly, our current data suggested that expression of Foxp3 at the endogenous level in Treg cells is not sufficient for their suppressive function and normal signaling through LAT is also required.

Many studies have been done to elucidate the mechanisms that enable regulatory T cells to suppress the proliferation of effector T cells. Although it is well known that regulatory T cells are able to produce suppressive cytokines, such as IL-10 and TGF-β, there are conflicting reports on the importance of these cytokines in Treg cell function. While some *in vitro* data do not support a role for these cytokines in the suppressive function of Treg cells (Takahashi et al., 1998; Thornton and Shevach, 1998), several *in vivo* studies have revealed their importance in dampening inflammation caused by infectious agents, allergies, and environmental components (Asseman et al., 1999; Belkaid et al., 2002; Li et al., 2007; Maloy et al., 2003; Rubtsov et al., 2008). However,

studies using IL10$^{flox/flox}$ x Foxp3$^{YFP\text{-}CRE}$ mice demonstrate that IL-10 produced by Treg cells is not necessary for the regulation of systemic autoimmunity (Rubtsov et al., 2008). TGF-β is another suppressive cytokine that may be important in Treg cell function. In some models, such as IBD and type I diabetes, TGF-β is produced by Treg cells, but in other models, this suppressive cytokine is produced by different cell types (Belghith et al., 2003; Kullberg et al., 2005; Li et al., 2007; Wilson et al., 2005). Studies done using an autoimmune gastritis model show that TGF-β is not essential for Treg cell function (Piccirillo et al., 2002; Tang and Bluestone, 2008). Our data showed that the LAT-PLC-γ1 interaction is important in the production of IL-10 and TGF-β by Treg cells in response to TCR stimulation. Addition of IL-10 to the suppression assay did not rescue the function of ERCreLAT$^{f/m}$ Treg cells *in vitro* (data not shown), suggesting that the impaired production of these cytokines is not likely the reason for the defective function of these Treg cells. Given these conflicting data pertaining to the role of TGF-β and IL-10 in Treg cell-mediated suppression, it is still possible that these cytokines may contribute to the suppressive function of Treg cells *in vivo*.

ERCreLAT$^{f/m}$ CD4 T cells have a defect in the ability to flux calcium in response to TCR stimulation. Recent studies demonstrated the necessity of a robust calcium flux for the suppressive function of Treg cells (Oh-Hora et al., 2008). Therefore, it is possible that the impaired function of ERCreLAT$^{f/m}$ Treg cells can be explained by the importance of the LAT-PLC-γ1 interaction in TCR-mediated calcium signaling. Even though the

defects in TCR-mediated proliferation and IL-2 production by ERCreLAT$^{f/m}$ T cells could be corrected by stimulation with PMA and ionomycin (Figure 2), which bypasses TCR proximal signaling, ERCreLAT$^{f/m}$ Treg cells pre-activated with PMA and ionomycin for four hours still failed to suppress the proliferation of responder cells (data not shown). Thus, it is possible that continuous activation of Treg cells by engagement with APCs is required for efficient suppression or that the LAT-PLC-γ1 interaction is required for maintenance of Treg programming and function, which cannot be simply corrected by stimulation with PMA and ionomycin. Another mechanism through which Treg cells may induce the suppression of conventional T cells is termed "IL-2 sink." This process is described as the ability of Treg cells to absorb IL-2 from the surrounding environment via the IL-2Rα (CD25), thus depriving effector T cells of this cytokine that is necessary for proliferation and differentiation. Bim, a proapoptotic factor, is upregulated in effector T cells as a result of this IL-2 starvation, inducing CD4^{+}CD25^{-} T cells to undergo apoptosis (Pandiyan et al., 2007). Treg cells in ERCreLAT$^{f/m}$ mice after tamoxifen treatment express normal levels of CD25. In addition, ERCreLAT$^{f/m}$ T cells can expand *in vitro* in the presence of IL-2, indicating that CD25-mediated signaling is normal. This suggests that "IL-2 sink" is unlikely to be a mechanism of suppression in our experimental system.

Studies by Wing et al. demonstrated that, while the expression of CTLA-4 on regulatory T cells is not necessary for Treg cell development and survival, it is essential

for both their *in vivo* and *in vitro* suppressive capabilities (Wing et al., 2008). In these studies, mice with CTLA-4 specifically deleted in Treg cells (CKO) develop a lymphoproliferative syndrome similar to the condition observed here. While wildtype Treg cells are able to prevent the upregulation of CD80 and CD86, DCs cultured with CKO Treg cells still expressed high levels of these cell surface molecules (Cederbom et al., 2000; Oderup et al., 2006; Wing et al., 2008). This demonstrates that CTLA-4 is required to thwart DC activation and subsequently diminish the activation of conventional T cells through the CD28 coreceptor molecule (Wing et al., 2008). It has also been shown that high expression of CTLA-4 on regulatory T cells correlates to their ability to increase the activity of indoleamine 2,3-dioxygenase (IDO) in dendritic cells (Fallarino et al., 2003; Oderup et al., 2006). IDO is an immunosuppressive enzyme that has been linked to the suppression of effector cell proliferation (Fallarino et al., 2003; Munn et al., 2002). The activation of IDO initiates tryptophan catabolism, leading to the production of pro-apoptotic catabolites known as kynurenines, and the subsequent regulation of effector cells. Our data reveal a decrease in the protein expression of CTLA-4 in ERCreLAT$^{f/m}$ T reg cells. Whether this reduction in CTLA-4 expression in Treg cells is indeed the cause of the observed functional defect remains to be determined.

4. The importance of LAT in the negative regulation of T cell homeostasis

4.1 Introduction

Early studies using LAT-deficient Jurkat cells clearly indicate the importance of LAT in TCR-mediated signaling; however, not many studies have focused on the function of LAT in primary T cells. This issue is complicated by the fact that mature αβ T cells are absent in $LAT^{-/-}$ mice (Zhang et al., 1999b). Additionally, LAT^{4YF} knockin mice harboring mutations of the tyrosines most essential for binding to other proteins (Y136, Y175, Y195, and Y235) exhibit the same phenotype as the $LAT^{-/-}$ mice (Nunez-Cruz et al., 2003; Sommers et al., 2001). In LAT^{3YF} knockin mice with mutations at the Y175, Y195, and Y235 residues, αβ T cell development is also totally blocked at the DN3 thymocyte stage (Nunez-Cruz et al., 2003).

As discussed in Chapter 3, studies using $CD4^+$ T cells from LATY136F mice show that the loss of the LAT-PLC-γ1 interaction leads to diminished PLC-γ1 activation and calcium mobilization (Aguado et al., 2002; Sommers et al., 2002). Due to the development of a lymphoproliferative syndrome in LATY136F mice, it is hypothesized that this specific interaction plays a role in negatively regulating TCR-mediated signaling. However, while we and others have now identified the importance of the LAT-PLC-γ1 interaction in T cells, the role of total LAT-mediated signaling in mature primary T cells has yet to be fully defined.

The data presented in Chapter 3 also demonstrated that the LAT-PLCγ1 interaction is important in mature T cell signaling and T cell function, particularly regulatory T cell function. Treg cells are defined by their expression of Foxp3, a transcription factor essential for establishing the Treg repertoire. Studies have revealed that the ablation of Foxp3 from Treg cells strips these cells of their functional phenotype; in addition, the cell gene expression profile is transformed, including a decrease in CTLA-4 expression (Kim et al., 2009). In fact, due to the instability of Foxp3, Treg cells can differentiate into pathogenic T cells (Zhou et al., 2009). While it is known that TCR-mediated signaling is important for Treg activation, the role of LAT-mediated signaling in stabilizing Foxp3 expression has not been examined.

To investigate the function of LAT in mature T cells, including regulatory T cells, we used the ERCre transgenic system to induce deletion of LAT in mature T cells. In the proceeding section, we present data that demonstrates that the loss of LAT in mature regulatory T cells led to the dysregulation of these cells; their suppressive function was impaired and Foxp3 expression was decreased. Furthermore, over time, the ablation of LAT in peripheral T cells induced a LATY136F-like lymphoproliferative syndrome.

4.2 Results

4.2.1 Reconstitution of $LAT^{-/-}$ mice with $ERCreLAT^{f/-}$ T cells

Despite being widely regarded as a positive regulator of TCR-mediated signaling, studies on LATY136F knockin mice suggest that LAT may have a negative

role in T cell homeostasis. $CD4^+$ T cells from these mice undergo an uncontrolled expansion and produce large amounts of Th2 cytokines. It has been suggested that the Y136F mutation may disrupt a delicate balance between LAT-mediated positive and negative signaling pathways (Aguado et al., 2002; Sommers et al., 2002). In order to test this hypothesis, we examined whether such a lymphoproliferative disease would arise in the absence of LAT.

To avoid possible complications arising from T cells newly emigrated from the thymus from $ERCreLAT^{f/-}$ mice, we reconstituted $LAT^{-/-}$ mice with 2×10^7 enriched T cells (more than 75% pure) from untreated $ERCreLAT^{f/-}$ or $ERCreLAT^{f/+}$ mice. We waited five weeks to allow for homeostatic expansion of T cells in these mice, after which similar percentages of $CD4^+$ and $CD8^+$ T cells were found in the peripheral blood of these mice (data not shown). To delete LAT, these mice were first treated with tamoxifen on two consecutive days and were then treated once every week for 4 weeks. After 4 weeks of tamoxifen treatment, $LAT^{-/-}$ mice reconstituted with $ERCreLAT^{f/-}$ splenocytes exhibited splenomegaly, while mice that received $ERCreLAT^{f/-}$ splenoctyes had normal sized spleens (not depicted). Similar to the disease observed in LATY136F mice, the splenomegaly in mice reconstituted with $ERCreLAT^{f/-}$ cells was caused by the expansion of $CD4^+$ T cells. As shown in Figure 4.1a, there was an increase in the ratio of CD4:CD8 cells; the total number of $CD4^+$ cells was increased in the spleen of LAT-deficient mice receiving $ERCreLAT^{f/-}$ cells as compared to the control (Figure 4.1b). Most of these

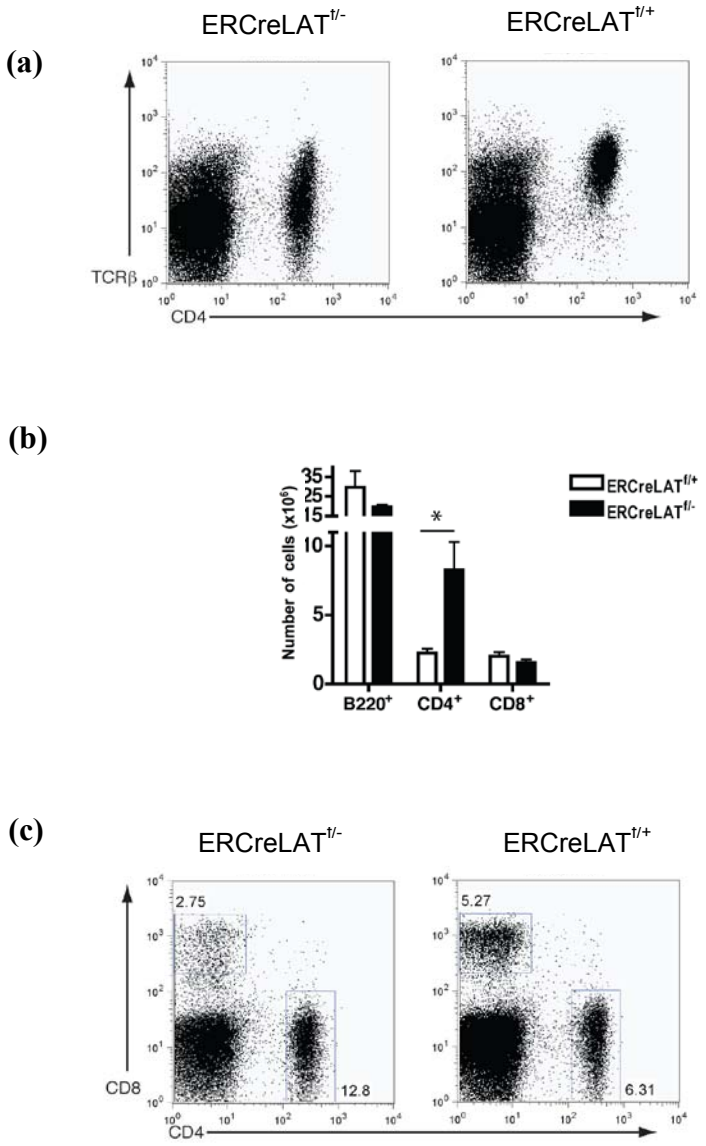


Figure 4.1 LAT ablation in peripheral T cells results in expansion of CD4 T cells
2×10^7 enriched T cells from untreated ERCreLAT$^{f/-}$ littermates were adoptively transferred to syngeneic LAT$^{-/-}$ recipients; ERCreLAT$^{f/+}$ splenocytes were used as controls. Five weeks later, the recipients were treated with tamoxifen on two consecutive days. Tamoxifen treatment was then repeated once a week for 4-5 weeks. Data are representative of two independent experiments with three mice per genotype in each experiment. (a) Expression of CD4 vs TCRβ in the spleen. (b) Total number of CD4$^+$, CD8$^+$, and B220$^+$ cells in the spleen. A two-tailed student t-test analysis was performed; * represents $p< 0.05$. Error bars on the bar graph represent the standard error of the mean of three mice analyzed in an independent experiment. (c) Expression of CD4 vs CD8 in the spleen.

$CD4^+$ T cells were GFP^+, suggesting that deletion of LAT was efficient. Interestingly, the majority of $ERCreLAT^{f/-}$ $CD4^+$ T cells had downregulated TCR surface expression, as compared with the T cells from control mice (Figure 4.1c). These data indicated that, similar to LATY136F T cells, LAT-deficient $CD4^+$ T cells were hyperproliferative. In addition, surface MHC Class II expression was increased on B cells from LAT-deficient recipients reconstituted with $ERCreLAT^{f/-}$ T cells (Figure 4.2a). Futhermore, the percentage of IgD^-IgM^- B cells in these mice was increased (Figure 4.2b), suggesting that these B cells had undergone maturation and isotype switching. Consequently, high levels of serum IgE and IgG_1 were detected in these mice (Figure 4.2c).

We next assessed the effect of deleting LAT on the presence of regulatory T cells in the periphery. As shown in Figure 4.3a, Treg cells were indeed present in the spleens and lymph nodes of $LAT^{-/-}$ recipient mice receiving $ERCreLAT^{f/-}$ T cells, albeit at a reduced percentage compared to $LAT^{f/-}$ controls. The decrease in Treg cells was even more obvious when CD4 T cells were analyzed (Figure 4.3b). Correspondingly, the deletion of LAT in peripheral regulatory T cells led to a decrease in their total number (Figure 4.3c). Altogether, our data showed that the deletion of LAT in peripheral T cells could induce a similar autoimmune-like lymphoproliferative disorder to that observed in LATY136F mice; our data also suggests a role for LAT in supporting the survival of Treg cells in the periphery.

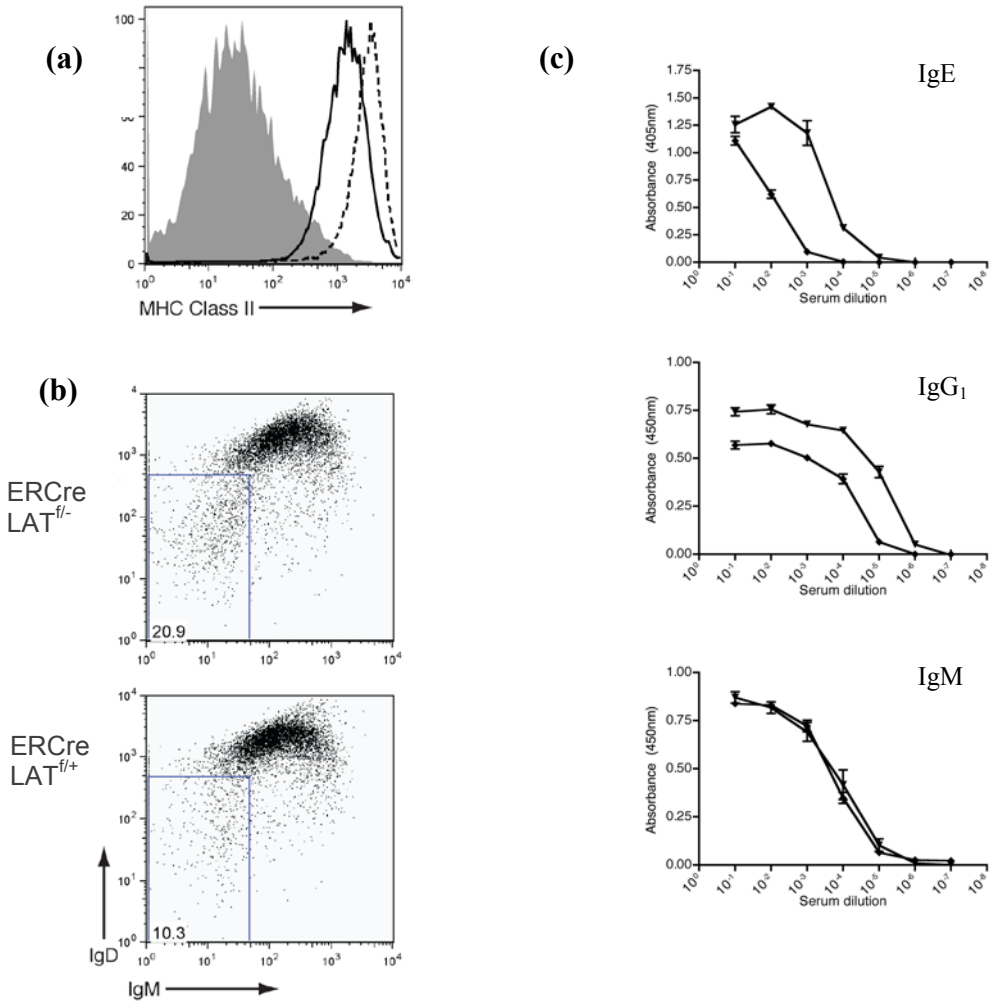


Figure 4.2 The deletion of LAT leads to the hyperactivation of B cells.
LAT$^{-/-}$ mice were reconstituted with ERCreLAT$^{f/-}$ or ERCreLAT$^{f/+}$ T cells and injected with tamoxifen for 4 weeks. (a) Expression of MHC Class II on B220^{+} cells in lymph nodes. ERCreLAT$^{f/+}$ is represented by the black line, ERCreLAT$^{f/-}$ is represented by the dotted line, and a negative control (GFP^{+} cells) is represented by the gray shaded area. (b) The expression of IgD vs IgM on B220^{+} lymph node cells. (c) Blood serum levels of IgE, IgG$_1$, and IgM from LAT$^{-/-}$ recipients injected with T cells from ERCreLAT$^{f/-}$ (triangles) and ERCreLAT$^{f/+}$ (diamonds) mice. Data are representative of two independent experiments with three mice per genotype in each experiment.

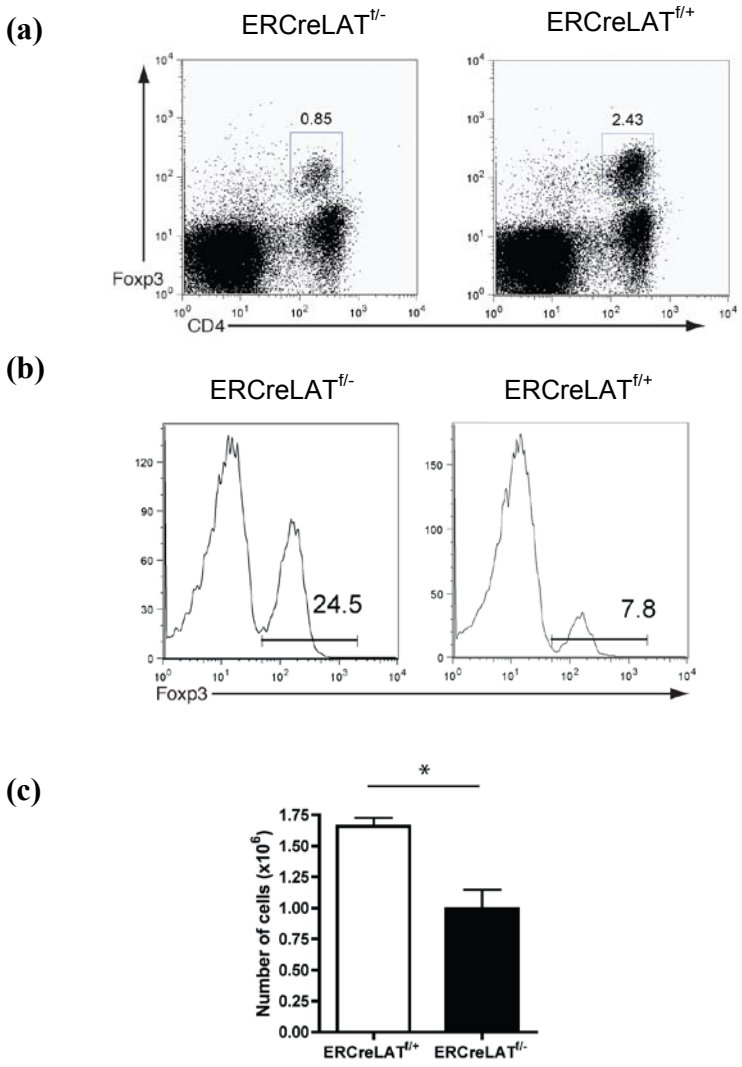


Figure 4.3 Decreased Foxp3 expression in ERCreLAT$^{-/-}$ Treg cells

LAT$^{-/-}$ mice were reconstituted with ERCreLAT$^{f/-}$ or ERCreLAT$^{f/+}$ T cells and injected with tamoxifen for 4 weeks. (a) Expression of CD4 vs Foxp3 in the spleen. (b) The expression of Foxp3 in CD4^{+} splenocytes. (c) Total number of CD4^{+}Foxp3^{+} cells in the spleen. Data are representative of two independent experiments with three mice per genotype in each experiment. Error bars on bar graphs represent the standard error of the mean of three mice analyzed in one independent experiment.

4.2.2 Comparison of the severity of the LATY136F phenotype between ERCreLAT$^{f/m}$ and ERCreLAT$^{f/-}$ mice

Our data in figures 4.1-2 showed that deletion of LAT in peripheral T cells caused the development of an autoimmune-like syndrome similar to the one observed in LATY136F mice. To directly compare the differences of the syndromes caused by LAT deficiency or the Y136F mutation, we treated ERCre^{+}LAT$^{f/-}$ and ERCre^{+}LAT$^{f/m}$ mice with tamoxifen to induce deletion of LAT. LAT$^{f/-}$ mice were used as controls. Mice were injected with tamoxifen once a week for 5 weeks before analysis to ensure efficient and continuous deletion of LAT. As shown in Figure 4.4a, ERCre^{+}LAT$^{f/m}$ mice had severe splenomegaly. The weight of their spleens (407±39mg) was approximately four times that of LAT$^{f/-}$ mice (86±7mg) (Figure 4.4b). In contrast, the spleens of ERCre^{+}LAT$^{f/-}$ mice were only slightly enlarged (137±30mg). The numbers of splenocytes were increased in these mice, as expected (Figure 4.5a). A similar increase of cells was also seen in lymph nodes. While the numbers of B220^{+} cells in the spleens of ERCre^{+}LAT$^{f/-}$, ERCre^{+}LAT$^{f/m}$, and LAT$^{f/-}$ mice were similar, the numbers of CD4^{+} T cells were drastically different (Figure 4.5b). ERCre^{+}LAT$^{f/m}$ mice had ~5 times more CD4^{+} T cells than LAT$^{f/-}$ mice (55±7x10^{6} vs. 11±2x10^{6}), while ERCre^{+}LAT$^{f/-}$ mice had ~60% more (18±5x10^{6}). In contrast, the number of ERCre^{+}LAT$^{f/-}$ CD8^{+} T cells was reduced to 50% of that in LAT$^{f/-}$ mice.

Further analysis of T cells from these mice showed that there was a corresponding increase in the CD4:CD8 ratio in ERCreLAT$^{f/-}$ mice (Figure 4.6a). In

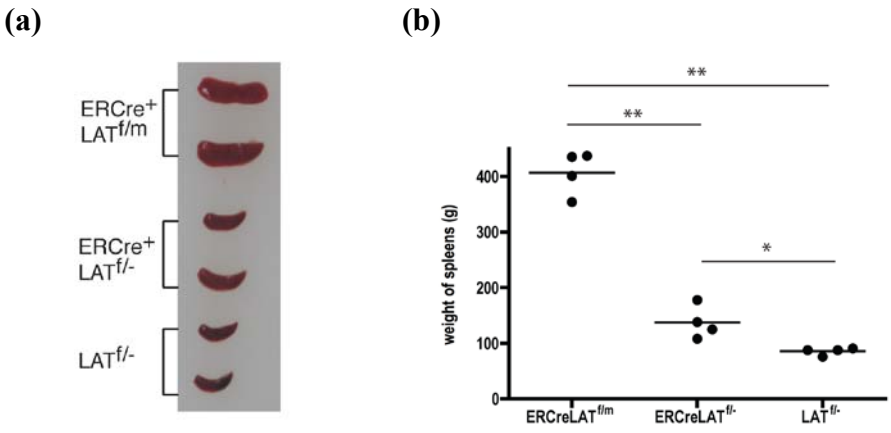


Figure 4.4 Enlarged spleens in ERCre^{+}LAT$^{f/m}$ and ERCre^{+}LAT$^{f/-}$ mice

ERCre^{+}LAT$^{f/m}$, ERCre^{+}LAT$^{f/-}$, and LAT$^{f/-}$ mice were treated with tamoxifen for 5 weeks before analysis. (a) A picture of spleens from two mice of each genotype. (b) Weight of spleens. Two-tailed t-test; *, $p \leq 0.05$; **, $p \leq 0.001$. Data shown are representative of four mice analyzed.

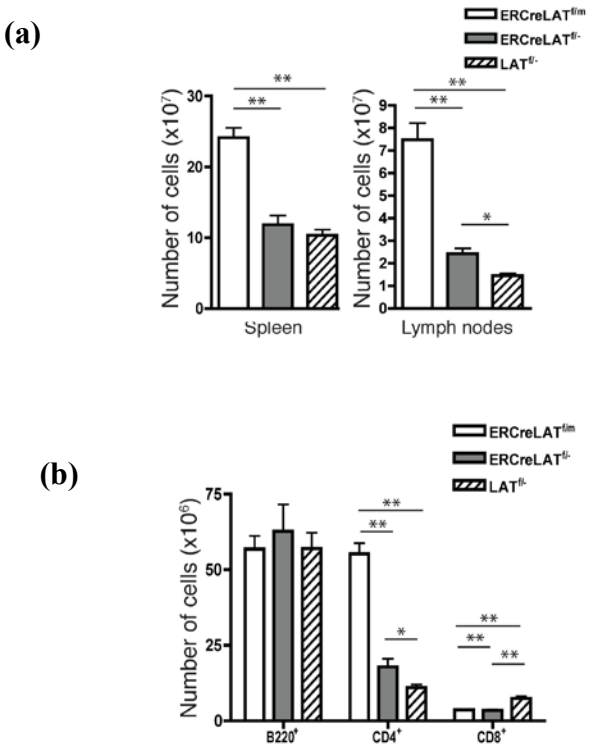


Figure 4.5 Increased total number of cells and CD4⁺ cells in ERCre⁺LAT$^{f/m}$ and ERCre⁺LAT$^{f/-}$ mice

ERCre⁺LAT$^{f/m}$, ERCre⁺LAT$^{f/-}$, and LAT$^{f/-}$ mice were treated with tamoxifen for 5 weeks before analysis. (a) Total numbers of cells in spleens and lymph nodes (n=4). Two-tailed t-test; *, p≤ 0.01; **, p≤ 0.001. (b) Total numbers of B220⁺, CD4⁺, and CD8⁺ splenocytes (n=4). Two-tailed t-test; *, p≤ 0.01; **, p≤ 0.001. Error bars on bar graphs represent the standard error of the mean of four mice analyzed in an independent experiment.

addition, there was a downregulation of TCR expression on $CD4^+$ T cells from $ERCre^+LAT^{f/m}$ mice similar to that observed in the LATY136F mice. This downregulation of the TCR was also seen in $ERCre^+LAT^{f/-}$ T cells, although a large percentage of $CD4^+$ T cells still retained a normal level of TCR expression (Figure 4.6b). We also analyzed cytokine production by $CD4^+$ T cells from these mice. As shown in Figure 4.6c, T cells from both $ERCre^+LAT^{f/m}$ and $ERCre^+LAT^{f/-}$ mice produced large amounts of cytokines, such as IL-4 and IFN-γ, compared with those from $LAT^{f/-}$ mice. However, more $ERCre^+LAT^{f/m}$ T cells produced IL-4 compared with $ERCre^+LAT^{f/-}$ T cells. In addition, the fluorescence intensity of anti-IL-4 staining in T cells from $ERCre^+LAT^{f/m}$ mice appeared higher, indicating that they produced more IL-4. In LATY136F mice, B cells are activated and undergo maturation and isotype switching (Aguado et al., 2002). Similar activation was also seen in $ERCre^+LAT^{f/m}$ and $ERCre^+LAT^{f/-}$ treated with tamoxifen. Among splenocytes from $ERCre^+LAT^{f/-}$ and $ERCre^+LAT^{f/m}$ mice, more B cells had downregulated B220 and upregulated MHC Class II expression when compared to controls (Figure 4.7a).The percentage of IgM^-IgD^- B cells were 18.0% and 51.8%, respectively (Figure 4.7b), indicating that more B cells in $ERCreLAT^{f/m}$ mice were activated and had undergone isotype switching. Analysis of Foxp3 expression in $CD4^+$ T cells showed that $ERCre^+LAT^{f/m}$ mice had the lowest percentage of Treg cells. The percentages of Treg cells in $ERCre^+LAT^{f/m}$, $ERCre^+LAT^{f/-}$, and $LAT^{f/-}$ mice were 0.71%, 1.55%, and 2.64%, respectively (Figure 4.7c). Although the percentage of Treg cells was low in

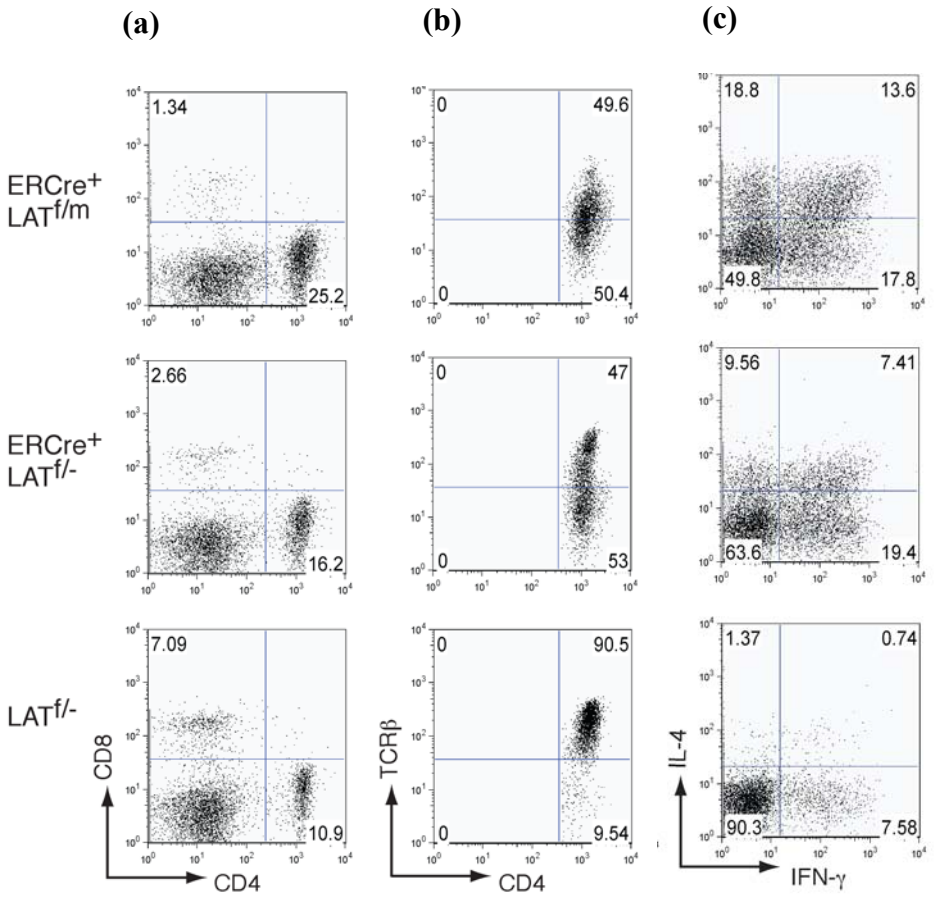


Figure 4.6 Increased activation of ERCreLAT$^{f/m}$ and ERCreLAT$^{f/-}$ CD4^{+} T cells
Mice were treated with tamoxifen for 5 weeks splenocytes were analyzed via FACS analysis. (a) CD8 vs CD4 expression on total cells (b) TCRβ expression on CD4^{+} cells (c) IL-4 vs IFNγ expression in CD4^{+} T cells after 4hr P+I stimulation. The numbers on the FACS plots represent the percentages of the gated populations. FACS plots shown are representative of four mice per genotype.

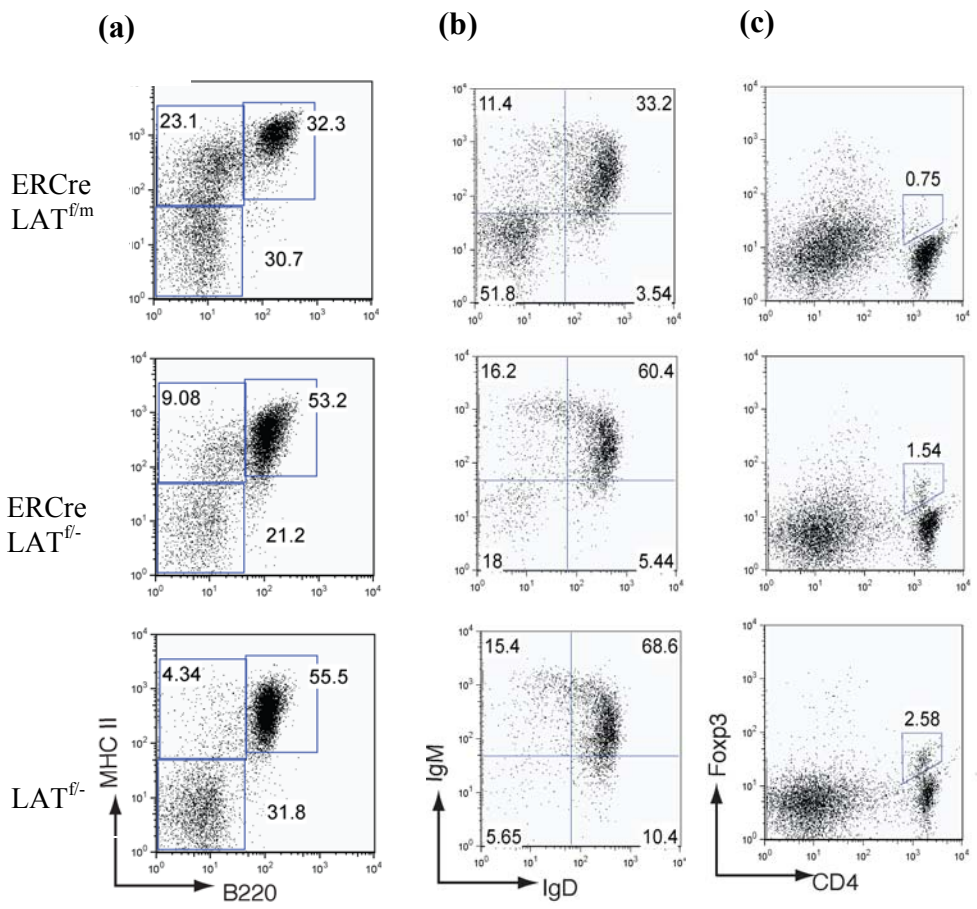


Figure 4.7 Decreased proportion of Treg cells and increased activation of B cells in ERCreLAT$^{f/m}$ and ERCreLAT$^{f/-}$ mice

Mice were treated with tamoxifen for 5 weeks before splenocytes were analyzed via FACS analysis. (a) B220 vs MHC Class II expression on total cells (b) IgM vs IgD expression on B220^{+} cells (c) Foxp3 vs CD4 expression on total cells. The numbers on the FACS plots represent the percentages of the gated populations. FACS plots shown are representative of four mice per genotype.

ERCre^{+}LAT$^{f/m}$ mice, the number of Foxp3^{+} Treg cells in these mice was similar to that in LAT$^{f/-}$ mice (2.5x10^{6} vs 2.1x10^{6} per spleen). On the other hand, the number of Foxp3^{+} Treg cells in the spleen of ERCre^{+}LAT$^{f/-}$ mice was only ~1.2x10^{6}. Together, these results indicated that, although mice with LAT-deficient T cells developed a similar autoimmune-like syndrome, this disease is less severe than the disease that develops in LATY136F mice.

4.2.3 Effect of LAT deficiency on regulatory T cell survival, maintenance and function

Studies done in our lab have shown that LAT-deficient T cells were defective in both long-term survival and lymphopenia-driven homeostatic proliferation (Shen et al, submitted). These data seemed to contradict the findings that these cells could also drive the development of a LATY136F-like lymphoproliferative disease. It is possible that LAT deletion caused a breakdown in peripheral tolerance by affecting Treg cell maintenance or survival. As shown earlier in Figure 4.3, among CD4^{+} T cells from the LAT$^{-/-}$ mice reconstituted with ERCre^{+}LAT$^{f/-}$ splenocytes, only ~8% of them expressed Foxp3 as compared with ~25% by control cells. Therefore, deletion of LAT leads to a decrease in the frequency and number of Treg cells.

To confirm that the decrease in total Treg cells upon treatment with tamoxifen was directly caused by the loss of LAT and not by the autoimmune-like environment, we examined the effects of LAT deletion on Treg cells before the onset of the disease.

Figure 4.8 Lack of lymphoproliferative disorder ten days after tamoxifen treatment

ERCre^{+}LAT$^{f/-}$ and LAT$^{f/-}$ littermates were treated with tamoxifen on two consecutive days. Ten days after the initial treatment, splenocytes were harvested and analyzed by FACS. (a) A picture of spleens from two mice of each genotype. (b) Total number of cells in the spleen. The graph is a one representative of three independent experiments. Error bars on the bar graph represent the standard error of the mean of the mice analyzed in an independent experiment. (c) CD4 vs CD8 expression. (d) TCRβ expression on CD4+ cells. ERCreLAT$^{f/-}$ is represented by the dashed line, LAT$^{f/-}$ is represented by the black line, and the shaded region represents a GFP- negative control. (e) B220 vs MHC Class II expression. Data shown are representative of three independent experiments using two mice per genotype in each experiment.

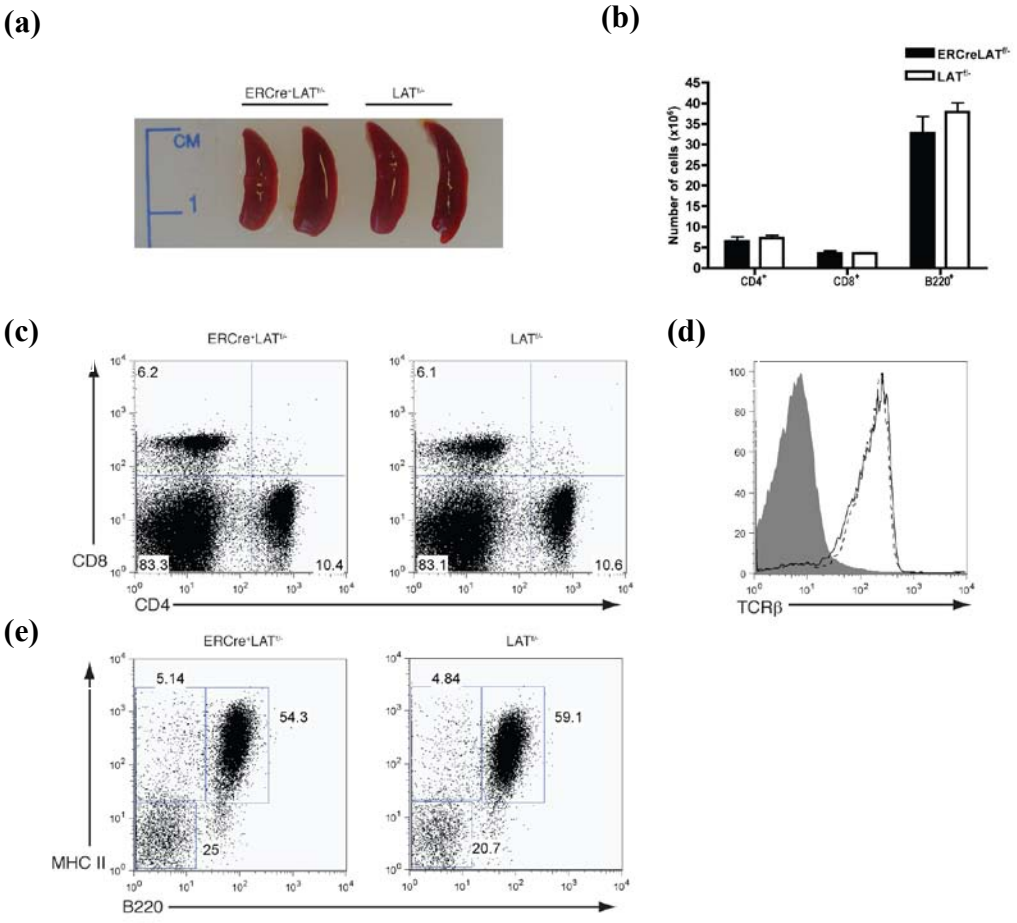

(a)
(b)
(c)
(d)
(e)
CM
1
Number of cells (x10⁶)
CD4⁺
CD8⁺
B220⁺
CD8
CD4
6.2
83.3
10.4
6.1
83.1
10.6
TCRβ
MHC II
B220
5.14
54.3
25
4.84
59.1
20.7

Figure 4.8

ERCre^{+}LAT$^{f/-}$ and LAT$^{f/-}$ littermates were treated with tamoxifen on two consecutive days. Ten days after the first treatment, spleens from both groups of mice remained a normal size (Figure 4.8a), and the total number and percentages of CD4^{+} T cells were comparable (Figure 4.8b and 4.8c). In addition, TCRβ expression was normal in ERCreLAT$^{f/-}$ mice compared to LAT$^{f/-}$ controls (Figure 4.8d); B cells from these mice were also similar in their level of B220 and MHC Class II expression (Figure 4.8e). Cumulatively, these data suggest that 10 days after tamoxifen treatment, the lymphoproliferative disorder had not yet developed. Interestingly, the percentage of CD4^{+}Foxp3^{+} cells was decreased in ERCre^{+}LAT$^{f/-}$ spleens; this decrease was even more drastic in the lymph nodes (Figure 4.9a). There was also a slight decrease in the total number of ERCreLAT$^{f/-}$ Treg cells compared to the control (Figure 4.9b). We next sought to assess the function of LAT-deficient Treg cells 4-5 days after tamoxifen treatment. These Treg cells were not able to suppress the expansion of conventional T cells *in vitro* (Figure 4.9c), indicating the importance of LAT in Treg cell function. Our data suggests that the deletion of LAT from regulatory T cells occurred before the onset of the autoimmune-like syndrome, implicating the lack of regulatory T cell suppression as one cause for the development of the disorder.

4.2.4 Reduced expression of Foxp3 in ERCreLAT$^{f/-}$ regulatory T cells

Our data suggests that the requirement for LAT in the function of regulatory T cells is possibly the main cause of the development of the lymphoproliferative disease in

Figure 4.9 Decreased number of Treg cells in the periphery ten days after tamoxifen deletion

(a-b) $ERCre^{+}LAT^{f/-}$ and $LAT^{f/-}$ littermates were treated with tamoxifen on two consecutive days. Ten days after the initial treatment, splenocytes were harvested and analyzed. (a) Foxp3 expression in $CD4^{+}$ lymph node cells (left) and total numbers of $CD4^{+}Foxp^{+}$ cells in the lymph node. (b) Foxp3 expression in $CD4^{+}$ splenocytes (left) and total numbers of $CD4^{+}Foxp^{+}$ cells in the spleen. The figures shown are a representative of 3 independent experiments. Error bars on bar graphs represent the standard error of the mean of three mice analyzed in an independent experiment. (c) *In vitro* suppression assay using 1:1 ratio of $CD4^{+}CD25^{-}$ responder cells to $CD4^{+}CD25^{+}$ T_{reg} cells from $ERCre^{+}LAT^{f/-}$ (upper panel) and $LAT^{f/-}$ mice (lower panel) 4-5 days after tamoxifen treatment. Proliferation of wildtype $Thy1.1^{+}CD4^{+}CD25^{-}$ responder cells is shown as indicated by CFSE dilution (gray shaded region represents responder cells cultured with no Treg cells). The figure shown is one representative of 3 experiments performed.

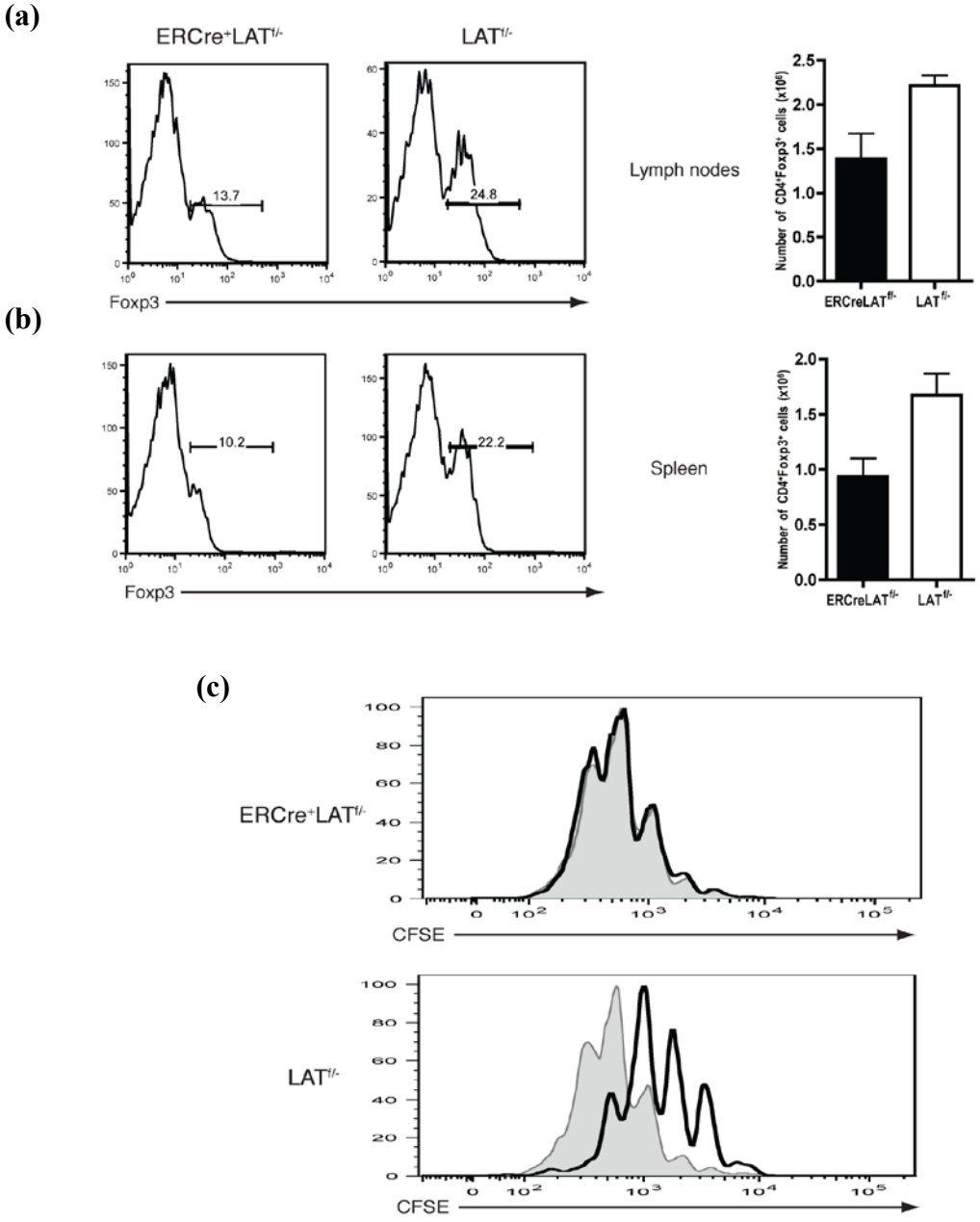

(a)
ERCre+LATf/-
LATf/-
13.7
24.8
Foxp3
Lymph nodes
Number of CD4+Foxp3+ cells (x10⁶)
ERCreLATf/-
LATf/-
(b)
10.2
22.2
Foxp3
Spleen
Number of CD4+Foxp3+ cells (x10⁶)
ERCreLATf/-
LATf/-
(c)
ERCre+LATf/-
CFSE
LATf/-
CFSE

Figure 4.9

ERCreLAT$^{f/-}$ mice treated with tamoxifen over the course of four weeks. We sought to further characterize the defects in ERCreLAT$^{f/-}$ Treg cells. Detailed analysis of these cells revealed a role for LAT in the expression of Foxp3. As shown in Figure 4.10a, deletion of LAT led to a reduction in the mean fluorescence intensity (MFI) of Foxp3 in Treg cells, just 10 days after tamoxifen treatment. CTLA-4 and CD25 are proteins expressed by Treg cells. The transcription of these proteins is regulated by Foxp3, the "master" transcription factor of Treg cells. Accordingly, we observed a decrease in expression of CD25 and CTLA-4 in GFP^{+} CD4^{+}Foxp3^{+} Treg cells from ERCreLAT$^{f/-}$ mice (Figures 4.10b and 4.10c). However, analysis of the mRNA expression of Foxp3 in ERCreLAT$^{f/-}$ and LAT$^{f/-}$ Treg cells demonstrated that there was no notable difference in Foxp3 expression at the transcriptional level (Figure 4.10d). This suggests that LAT plays a role in the regulation of Foxp3 expression at the post-transcriptional level. Together, our data showed that the deletion of LAT caused a reduction of Foxp3 expression in Treg cells, as well as a decrease in the number of Treg cells in the periphery indicating that LAT plays a critical role in Treg cell maintenance or survival. As Treg cells play an essential role in maintaining peripheral tolerance, the autoimmune-like disorder observed in mice with LAT-deficient T cells is likely caused by a lack of suppression by Treg cells.

4.3 Discussion

Our studies revealed that LAT-deficient CD4^{+} T cells were capable of causing a lymphoproliferative syndrome and producing large amounts of cytokines, such as IL-4.

Figure 4.10 Decreased expression of Foxp3, CTLA-4, and CD25 in LAT-deficient Treg cells

$ERCre^{+}LAT^{f/-}$ and $LAT^{f/-}$ littermates were treated with tamoxifen on two consecutive days. Ten days after the initial treatment, cells from the lymph node and spleen were harvested and analyzed. (a) Foxp3 expression in $CD4^{+}$ lymph node cells (left), and spleen (middle); Relative mean fluorescence intensity (MFI) of $CD4^{+}Foxp3^{+}$ splenocytes (right). (b and c) CD25 (b) and CTLA-4 (c) expression in $CD4^{+}Foxp3^{+}$ lymph node cells (left), and splenocytes (middle); Relative mean fluorescence intensity (MFI) of $CD4^{+}Foxp3^{+}$ splenocytes (right). $ERCreLAT^{f/-}$ samples were gated on GFP^{+} cells. The histograms shown are representative of 3 independent experiments performed, each using two mice per genotype. Error bars on bar graphs represent the standard error of the mean of five mice analyzed. Student t-test: **<0.001. (d) RNA levels of Foxp3 in $ERCreLAT^{f/-}$ and $LAT^{f/-}$ $CD4^{+}CD25^{+}$ cells. Samples were serially diluted by two fold prior to PCR. The figure represents three independent experiments.

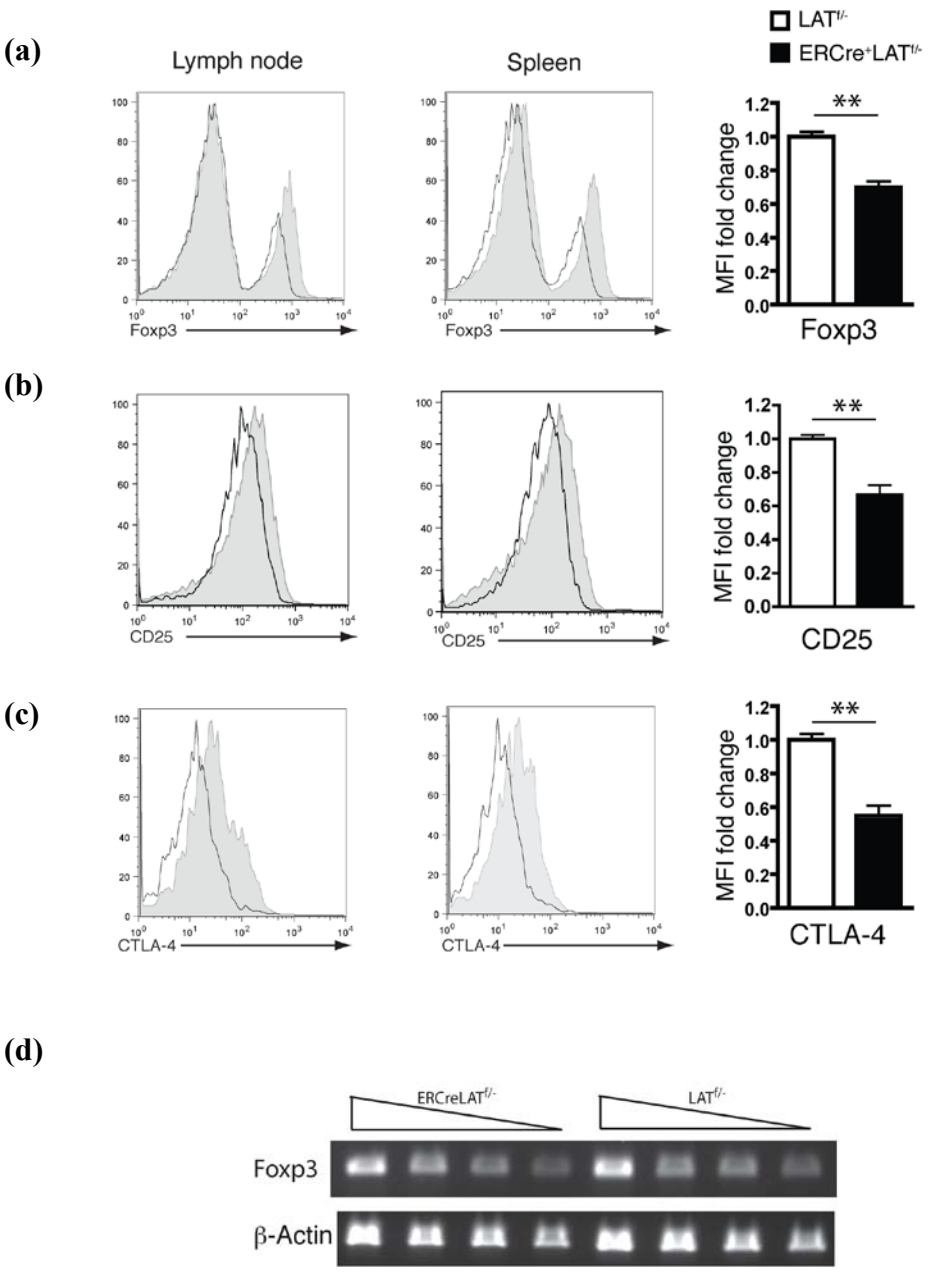

(a)
Lymph node
Spleen
LAT f/-
ERCre+LAT f/-
Foxp3
Foxp3
MFI fold change
**
Foxp3
(b)
CD25
CD25
MFI fold change
**
CD25
(c)
CTLA-4
CTLA-4
MFI fold change
**
CTLA-4
(d)
ERCreLAT f/-
LAT f/-
Foxp3
β-Actin

Figure 4.10

After tamoxifen treatment, $LAT^{-/-}$ mice reconstituted with $ERCre^{+}LAT^{f/-}$ T cells developed a Th2-type autoimmune-like syndrome characterized by splenomegaly and IgG_1/IgE hypergammaglobulinemia. This phenotype is very similar to the one seen in LATY136F knockin mice, in which the LAT-PLC-γ1 interaction is abolished (Aguado et al., 2002; Sommers et al., 2002). As discussed in Chapter 3, and as shown by others (Archambaud et al., 2009), this disease arises independently of abnormal thymic selection in the knockin mice. Instead, the LATY136F mutation in peripheral T cells is sufficient to confer pathogenicity and induce a similar disease. Moreover, LAT^{3YF} knockin mice, which have mutations at Y175, Y195, and Y235, also exhibit a similar syndrome mediated by mutant γδ T cells (Nunez-Cruz et al., 2003). These data seem to support the assertion that, despite being a critical positive regulator in TCR activation, LAT may also play a negative role.

While both $ERCre^{+}LAT^{f/m}$ and $ERCre^{+}LAT^{f/-}$ $CD4^{+}$ T cells produce abnormally high levels of cytokines and undergo seemingly uncontrolled expansion, our data showed that $ERCre^{+}LAT^{f/m}$ T cells were more hyperactive and triggered a more drastic disease. What causes the difference in the severity of the autoimmune syndromes is not clear. Such a negative role of LAT, if it exists, should be missing in T cells from $ERCre^{+}LAT^{f/m}$ and $ERCre^{+}LAT^{f/-}$ mice after tamoxifen treatment. It is possible that, in the absence of this negative regulation by LAT, the LATY136F mutant is able to drive more

expansion of $CD4^+$ T cells. The signals that drive this expansion remain to be determined. Previous studies have shown that $CD4^+$ T cells expressing the LATY136F mutant form of LAT can expand in the absence of MHC Class II (Wang et al., 2008), indicating that the engagement of the TCR with MHC-self-peptide complexes is likely not involved in the development of this syndrome. This speculation is supported by the fact that TCR-mediated LAT and PLC-γ1 phosphorylation is severely impaired in LATY136F T cells (Sommers et al., 2002). However, it is still possible that LAT-mediated tonic signals from the TCR, which are absent in $ERCre^+LAT^{f/-}$ T cells, are able to drive faster expansion of LATY136F T cells. Even though the LATY136F mutant fails to bind PLC-γ1, it still binds to Grb2 and Gads, thus partially functioning in TCR-mediated signaling (Zhang et al., 2000). This partial or unbalanced signaling is supported by data showing that Erk activation in the mutant T cells is enhanced compared with that in normal T cells (Sommers et al., 2002). Not only did the LATY136F T cells, as those in $ERCre^+LAT^{f/m}$ mice, expand faster, they also produced more cytokines, such as IL-4, than $ERCre^+LAT^{f/-}$ T cells (Figure 4.7). This dysregulation is likely responsible for driving the expansion of these mutant T cells, as well as the maturation and isotype switching of B cells.

Furthermore, our data showed that, after tamoxifen treatment, the percentage and number of $CD4^+Foxp3^+$ Treg cells in $ERCre^+LAT^{f/-}$ mice were decreased. Additionally, Foxp3 expression in Treg cells, as well as CTLA-4 and CD25 expression,

was reduced. Moreover, the effect of LAT deficiency on the expression of these proteins occurred within 10 days of tamoxifen treatment, well before the onset of the lymphoproliferative disorder. These data suggested that the mechanisms behind the T cell hyperproliferation are complex and may involve a breakdown in Treg-mediated peripheral tolerance due to the loss of LAT.

We have previously shown that the LAT-PLC-γ1 interaction plays a critical role in the development of $CD4^+CD25^+$ Treg cells, as LATY136F mice lack this population (Koonpaew et al., 2006). However, the data from this study suggested that LAT is not only important in the development of Treg cells, but also in the maintenance and/or survival of mature Treg cells. How TCR signaling regulates the expression of Foxp3 during Treg development has been answered with some satisfaction; however, the role of TCR signaling in regulating mature Treg homeostasis is not yet clear. Interestingly, Kim et al. recently reported that specific abrogation of TCR signaling in Treg cells by inactivation of $p56^{Lck}$ resulted in defective Treg turnover, loss of suppressive function, and an altered gene expression profile, including reduced CTLA-4 expression (Kim et al., 2009). These data, together with our results, suggest that mature Treg homeostasis in the periphery is dependent on sustained TCR signaling. Intriguingly, a minimal decrease of Foxp3 expression was observed in $p56^{Lck}$-inactivated Treg cells (Kim et al., 2009), while a noticeable reduction of Foxp3 expression was consistently observed in our LAT-depleted Treg cells. As a transcription factor, Foxp3 has long been established as

the master regulator of Treg cell development and function (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Interestingly, recent data demonstrate that Foxp3 expression in mature Treg cells is not stable (Zhou et al., 2009). Treg cells with transient Foxp3 expression can convert into inflammatory effector T cells. This finding highlights the importance of understanding the precise mechanisms controlling Foxp3 expression. Our results implicate a potential role of the loss of LAT in disrupting the maintenance of Foxp3 expression in Treg cells.

5. General discussion and future direction

5.1 What causes the LATY136F phenotype?

LATY136F mice develop a lymphoproliferative disease characterized by hyperactivated T cells that expand uncontrollably in the periphery (Aguado et al., 2002; Sommers et al., 2002). The factors driving the development of this disorder have been a subject of controversy. The initial analysis of LATY136F mice demonstrated that T cells from these mice express low levels of TCR and exhibit defective TCR-mediated signaling; upon TCR stimulation, LAT and PLC-γ1 phosphorylation are decreased while Ca^{2+} mobilization and T cell proliferation are also impaired (Aguado et al., 2002; Sommers et al., 2002). The weak signals emanating from the TCR of LATY136F T cells suggest that the uncontrolled expansion of these mutant conventional T cells occurs independently of TCR-MHC Class II interaction.

Subsequent studies by Sommers et al. have demonstrated that central tolerance is defective in these mice. Specifically, thymocytes do not undergo normal negative selection, thereby allowing for the development of autoreactive T cells upon exiting the thymus (Sommers et al., 2005). This evidence implicates a defect in thymic selection as the primary cause of the development of the LATY136F phenotype. However, it was shown that there is development of the lymphoproliferative disorder in T cell- and MHC Class II- double deficient mice that were reconstituted with LATY136F CD4 T cells (Wang et al., 2008). This finding definitively confirms that this disease occurs in an MHC Class II-independent manner and therefore virtually excludes the possibility that this disease is caused entirely by defects in negative selection.

Investigations done in our laboratory have demonstrated that $CD4^{+}Foxp3^{+}$ Treg cells are absent from the periphery of these mice. Moreover, reconstitution of LATY136F neonates with wildtype natural Treg cells rescued these mice from the disorder (Koonpaew et al., 2006). A group from Malissen's laboratory also evaluated this issue using Foxp3EGFP reporter mice. While their findings did not show a complete loss of $Foxp3^{+}$ Treg cells, this cell population was drastically reduced in the periphery (Wang et al., 2008). In addition, the LATY136F phenotype can be rescued by adoptive transfer of wildtype Treg cells. However, the phenotype is not fully suppressed in this model, but, rather, delayed, likely due to a dearth of wildtype Treg cells adoptively transferred into

the LATY136F mice. Nevertheless, these experiments collectively stress the importance of Treg cells in the development of the LATY136F disease.

The results presented in this thesis show that, even with proper thymic development, the defect in peripheral tolerance in these mice is sufficient to cause the development of the disorder. Using an ERCre conditional knock-in mouse model, our data demonstrate that, despite normal T cell development, and thymic selection processes, mature LATY136F mutant T cells were still able to recapitulate the lymphoproliferative disease. Treg cells were present in these mice but were non-functional, allowing for the uncontrolled expansion of the mutant conventional $CD4^+$ T cells. Furthermore, we observed a drastic increase in the number of $CD11c^+MHCII^+$ DCs in $ERCreLAT^{f/m}$ mice.

Recent studies have implicated a role for dendritic cells in regulating the homeostasis of Treg cells (Darrasse-Jeze et al., 2009). The proposed homeostatic feedback loop (shown in Figure 5.1) consists of an increase in dendritic cells, which results in a higher number of Treg cells. This spike in Treg cells causes an increase in IL-10 and TGF-β production and CTLA-4 expression, which in turn causes a decline in the number of dendritic cells. $ERCreLAT^{f/m}$ mice treated with tamoxifen for 4 weeks exhibited increased numbers of $CD11c^+$MHC Class II^+ cells with increased total numbers of Treg cells. However, these Treg cells had decreased CTLA-4 protein expression and decreased IL-10 and TGF-β RNA expression after *in vitro* TCR stimulation. These defects

Figure 5.1 Proposed model for the observed autoimmune syndrome caused by ERCreLAT$^{f/m}$ T cells

(a) As proposed by Darrasse-Jeze et al., dendritic cells (DCs) are activated by a growth factor signal, such as through Flt3 ligand, which leads to an increase in regulatory T (Treg) cells. This is dependent on the expression of surface MHC Class II molecules by DCs. As a result of increased Treg cells and subsequent increased expression of anti-inflammatory molecules, the number of DCs decreases followed by a similar decrease in Treg cells. (b) In ERCreLAT$^{f/m}$ mice, an unknown cell type, probably conventional T cells, produces a growth signal that induces the increased number and activation of DCs. As a result, Treg cells increase in number. However, due to the failure of Treg cells to express normal levels of CTLA-4, IL-10, and TGF-β, DC numbers do not decrease, but rather continue to increase. This likely plays a role in inducing the lymphoproliferative disease characteristic of LATY136F mice. (Adapted from (Darrasse-Jeze et al., 2009))

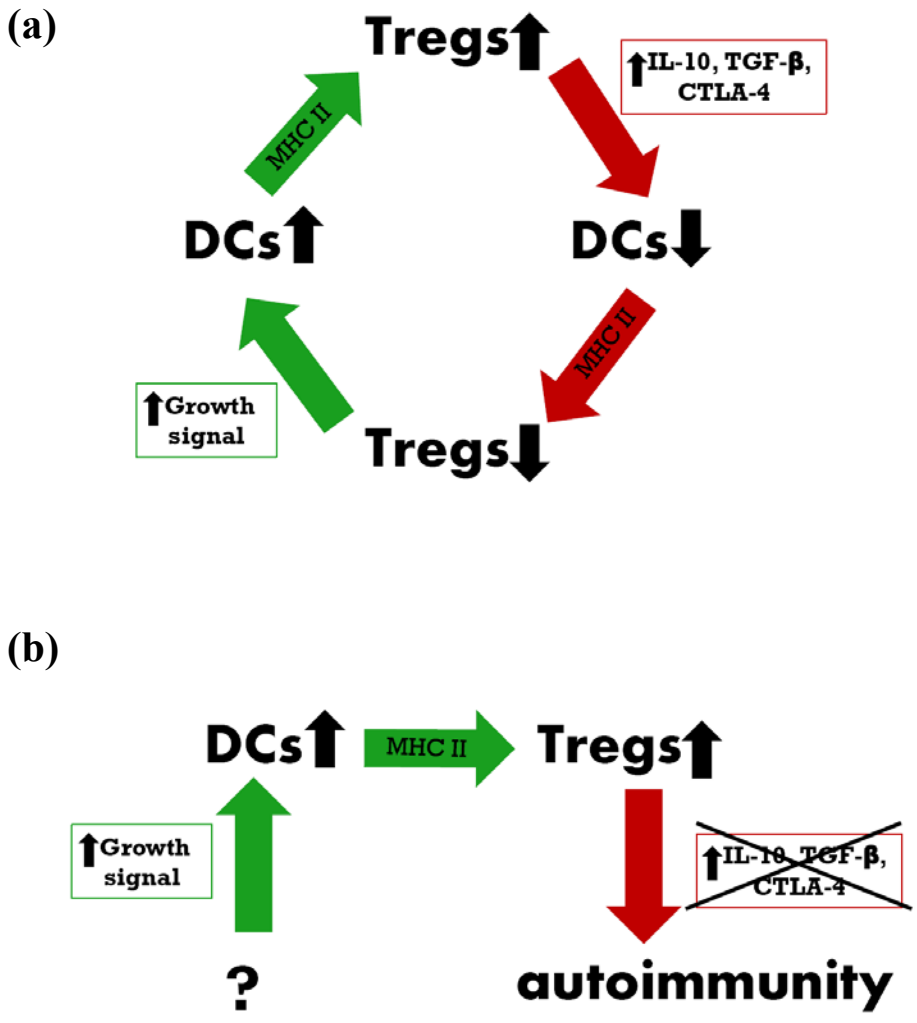

(a)
Tregs
IL-10, TGF-β, CTLA-4
DCs
MHC II
Tregs
Growth signal
DCs
MHC II
(b)
DCs
MHC II
Tregs
Growth signal
IL-10, TGF-β, CTLA-4
?
autoimmunity

Figure 5.1

may disrupt the feedback loop, thereby not allowing for the decrease in dendritic cell numbers that would normally follow; observed in ERCreLAT$^{f/+}$ control mice no increase in dendritic cells numbers. Furthermore, splenic CD11c^{+}MHC Class II^{+} cells in ERCreLAT$^{f/m}$ mice were highly activated, expressing high levels of CD40, CD86, and CD80. This is similar to findings showing that a lack of CTLA-4 expression in Foxp3^{+}CD4^{+} cells rendered these cells unable to attentuate CD80 and CD86 expression on DCs (Wing et al., 2008). It is likely that the highly activated state of the DCs in ERCreLAT$^{f/m}$ mice induces the aberrant expansion of conventional T cells. In order to test this, dendritic cells can be ablated from the periphery prior to induction of the lymphoproliferative disorder. This experimental setup is diagrammed in Figure 5.2. This model utilizes CD11cDTR mice to achieve the deletion of dendritic cells.

While our data suggested that the number of dendritic cells was increased in ERCreLAT$^{f/m}$ mice, studies have shown that cells other than DCs also express CD11c. One such subset that expresses low levels of CD11c is a newly identified population of autoimmune B cells (ABCs) (Isnardi et al., 2010). It is possible that the relatively large population of CD11c^{int}MHC Class II^{+}B220^{+} cells that was characterized as pDCs in Section 3.2.4 could in fact be ABCs. In order to investigate this possibility, CD11c^{int} cells from tamoxifen-treated ERCreLAT$^{f/m}$ mice will have to be analyzed for the expression of molecules that phenotypically define these ABCs; these cells are CD11c$^{int/+}$ B220^{+}MHCII^{hi}IgM^{+}CD80^{+}CD86^{+}.

Figure 5.2 Proposed experimental model for the analysis of the role of dendritic cells in the development of the observed lymphoproliferative syndrome mediated by ERCreLAT$^{f/m}$ T cells

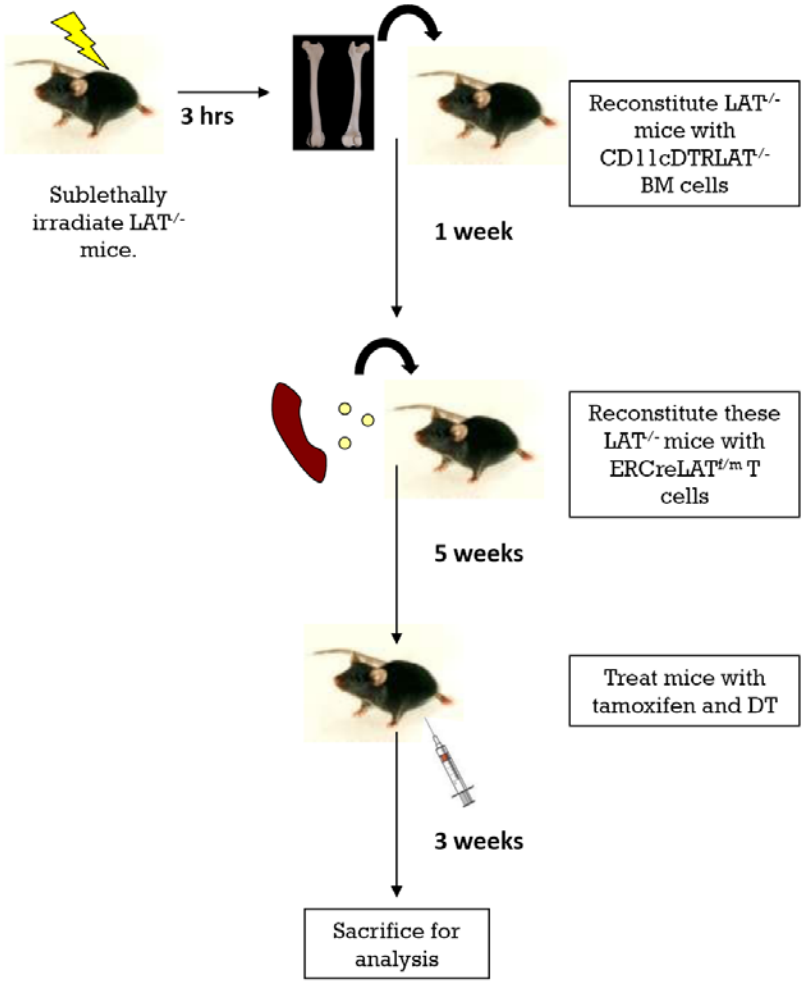

3 hrs
Sublethally irradiate LAT-/- mice.
Reconstitute LAT-/- mice with CD11cDTRLAT-/- BM cells
1 week
Reconstitute these LAT-/- mice with ERCreLAT f/m T cells
5 weeks
Treat mice with tamoxifen and DT
3 weeks
Sacrifice for analysis

Figure 5.2

5.2 What is the role of LAT in Treg cell survival and maintenance?

LATY136F mice lack peripheral natural regulatory T cells (Koonpaew et al., 2006). However, when T cells undergo normal development and selection processes, as in ERCreLAT$^{f/m}$ mice, these LAT-mutated Treg cells are present in the periphery. Furthermore, there is a slight increase in the number of Treg cells in these mice when compared to controls. This evidence supports the conclusion that the LAT-PLC-γ1 interaction is not essential for the survival or maintenance of Treg cells. In contrast, the number of Tregs from ERCreLAT$^{f/-}$ mice was significantly decreased early as 10 days after tamoxifen treatment. Collectively, these findings suggest that LAT-mediated signaling is indeed important for Treg cell maintenance or survival; however, the role of LAT in this context is not dependent on with the binding of LAT to PLC-γ1. It is therefore likely that the association of the Grb2-Sos complex with LAT is responsible for the presence of Treg cells in the periphery of ERCreLAT$^{f/m}$ mice and, conversely their absence in ERCreLAT$^{f/-}$ mice. The binding of Grb2-Sos to the cytoplasmic tail of LAT activates the Ras-Erk signaling cascade. As Erk is a well-documented mediator of survival within many different cell types, this is the most likely mechanism through which LAT plays a role in Treg cell survival.

The decrease in Treg cell numbers observed in ERCreLAT$^{f/-}$ mice also raises the question about the survival requirements of these cells once they have exited the thymus. Our data suggest that Treg cells need LAT-mediated signals for survival. These

signals are likely in the form of constant, weak TCR-mediated signals initiated by the binding of ubiquitous self-ligands. It is also notable that ERCreLAT$^{f/-}$ conventional CD4^{+} T cells did not exhibit *in vivo* survival defects at the same level of severity as those observed in Treg cells. These data support the hypothesis that there are differential survival requirements for the maintenance of these two T cell subsets.

It is also possible that the decrease in total numbers of LAT-deficient Treg cells (shown in Figure 4.9) is not a result of a survival defect. Studies have shown that in healthy mice, but moreso in autoimmune mouse models, a small percentage of Foxp3^{+} T cells downregulate Foxp3 expression and convert to "autoaggressive" T cells with effector function capabilities; these cells are called exFoxp3 cells (Zhou et al., 2009). Similarly, LAT-deficient Treg cells may have increased instability of Foxp3 expression-enough to convert Foxp3^{+}CD4^{+} cell to effector cytokine-producing exFoxp3 cells. This will further support our data (shown in Figure 4.9) demonstrating that the expression, of Foxp3 is downregulated in the absence of LAT. In order to assess whether the decrease in total Treg cell numbers observed was due to a survival defect or an increased rate of conversion into exFoxp3 cells, we can employ a mixed reconstitution model. Thy1.1^{+} wildtype conventional T cells can be adoptively transferred into LAT$^{-/-}$ mice with either Thy1.2^{+}ERCreLAT$^{f/-}$ Treg cells or control Thy1.2^{+}LAT$^{f/-}$ Treg cells. The use of congenic markers allows for the tracking of the Treg populations. If after reconstitution and tamoxifen treatment a large percentage of Thy1.2^{+}ERCreLAT$^{f/-}$ cells are Foxp3^{-}, it is likely

that these cells have converted to exFoxp3 cells. Cytokine production by these cells can be measured to confirm this conclusion. This result would further support our finding that LAT-mediated signaling is involved in regulating Foxp3 expression.

5.3 How does LAT regulate Foxp3 expression?

Foxp3 acts as a master regulator of the regulatory T cell lineage: This transcription factor is responsible for the distinct genetic program that defines cells as Treg cells. When Foxp3 is ablated in peripheral Treg cells, these cells lose their suppressive function and essentially undergo transcriptional deprogramming (Williams and Rudensky, 2007). This occurs because Foxp3 regulates other genes that are important for regulatory T cell function and homeostasis. While many target genes that are regulated by Foxp3 have been identified, not much is known about what regulates Foxp3 expression, specifically in the periphery. As shown in Chapter 4, LAT-deficient regulatory T cells had significantly reduced Foxp3 expression; similarly, CTLA-4 and CD25 expression are decreased, which is consistent with accumulating evidence that Foxp3 regulates the transcription of these genes. While the protein level of Foxp3 was decreased, a difference in the RNA level was not detected. These data suggest that LAT-mediated signaling is involved in regulating Foxp3 expression at a post-transcriptional level.

As transcription of Foxp3 is seemingly unaffected by LAT deficiency, it is possible that the decrease in Foxp3 at the protein level is caused by defective translation.

Indeed, upon TCR crosslinking of ionomycin treatment, T cells upregulate the expression of eukaryotic translation initiation factor 4 E (eIF4E). This protein is a member of the eIF4F complex that is commonly the target of translational control. Ras, Erk, and MAPK mediate the phosphorylation of eIF4E which usually correlates to translation activity. Whether LAT plays a role in translational control of Foxp3, and the potential mechanism that could enable this process, requires further investigation.

Ubiquitination is a form of post-translational regulation that most commonly leads to the proteosomal degradation of proteins. How (or if) this process is involved in the maintenance of Foxp3 protein expression is not yet known. It is possible that LAT-mediated signaling prevents the direct ubiquitination of Foxp3 thereby sustaining its stability. Published studies have demonstrated that Foxp3 protein expression is indirectly regulated by this process. The E3 ubiquitin ligase Itch ubiquitinates the transcription factor TIEG1 which in turn allows for TGFβ-induced Foxp3 expression. LAT-mediated signaling may play a role in stabilizing Foxp3 expression by regulating molecules involved in ubiquitination; this could include the ubiquitination of Foxp3 itself or other upstream molecules. However, other post-translational modifications, such as acetylation, poly ADP-ribosylation, and methylation, may also directly or indirectly affect the stability of Foxp3.. Whether LAT plays a role in Foxp3 stability can be tested by doing a pulse-chase assay using ERCreLAT$^{f/-}$ Treg cells. Alternatively, these

cells can be cultured in the presence of a protein synthesis inhibitor in order to analyze whether Foxp3 degrades at a faster rate without LAT.

It is also possible that there is a difference in Foxp3 RNA level that is too small for detection but is still significant in its impact on the level of protein expression. The Foxp3 gene has a promoter, an upstream enhancer, and an intronic enhancer. The proximal promoter region of *Foxp3* contains AP-1 and NFAT binding sites which potentially play a role in regulating the transcription of this gene. The abrogation of LAT-mediated signaling in ERCreLAT$^{f/-}$ Treg cells likely affects the activation of AP-1 and NFAT and may therefore influence the binding of these transcription factors to the *Foxp3* promoter. In order to address this question a chromatin immunoprecipitation (ChIP) assay can be performed. Published studies have also revealed multiple sites of epigenetic regulation via methylation and acetylation within the Foxp3 promoter and/or enhancer regions. Comparison of these regions in ERCreLAT$^{f/-}$ versus LAT$^{f/-}$ Treg cells may reveal differences that explain the decrease in Foxp3 protein expression observed in the LAT-deficient Treg cells. Such studies may reveal a mechanism through which LAT exerts its influence on Foxp3 expression and stability.

In this dissertation, I have presented data that demonstrate the importance of the LAT-PLC-γ1 interaction in the suppressive function of regulatory T cells. Conventional CD4$^+$ T cells expressing a mutation that disrupts this association mediated the development of a lymphoproliferative disorder despite undergoing normal thymocyte

development and negative selection. This finding proves that impaired central tolerance is not solely responsible for the development of the LATY136F lymphoproliferative syndrome. Our data strongly suggest that this phenotype is at least partially caused by defective peripheral tolerance, specifically lack of suppression by regulatory T cells. I have also presented data that demonstrate that this disorder, though less severe than the disorder mediated by LATY136F-mutated T cells, develops in mice in which total LAT is ablated. Regulatory T cells from these mice are also nonfunctional and, additionally, exhibit lower levels of Foxp3 and a decrease in their total numbers, suggesting that LAT is important for the maintenance of Foxp3 expression and Treg cell survival. Collectively, these studies illuminate the importance of LAT in positively mediating regulatory T cell function and negatively regulating conventional T cell homeostasis.

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Biography

I was born on the 9th of January, 1984 in St.Andrew, Jamaica where I lived for 17 years. I attended Brown University from August 2001 until December 2004; at this time I was awarded my Bachelor of Science degree with Honors in Biology. From the spring of 2004 to the summer of 2005, I conducted research on xenogeneic graft versus host disease at the Rhode Island Hospital under the mentorship of Dr. Loren Fast. My education in Jamaica, my courses at Brown University, and my research with Dr. Fast collectively cultivated an appreciation for the study of the immunological system. Fortunately, I next chose to attend Duke University in order to earn my doctoral degree in Immunology. For the last 5 years, I have studied under the guidance of the proficient Dr. Weiguo Zhang. My research in his lab was focused on the study of adaptor proteins in lymphocyte signaling.

Publications:

- Shen S*, **Chuck M***, Zhu M, Zhang W. *The Importance of Linker for Activation of T cells in T Cell Activation, Homeostatic Proliferation, and Cell Survival*. [submitted]; *denotes shared first co-authorship.
- **Chuck M**, Zhu M, Shen S, Zhang W. *The role of the LAT-PLC-γ1 interaction in T regulatory function.* J Immunol. 2010 Mar 1;184(5):2476-86
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