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Regulation of Lymphocyte Development and Activation by the LAT Family of Adaptor Proteins

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The LAT Family of Adaptor Proteins

Transmembrane adaptor proteins (TRAPs) are critical components of signaling pathways in lymphocytes, linking antigen receptor engagement to downstream cellular processes. While these proteins lack intrinsic enzymatic activity, their phosphorylation following receptor ligation allows them to function as scaffolds for the assembly of multi-molecular signaling complexes. Many TRAPs have recently been discovered and numerous studies demonstrate their roles in the positive and negative regulation of lymphocyte maturation, activation, and differentiation. One such example is the LAT (linker for activation of T cells) family of adaptor proteins. While LAT has been shown to play an indispensable role in T cell and mast cell function, the other family members, LAB (linker for activation of B cells) and LAX (linker for activation of X cells), are necessary to fine-tune immune responses. In addition to its well-established role in the positive regulation of lymphocyte activation, LAT exerts an inhibitory effect on TCR (T cell receptor)-mediated signaling. Furthermore, LAT, along with LAB and LAX, plays a crucial role in establishing and maintaining tolerance. Here, we review recent data concerning the regulation of lymphocyte development and activation by the LAT family of proteins.

Keywords

TRAP (transmembrane adaptor protein); tyrosine phosphorylation; autoimmune lymphoproliferative disease; T cell homeostasis; PLC- γ

Introduction

The immune system depends upon a number of finely tuned signals to maintain homeostasis and to drive the development, differentiation, and activation of its many cellular components. Most of these processes are regulated by antigen receptors, such as the T and B cell receptors (TCR and BCR), on the surface of lymphocytes (1). These antigen receptors are indispensable for the integration of the different signals that guide immune responses. A key element to bridging the initial engagement of an antigen receptor with downstream cellular processes is the TRAP (transmembrane adaptor protein) (2).

Upon receptor engagement, immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic tails of receptor-associated subunits are phosphorylated by Src family kinases, such as Lck, Lyn, and Fyn (3–5). The phosphorylated tyrosines within ITAM motifs serve as docking sites for the Syk family kinases, Syk and Zap-70 (ζ -chain-associated protein kinase of 70 kDa) in B and T cells, respectively. Syk and Zap-70 are then phosphorylated and activated by the Src kinases, initiating a cascade of downstream signaling pathways by phosphorylating other proteins in close proximity to the receptor

complex (6). Signals may be amplified by the presence of multiple ITAMs within antigen receptors and by the compartmentalization of receptors, co-receptors, and co-stimulatory molecules within specific regions of the plasma membrane called lipid rafts (7–9).

TRAPs are essential for the integration of extrinsic signals into a cellular output by organizing multi-molecular protein complexes at the plasma membrane. Although they typically have no intrinsic enzymatic activity, TRAPs are phosphorylated and serve as scaffolds to recruit critical downstream effector proteins (10–12). Many adaptor proteins have been recently discovered and the crucial roles that TRAPs play in antigen receptor-mediated signaling have been highlighted in studies using cell lines and genetically-deficient mice. Recent advances show that LAT (linker for activation of T cells) is one of the most important TRAPs in hematopoietic cells. LAT serves to nucleate a large signaling complex upon TCR engagement that is essential for T cell development and function (13–14). While LAT plays an indispensable role in TCR- and FcεRI-mediated signaling, two other LAT family adaptor proteins, LAB (linker for activation of B cells)/NTAL (non-T cell activation linker) and LAX (linker for activation of X cells) are important for the fine-tuning of lymphocyte activation (15–16). The LAT family of proteins is able to exert both positive and negative effects on the complex signaling pathways that regulate immune responses in a variety of hematopoietic cells.

In addition to the LAT family of adaptor proteins, other TRAPs are critical for immune system signaling. For example, SIT (SH2 domain-containing phosphatase 2-interacting TRAP) and TRIM (TCR-interacting molecule) negatively regulate TCR-mediated signals and are important for the development of thymocytes. The loss of both of these adaptor proteins leads to enhanced positive selection, including an up-regulation of CD5, CD69, and TCR-β surface expression and strong MAPK (mitogen activated protein kinase) activation (17). PAG (phosphoprotein associated with glycosphingolipid-enriched domains) is able to inhibit Src kinase activity and Ras activation through its recruitment of Csk (C-terminal Src kinase) and RasGAP (RasGTPase activating protein) to the membrane (18–20). LIME (Lck-interacting molecule) is an adaptor protein known to associate with Lck, Gads (Grb2-related adaptor protein), and Grb2 (growth factor receptor-bound protein 2), leading to the activation of ERK1/2 (extracellular-signal regulated kinase) and JNK (Jun N-terminal kinase) (21). This review, however, will focus on the more recent advances from studies concerning the roles of LAT, LAB, and LAX in immune system signaling.

LAT

Many details of the protein later to be named LAT were known before this protein was actually discovered. In the search for a molecule that could bridge TCR engagement to the downstream activation of signaling pathways, attention focused on a 36–38 kDa protein that is heavily tyrosine-phosphorylated after TCR stimulation (22). Further investigation showed that this protein localizes in the plasma membrane and is able to bind to the SH2 domains of Grb2, Grap (Grb2-related adaptor protein), PLC-γ1 (phospholipase C), and the p85 subunit of PI3K (phosphatidylinositol 3-kinase) (23–27). Therefore, this protein seemed to be an ideal candidate for linking initial TCR engagement to a complex orchestration of downstream signaling pathways.

Cloning of LAT revealed that it is a transmembrane protein containing a short extracellular domain, transmembrane domain, and cytoplasmic tail with nine conserved tyrosine motifs. It is expressed in thymocytes, mature T cells, NK cells, mast cells, megakaryocytes, and pre-B cells (13, 28–30). The juxtamembrane region of LAT contains two cysteine residues, C26 and C29 in humans, which are critical for LAT palmitoylation, raft localization, and function. Mutation of these cysteines, especially C26, abolishes LAT localization to lipid

rafts and severely diminishes phosphorylation of LAT in response to TCR ligation (31). Although the physiological relevance of LAT localization to lipid rafts is still controversial (32), LAT palmitoylation is undeniably essential for its function. Studies examining antigen-primed anergic T cells show that LAT palmitoylation is impaired in these cells, leading to the disruption of downstream signaling events, such as PLC- γ 1 phosphorylation. Signaling events upstream of LAT, such as ZAP-70 phosphorylation, remain intact in these cells, implicating the LAT palmitoylation defect as the initiating event in the establishment of anergy in T cells (33). However, how anergy induction in T cells is affected by LAT palmitoylation is still unclear.

The functional importance of LAT was clearly demonstrated in LAT-deficient Jurkat T cells, in which TCR-mediated calcium mobilization, Erk activation, CD69 upregulation, and AP (activator protein)-1- and NFAT (nuclear factor of activated T cells)-mediated gene transcription are severely impaired (34). To translate these *in vitro* results into a physiological role for LAT in T cells, LAT-deficient mice were generated. These mice show normal B cell populations but a complete lack of peripheral T cells. Closer examination of thymocytes from LAT^{-/-} mice reveals that LAT is essential for the generation of DP and SP thymocytes, as these mice have a block at the DN3 stage of thymocyte development. Additionally, $\gamma\delta$ T cells are absent in the periphery. These studies demonstrate the critical role of LAT in pre-TCR signaling and during thymocyte development (35).

However, because LAT-deficient mice have a severe block at the DN3 stage, the role of LAT in the later stages of thymic development could not be elucidated from analysis of LAT^{-/-} thymocytes. Therefore, our lab recently generated LAT knock-in mice in which the *lat* gene could be deleted by the Cre recombinase. Deletion of LAT by Cre under the control of the CD4 proximal promoter allows for the generation of DP thymocytes; however, the transition from DP to SP thymocytes is severely blocked. Therefore, LAT plays an irreplaceable role in both the early and late stages of thymic development (36).

LAT Phosphorylation and Interaction with other Signaling Proteins

As an adaptor protein lacking any intrinsic enzymatic activity, the ability of LAT to transmit signals depends upon its phosphorylation, which initiates the recruitment of a number of other signaling proteins. Upon TCR engagement, phosphorylation of LAT allows it to interact with several SH2 domain-containing proteins, such as Grb2 and PLC- γ 1 (13). Studies reconstituting LAT-deficient Jurkat cells with LAT mutants unable to bind PLC- γ 1 and Grb2 show the necessity of the association of LAT with both of these proteins for TCR-mediated Ras activation, calcium flux, and NFAT activation. Correspondingly, these interactions are necessary for thymocyte development (37). LAT also contains two binding sites for Gads. LAT association with Gads is needed for full activation of T cells, although reconstitution of LAT-deficient Jurkat cells with a LAT mutant unable to bind Gads shows slight restoration of calcium flux and NFAT activation (37). Additionally, Gads is constitutively associated with SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa), allowing LAT to indirectly bind to SLP-76, a cytosolic adaptor (13, 30, 38). SLP-76 has been shown to be indispensable for TCR signaling through its regulation of actin polymerization following receptor engagement. Its essential role *in vivo* is demonstrated in SLP-76-deficient mice, in which thymocytes are unable to progress past the DN3 stage of development (39).

Our published data indicate that Grb2, Gads, and PLC- γ 1 may bind cooperatively to LAT (40). Only LAT mutants that are capable of binding Grb2 and PLC- γ 1 are able to reconstitute T cell activation and thymocyte development, highlighting the importance of these tyrosine residues in LAT function (37). Not surprisingly, knowing the critical role of LAT as a docking protein, mutating the four distal tyrosine residues on LAT, which mediate

binding to Gads, Grb2, and PLC- γ 1, renders Jurkat T cells completely unresponsive to receptor engagement (40). These results were made manifest in thymocyte development as well. LAT knock-in mice harboring mutations at the four distal tyrosines – Y136, Y175, Y195, and Y235 in mice – have an identical phenotype to LAT^{-/-} mice (41).

The importance of the LAT-PLC- γ 1 interaction has been revealed by recent studies that indicate a potential role for LAT in T cell homeostasis and the regulation of autoimmunity. Experiments using Jurkat T cells expressing LAT with a Y136F mutation at the PLC- γ 1 binding site show the necessity of this residue for the mobilization of calcium and the activation of NFAT, demonstrating that the LAT-PLC- γ 1 interaction is critical for the emanation of signals originating from the TCR (42, 43). To elucidate the importance of the LAT-PLC- γ 1 interaction *in vivo*, two groups independently generated LATY136F knock-in mice, which harbor a point mutation in the PLC- γ 1 binding site of LAT (44, 45). LATY136F mice have a partial block at the DN3 stage of thymocyte development; however, a small percentage of cells are able to mature into DP and SP cells. Surprisingly, after maturation and entry into the periphery, these T cells become hyperactive and quickly initiate a fatal lymphoproliferative disease (44–46).

The autoimmune-like disease observed in LATY136F mice is T_H2-skewed, characterized by tissue eosinophilia and massive production of IgE and IgG₁. These mice have severe tissue infiltration that causes lymphadenopathy, splenomegaly, and lesions in the lung, liver, and kidney. The infiltrating T cells are characterized by a memory/activated phenotype with high surface expression of CD44 and downregulated expression of CD62L. Furthermore, these T cells are resistant to TCR-mediated cell death. B cells from these mice are hyperactivated, resulting in elevated autoantibody serum titers and systemic autoimmunity marked by the incredible production of IgE and IgG₁ (44–46). These findings raise the possibility that LAT could have a negative effect on T cell homeostasis in addition to its established role as a critical positive regulator of the immune response.

Further analysis of LATY136F mice using the HY-TCR transgenic system, which is routinely used to examine positive and negative thymic selection events, show that both thymic selection processes are severely impaired in LATY136F mice. To this end, autoreactive T cells that would normally be deleted are now able to escape central tolerance, perhaps contributing to their uncontrolled expansion in the periphery (47). However, adoptive transfer of LATY136F T cells into MHC-deficient hosts does not impair the slow and sustained proliferation of the mutant T cells. Therefore, the LATY136F T cells are able to expand in the absence of MHC molecules and the presentation of self ligands by antigen presenting cells (48). This observation shows the completely atypical nature of these T cells and the complex characteristics of this autoimmune disorder.

In addition to the inability of LATY136F mice to properly select for T cells in the thymus, our results show that LATY136F mice fail to develop natural CD4⁺CD25⁺ T regulatory cells (T_{regs}). Foxp3 expression is significantly reduced at the RNA level and Foxp3 protein expression is not detectable by intracellular antibody staining in thymocytes and mature T cells from these mice. This lack of CD4⁺CD25⁺ T_{regs} plays a role in the development of the LATY136F disease as adoptive transfer of normal T_{reg} cells into neonatal LATY136F mice prevents the development of the lymphoproliferative disease. Additionally, ectopic expression of Foxp3, which converts conventional CD4⁺25⁻ T cells into CD4⁺CD25⁺ regulatory T cells (49), is able to confer suppressor function onto LATY136F T cells (50).

In contrast to our results, Wang *et al* employed a system using *Foxp3EGFP* reporter mice to study the contribution of defective T_{regs} to the LATY136F phenotype (48). Their study shows that Foxp3⁺ T cells are actually present in LATY136F mice but are nonfunctional.

Furthermore, they assert that conventional LATY136F T cells are able to escape the control of wildtype regulatory T cells. Since we fail to detect the presence of Foxp3⁺ cells in these mice by intracellular staining, it is very possible that the Foxp3 expression in their GFP⁺ cells is low, leading to impaired regulatory function. While our study correctly concludes that normal CD4⁺CD25⁺Foxp3⁺ T_{regs} are absent in LATY136F mice, some Foxp3⁺ cells are able to develop in these mice. However, whether natural regulatory T cells are able to suppress the autoimmune syndrome in a long-term situation in these mice remains to be determined.

The LATY136F lymphoproliferative disease is characterized by tremendous amounts of IL-4 and T_H2-type skewing. Interestingly, a recent study indicates that the lymphoproliferative disease is not dependent upon the assumption of a T_H2 lineage fate. In the absence of STAT6, the transcription factor responsible for IL-4 production and T_H2 differentiation, STAT6^{-/-}LATY136F mice also develop a lymphoproliferative disease of the same timing and magnitude as LATY136F mice; however, this autoimmunity involves CD8⁺ and T_H1 cells, culminating in IgG_{2a} and IgG_{2b} hyperagammaglobulinemia (51). Therefore, independent of cytokine profiles and T helper cell differentiation, the lymphoproliferative disease that develops in these mice is dependent upon intrinsic T cell defects that result from the total abrogation of the LAT-PLC- γ 1 interaction. The T_H2-driven disorder in LATY136F mice seems to be a default pathway and, when this pathway is blocked, the T cells that are able to develop can assume a similar hyper-reactive profile regardless of their T cell lineage fate.

Similar to LATY136F mice, LAT3YF mice, which harbor mutations at tyrosines 175, 195, and 235, contain a form of LAT protein that retains only partial docking function. Mutation of these residues abolishes association with Gads and Grb2, whereas PLC- γ 1 may potentially still bind to the Y136 residue upon phosphorylation. LAT3YF mice show a complete block in $\alpha\beta$ T cell development but accumulate $\gamma\delta$ T cells in peripheral lymphoid organs, suggesting differential requirement of LAT in $\alpha\beta$ and $\gamma\delta$ T cell development. Unexpectedly, these mice also develop a severe lymphoproliferative disease characterized by T_H2-like $\gamma\delta$ T cells and IgG₁/IgE hyperagammaglobulinemia (52). The LAT3YF mice similarly lack T_{regs} but their disease onset cannot be attributed solely to the absence of this population because TCR- β ^{-/-} and E β ^{-/-} mice also lack all $\alpha\beta$ T cell lineages but do not develop disease (53, 54). As with all areas of scientific research, this field continues to make advances, forcing re-interpretation of previous studies. The LATY136F and LAT3YF phenotypes are certainly no exception to this paradigm and future work will be aimed at reaching a clearer understanding of the phenomena observed in these mice. Nevertheless, despite not knowing the exact mechanisms by which these diseases arise, the striking similarities between the disorders observed in both strains of LAT mutant mice emphasize the critical role of this adaptor protein in T cell development and homeostasis.

LAT as a Negative Regulator

As previously mentioned, the development of the severe lymphoproliferative disease in LATY136F and LATY3F mice suggests a role for LAT in the negative regulation of TCR signaling. Indeed, Gab2 (Grb2-associated binding protein 2), which is able to constitutively associate with Gads/Grb2, was shown to be recruited to lipid rafts by LAT upon TCR ligation in Jurkat cells. Gab2 exerts a negative effect on T cell signaling through its recruitment of SHP (Src homology 2 domain-containing tyrosine phosphatase)-2, which induces the de-phosphorylation of crucial signaling components, such as CD3 ζ . Additionally, Gab2 may negatively impact signaling by competing with SLP-76 for Gads/Grb2 binding. Thus, the inhibitory effect of Gab2 relies upon its inducible association with LAT (55, 56).

Additional work in cell lines showed that, upon TCR ligation, LAT associates with a complex containing Grb2, SHIP (Src homology 2 domain-containing inositol polyphosphate 5'-phosphatase)-1, and Dok (downstream of kinase)-2. This interaction then leads to the tyrosine phosphorylation of SHIP-1 and Dok-2, the latter of which serves as a negative regulator of TCR-mediated IL-2 production and Zap-70 activation. Therefore, LAT also serves as a source from which negative signals can propagate in T cells, presumably to dampen T cell responses and avoid hyperactivation (57).

In addition to the interactions between LAT and SHP2- and SHIP-containing complexes, the recruitment of the tyrosine phosphatase SHP (Src homology 2 domain-containing tyrosine phosphatase)-1 to lipid rafts and its interaction with LAT are markedly enhanced after TCR ligation (58). Another interesting study indicates that ERK and JNK are able to phosphorylate LAT at its Thr 155 residue following TCR engagement. The result of this phosphorylation event is a defect in the ability of LAT to recruit PLC- γ 1 and SLP-76, ultimately resulting in a decrease in calcium mobilization and MAP kinase activation (59). However, this threonine residue is only found in human LAT and a homologous phenomenon has not been discovered in mice. Nevertheless, these studies clearly imply a role for LAT in the negative regulation of T cell signaling, but the relevance and precise timing of these inhibitory mechanisms are yet to be fully understood.

LAT in Mast Cells

The critical role of LAT as an adaptor protein is not limited to T cell receptor signaling and indeed extends to Fc ϵ RI-mediated signaling. LAT^{-/-} mice, despite having normal numbers of mature mast cells, are resistant to IgE-mediated passive systemic anaphylaxis. LAT is also highly phosphorylated following Fc ϵ RI engagement and loss of this adaptor protein leads to a severe decrease in the activation and phosphorylation of its associated proteins, such as SLP-76, Vav, and PLC- γ 1/2. Consequently, bone marrow-derived mast cells from these mice have impaired MAPK activation, cytokine production, and degranulation (60).

In order to more fully investigate the role of the four distal tyrosines of LAT in Fc ϵ RI-mediated mast cell activation, LAT^{-/-} bone marrow-derived mast cells (BMMCs) were transduced with retroviruses expressing either wild-type or mutant forms of LAT. Analysis of mast cells harboring these mutations demonstrated that tyrosine phosphorylation, PLC- γ 1 phosphorylation, calcium mobilization, and degranulation after Fc ϵ RI engagement are decreased for all the LAT mutants. However, the cells containing LAT with mutations at the PLC- γ binding site (Y136F) only and with mutations at all four distal tyrosines have the most severe diminution in Ca²⁺ flux and degranulation. Furthermore, all four tyrosine sites are necessary for complete reconstitution of JNK, ERK, and p38 activation. An *in vivo* analysis of passive systemic anaphylaxis showed that mice lacking the four distal tyrosines have a marked decrease in histamine release while LAT^{Y136F} mice have no measurable histamine release, again highlighting the critical role of this residue *in vivo* (61).

Additional experiments using LAT-deficient BMMCs reconstituted with different LAT mutants show that tyrosine 136 is the most critical residue for degranulation, cytokine production, and calcium response. The three distal tyrosines, however, prove to be more essential for ERK activation. Although it is not known if LAT recruits the same negative signaling complexes in mast cells as it does in T cells, LAT phosphorylation may play a dual role in positively and negatively regulating mast cells. Consequently, much remains to be uncovered concerning the ways by which LAT may mask negative effects or integrate both positive and negative signals in mast cells (62).

LAB

Due to the crucial role of LAT in T cell development and activation, along with its specifically high expression in T cells, many speculated that an equivalent protein must exist in other immune cells, particularly B lymphocytes. The search for other adaptor proteins that could function in immune cell activation led to the discovery and cloning of LAB (linker for activation of B cells), also known as NTAL (non-T cell activation linker). Although it does not share any major sequence homology with LAT, LAB contains a short extracellular domain, a transmembrane domain, and a cytoplasmic tail with multiple tyrosine residues. In addition, LAB also has a CXXC palmitoylation motif similar to that in LAT. LAB is not detected in the thymus or in mature T cells but is highly expressed in B cells, mast cells, monocytes, and NK cells (63, 64).

Upon engagement of the BCR, Fc γ RI, or Fc ϵ RI, LAB is phosphorylated most likely by Syk kinase. As predicted from its five potential Grb2 binding motifs, LAB was shown to associate with Grb2, as well as Sos1 and Gab1, but not PLC- γ 1 or PLC- γ 2 (63, 64). Furthermore, reconstitution of a LAT-deficient Jurkat cell line with LAB leads to a partial rescue of ERK activation and calcium flux (64). To determine whether LAB can replace LAT in thymocyte development, LAT-deficient bone marrow cells were retrovirally transduced with LAB. Analysis of thymocyte development in mice that received transduced bone marrow cells revealed that LAB is able to compensate for the LAT deficiency and allow development of DP and SP cells. However, T cells from these mice are not able to upregulate activation markers or produce IL-2 upon receptor engagement. Therefore, although LAT and LAB have some redundant functions, they also have unique roles in lymphocyte function and activation (63, 64).

Considering the critical importance of tyrosine phosphorylation in LAT function, we generated a series of LAB mutants in which the nine conserved tyrosine residues were mutated into phenylalanines to determine the effect on LAB function. Results from these experiments clearly show that the three membrane-distal tyrosines, Tyr136, Tyr193, and Tyr233, are the main sites of LAB phosphorylation. Furthermore, these residues are most important for Grb2 binding and for the rescue of calcium flux and thymocyte development in LAT-deficient mice (65). Again, similar to LAT, the intrinsic function of LAB relies heavily upon the phosphorylation of its membrane-distal tyrosines.

To further study the functional differences between LAT and LAB, transgenic mice expressing LAB under the human CD2 promoter were generated. Although the adoptive transfer studies previously mentioned (63) showed that LAB can rescue thymocyte development, the LAT^{-/-}LAB^{Tg+} thymocytes demonstrate only partial rescue. Although a moderate percentage of cells are able to progress into the DP and SP stages, there is an accumulation of thymocytes at the DN3 stage. In the periphery, significant numbers of both CD4⁺ and CD8⁺ cells can be found, although the CD4 to CD8 ratio is vastly skewed in favor of CD4⁺ T cells. Furthermore, by 10 weeks of age, the LAB-transgenic thymuses have insignificant DP populations and instead are dominated by CD4 SP cells and increased numbers of B220⁺ cells. These mice uniformly develop organomegaly with excessive lymphocytic infiltration (66). The autoimmune disease phenotype seen in these mice is markedly similar to that seen in LAT^{Y136F} mice (44). As seen with the Y136F mutation, the expression of the LAB transgene in LAT-deficient cells is unable to fully restore calcium flux and ERK activation (66). Since LAB is reasonably similar to LAT except for its inability to bind PLC- γ , these studies further highlight the importance of the LAT-PLC- γ interaction for immune development, homeostasis, and activation.

LAB in T cells

Surprisingly, LAB^{-/-} mice show no gross abnormalities in lymphocyte development (67, 68). Despite the high expression of LAB in B cells, these mice have normal numbers of T1, T2, and follicular B cells with only a reduced number of marginal zone B cells. LAB-deficient B cells actually demonstrate a slightly enhanced calcium response while MAPK activation and humoral responses are unaffected. Upon analysis of mice that lack LAB and harbor the LATY136F mutation, it was shown that the autoimmune disease could develop in the absence of LAB. LAT^{-/-}LAB^{-/-} mice were also generated and mice with single or combined deletions of the two genes do not show defects in early or late B cell development. These studies show that LAB in B cells does not play an analogous role to LAT in T cells (68).

Despite relatively normal lymphocyte development in LAB^{-/-} mice, aged LAB-deficient mice develop an autoimmune disease marked by splenomegaly and anti-nuclear antibodies. While B cells in these mice are relatively normal, T cells are hyperactivated and produce high levels of cytokines, such as IL-2, IL-10, and IFN- γ . Furthermore, calcium flux and phosphorylation of PLC- γ 1, ERK, and Akt were increased in LAB^{-/-} T cells. Due to the lack of LAB expression in naïve T cells, these were highly unexpected results. However, further analysis of LAB expression shows that LAB is upregulated in T cells upon TCR stimulation. When crossed onto a LATY136F background, the LAB deficiency further enhances the lymphoproliferation and organomegaly caused by dysfunctional T cells (16). Together, these data demonstrate a role for LAB in regulating T cell activation and limiting autoimmune responses.

LAB in B cells

While these studies of B cells from LAB^{-/-} mice indicate a limited role in BCR-mediated signaling, one study using the DT40 B cell line yielded a different result. Upon BCR stimulation, phosphorylated LAB is able to recruit Grb2. In Grb2^{-/-} cells, the BCR-mediated calcium response is enhanced; it is hypothesized that, upon its association with LAB, Grb2 is recruited to lipid rafts and can no longer associate with a yet to be characterized Ca²⁺ inhibitor (69). Regardless of this evidence for a positive role for LAB in BCR-mediated signaling, the deletion of LAB alone or in concert with LAT has no effect on B cell development or function, indicating that LAB may play a limited role in B lymphocytes (68).

Although the aforementioned results indicate a limited role for LAB in B cells, one study was performed to determine if the differences in LAT-mediated and LAB-mediated signaling are dependent solely upon the inability of LAB to bind PLC- γ . SLP65^{-/-}LAT^{-/-} pre-B cells were reconstituted with LAB and a LAB mutant carrying the PLC- γ 1/2 binding motif. These results demonstrated that, despite being highly expressed in bone marrow-derived pre-B cells, LAB does not induce pre-BCR downregulation or calcium flux in this cellular subset, two events critical for B cell development. The form of LAB carrying the LAT-PLC- γ binding motif is functional but is unable to rescue B cell differentiation. Further experiments using chimeric swap mutants transduced into SLP65^{-/-}LAT^{-/-} pre-B cells show that the N terminus of LAB is inhibitory and prevents pre-B cell differentiation (70). Therefore, the major differences in LAT and LAB function cannot be attributed solely to the inability of LAB to associate with PLC- γ 1.

LAB in Mast Cells

LAB protein is highly expressed in mast cells and has been shown to play a complex role in Fc ϵ RI signaling. Upon Fc ϵ RI ligation, LAB is phosphorylated, allowing it to interact with Grb2. Interestingly, LAB phosphorylation is increased in LAT-deficient cells, perhaps in an

attempt to compensate for the loss of this critical protein, but LAB^{-/-} mast cells show enhanced degranulation. FcεRI-mediated PLC-γ phosphorylation, calcium mobilization, and ERK activation are also enhanced, implying a negative role for LAB in mast cell function. However, mast cells lacking both LAT and LAB have a more dramatic block in FcεRI signaling than the LAT^{-/-} mast cells, indicating that LAB may also exert a positive effect on FcεRI-mediated signaling (67, 71). Additionally, an electron microscopic analysis shows that LAB and LAT localize to independent microdomains in the plasma membrane before and after FcεRI activation. Thus, these two proteins seem to affect mast cell signaling through autonomous mechanisms (72).

Further studies designed to extrapolate the role of LAB in FcεRI signal transduction, employing RBL (rat basophilic leukemia) cells with reduced or enhanced LAB expression, indicate that LAB may function in different stages of mast cell signaling. Overexpression of LAB interferes with the phosphorylation of FcεRI subunits, LAT, and Syk. However, this study also shows that overexpressing LAB increases calcium uptake while knocking down LAB expression inhibits calcium mobilization. These data implicate dissimilar roles for LAB at different stages of FcεRI signaling; while LAB may interfere with the immediate assembly of signaling platforms, it also positively regulates calcium activity during later stages of mast cell activation (73). Studies using LAB-knock-down human mast cells demonstrate the necessity of FcεRI- and Kit-mediated LAB phosphorylation for degranulation. Kit is able to directly phosphorylate LAB at different residues than Lyn and Syk. Furthermore, this study shows inducible association of LAB with PLC-γ1 following phosphorylation by Syk. Therefore, various receptors may utilize LAB in different ways depending upon the signal being propagated, adding another dimension of flexibility in signaling through adaptor proteins (74).

As previously discussed, LAT has been shown to negatively impact signaling through the recruitment of the phosphatase SHIP1 (57, 62). Therefore, the absence of LAB in mast cells enhances LAT-dependent positive as well as LAT-dependent negative signaling events, including the recruitment of SHIP1. SHIP1 is able to negatively regulate FcεRI signaling in two ways. First, it is able to remove phosphate groups from PIP₃, thus disrupting membrane recruitment of signaling molecules that contain a pleckstrin homology domain. Also, SHIP1 can recruit RasGAP and Dok-1 to quell Ras activity. Consequently, LAB^{-/-} mast cells have enhanced recruitment of SHIP1 by LAT, resulting in decreased Akt phosphorylation and decreased survival (75). However, in contrast to these published results, our unpublished data show that Akt activation and cell survival are enhanced in LAB^{-/-} mast cells, although it is not clear what causes these different observations.

IgE-induced mast cell survival is known to be dependent upon Syk activity and sustained Erk activation (76). Analysis of LAB^{-/-}LAT^{-/-} BMMCs showed the contribution of both of these adaptor proteins to this process. Double-deficient cells have impaired survival, IL-3 induction, and Ras activation, but these defects are restored upon expression of membrane-targeted Sos, which bypasses its recruitment by Grb2. This pathway is distinct from that mediated by the LAT-Gads-SLP-76-PLC-γ1 signaling complex, as evidenced by the unaffected IgE-mediated survival of BMMCs from Gads-deficient mice (77). Therefore, LAT and LAB are both critical for mast cell survival through their recruitment of Grb2 and activation of the Ras pathway (78).

LAB in Innate Immunity

Similar to the adaptive branch of the immune system, the initiation of an innate immune response requires activating receptors to trigger an attack on invading pathogens. TREM-1 (triggering receptor expressed on myeloid cells), a glycoprotein on the surface of myeloid cells, is an activating receptor known to interact with DAP12 (DNAX activation protein of

12kDa). DAP12 is an important transmembrane adaptor protein that non-covalently associates with several receptors and contains ITAM motifs necessary for downstream signaling. Engagement of TREM-1 by its natural ligands, which are as of yet unidentified, initiates the phosphorylation of DAP12 ITAM motifs and activation of PLC- γ and ERK. This event also increases phosphorylation of LAB. By using myelomonocytic cell lines treated with RNA interference to knockdown LAB protein expression, it was demonstrated that LAB negatively modulates ERK1/2 activation, calcium flux, and production of TNF- α and IL-8 (79). In addition, TREM-2, which induces the upregulation of co-stimulatory molecules and chemokine receptors on dendritic cells, utilizes DAP12 to propagate its signal. It will be interesting to determine if LAB plays a similar role in regulating TREM-2-mediated signals (80).

LAT and LAB in NK Cells

Both LAT and LAB are expressed in natural killer cells. While LAT is phosphorylated upon CD16 crosslinking on NK cells or interaction with target cells (80, 81) LAT^{-/-} NK cells retain their ability to lyse susceptible target cells and mediate ADCC (antibody-dependent cell-mediated cytotoxicity) (35). A likely candidate to compensate for this lack of LAT is LAB and both of these adaptor proteins have been implicated in signaling through the NK receptor family Ly49D (83). A study aimed at elucidating the roles of LAT and LAB in NK cell signaling shows that, whereas resting LAT^{-/-} NK cells have intact responses, activated NK cells from LAT^{-/-} mice have an impaired response to NK1.1, an activating ITAM-coupled receptor. LAB^{-/-} NK cells, on the other hand, have enhanced NK1.1 signaling, recapitulating the negative role of LAB seen in mast cells. Moreover, the deletion of both LAT and LAB in resting and active NK cells causes a severe defect in NK1.1 signaling (84). Natural killer cells, then, are able to utilize LAT and LAB and the interplay between these two can add plasticity to the NK response.

LAX

As studies revealed the dire importance of LAT in lymphocyte development, activation, and homeostasis, the search for other potentially irreplaceable adaptor proteins continued. Linker for activation of X cells (LAX) was discovered due to the similarity of its tyrosine motifs to those of LAT. Human LAX contains ten tyrosine residues, five of which are within a Grb2-binding motif. LAX is expressed in both B and T cells, as well as NK cells and monocytes, and does not localize to lipid rafts in these cells. LAX is phosphorylated upon antigen receptor stimulation by Src and Syk family kinases, leading to its interaction with Grb2, Gads, and PI3K. However, unlike LAT, LAX inhibits p38 MAPK and AP-1 activation following TCR engagement; but the mechanisms by which LAX negatively functions in TCR-mediated signaling is unclear (86).

Although LAX^{-/-} mice show no gross abnormalities in lymphocyte development, they develop spontaneous germinal centers and have hyper-responsive T and B cells. TCR-mediated calcium flux, MAPK activation, and tyrosine phosphorylation are all increased in LAX-deficient cells, demonstrating a negative role for LAX in T cell activation (15). Furthermore, LAX was shown to be a negative regulator of mast cell activation as deficient cells have enhanced degranulation, p38 phosphorylation, cytokine production, and survival. Surprisingly, in these cells, LAB expression is decreased. This diminution in LAB may account for the observed increase in LAT localization to lipid rafts in LAX^{-/-} mast cells, adding to an already complex and muddled network of adaptor protein interactions (86).

LAX Interaction with other Adaptor Proteins

One of the ways by which LAX negatively regulates T and B cell activation could depend upon its recruitment of other negative regulators. Alternatively, LAX could sequester SH2 domain-containing proteins, such as Gads, Grb2, and/or PI3K, thus decreasing their interaction with positive regulators like LAT (15). In studying negative regulation of lymphocyte activation, Perchonock *et al* (87) demonstrated LAX association with ALX (adaptor in lymphocytes of unknown function, x). ALX contains a single SH2 domain, polyproline SH3-binding motifs, and several potential sites of tyrosine phosphorylation (88). The phenotype of ALX-deficient mice is remarkably similar to that of LAX knockout mice, thus prompting investigation as to whether these proteins could act in concert. Indeed, LAX and ALX were shown to interact with each other and inhibit the activation of p38 and the production of IL-2 (86).

Interestingly, ALX is able to mediate the phosphorylation of LAX in the absence of TCR stimulation through its recruitment of Lck to LAX, leading to an inhibition of NFAT signaling. Experiments using chimeric mutants showed that tyrosine 193 is critical for the ability of LAX to inhibit NFAT activation. However, the LAX-ALX complex is also able to negatively regulate RE/AP-1 (responsive element/activated protein-1) and this modulation is independent of the phosphorylation of LAX protein. Thus, LAX is able to negatively impact T cell activation by two unique, but interconnected, means (89).

Additional data implied a cooperative role for LAX with the negative adaptor protein SIT in lymphocytes (90). While the combined deletion of SIT and LAX does not affect the development and function of mature T cells or conventional B cells, double-knockout mice have enhanced numbers of B1 B cells, activated CD4⁺ T cells, and high levels of serum Ig (91). These studies highlight the complexity of orchestrating an optimal immune response as multiple adaptor proteins may interact and synergize during lymphocyte activation.

Future Work in the Field of Adaptor Proteins

Palmitoylation and phosphorylation are two critical types of protein modifications that can impact the localization and function of transmembrane adaptor proteins. Whereas the kinases involved in the phosphorylation of LAT, LAB, and LAX at specific tyrosine residues have been well characterized, the proteins responsible for the palmitoylation and depalmitoylation of LAT and LAB are still unknown. Palmitoylation is the reversible attachment of palmitate to a cysteine residue through a thioester link. This modification serves to target the protein of interest to the plasma membrane or even specific domains of the plasma membrane, such as lipid rafts (92, 93). Although the phenomenon of palmitoylation has been studied for over thirty years, the molecular machinery responsible is just beginning to be discovered.

Studies in yeast revealed a family of integral membrane palmitoyl-transferases (PATs) that have a core cysteine rich domain (CRD) containing a sequence of Asp-His-His-Cys (DHHC) (94–96). Now, attention has now turned to identifying the predicted twenty-three DHHC proteins that potentially reside within the mammalian genome (97, 98). Future work in this field will entail the identification of the specific DHHC proteins that palmitoylate LAT and LAB and, since LAT palmitoylation is impaired in anergic T cells, how these PATs are regulated during T cell activation and anergy induction.

A critical, and very complex, issue that remains controversial in this field is the LATY136F phenotype and the mechanisms that cause such severe autoimmunity. Our previously mentioned studies show that the lack of functional regulatory T cells in LATY136F mice are a major contributor to the onset of the lymphoproliferative disease (50). However, there is

still much to be uncovered concerning LATY136F T_{regs}. The pathways downstream of LAT-PLC γ that are required for and regulate T_{reg} development remain unknown. Furthermore, the potential role of the LAT-PLC- γ 1 interaction in regulating Foxp3 expression and function is unclear. It is also unknown if wildtype regulatory T cells would be able to suppress the LATY136F autoimmune disease in a long-term situation. Thus, much remains to be elucidated concerning the role of T_{regs} in the development of the LATY136F disease.

Despite the importance of T_{regs} in suppressing autoimmunity, they cannot be the only contributing factor to disease onset. LAT3YF mice develop a very similar lymphoproliferative disorder mediated by $\gamma\delta$ T cells, while TCR- $\beta^{-/-}$ mice also lack regulatory T cells but fail to develop disease (52, 53). The severity of the LATY136F disease is likely a combination of a multitude of events that are dysregulated, including the failure of T cells to undergo proper thymic selection, the lack of peripheral suppression, and the uncontrolled expansion of mutant T cells. Indeed, the completely aberrant nature of the LATY136F T cells was highlighted in studies showing that their hyperproliferation is IL-7 driven but independent of MHC (41). Therefore, these T cells do not need to be activated via self-antigen on antigen presenting cells and may have an inherent predisposition to become hyperproliferative due to the LAT mutation.

Moreover, STAT6-deficient LATY136F mice develop a disease of similar magnitude but mediated by T_H1 and CD8⁺ cells (51). These T cells are obviously pre-disposed to undergo hyperproliferation and activation despite their surrounding environment. Perhaps, then, the LAT-PLC- γ 1 interaction is essential for the maintenance of homeostasis by consistently exerting a negative influence on naïve T cells. Certainly, studies on LATY136F mice have revealed that the LAT-PLC- γ 1 interaction is critical for T cell function, although much more work needs to be done in order to clearly understand the mechanisms by which such severe lymphoproliferation occurs.

In addition to LAT, there is much more to learn about the other LAT family members, LAB and LAX. While LAB^{-/-} mice do not have a severe phenotype, LAB function in other hematopoietic cells, such as dendritic cells and macrophages, has yet to be revealed. Studies of LAB in TREM-1-mediated signaling suggest a potential role for LAB in the innate immune response, but whether LAB^{-/-} mice are able to mount effective innate and adaptive immune responses remains to be determined. Furthermore, it was very interesting to note that a deficiency in LAB leads to an increase in LAT palmitoylation and raft localization; the mechanism and significance of this phenomenon are yet to be fully understood. Similarly, there is much to be uncovered concerning the role of LAX in potentially recruiting other negative adaptors and impacting antigen receptor-mediated signaling. As this field of study moves forward, we will certainly begin to reveal more complex adaptor protein relationships and the critical role these interactions play in modulating the immune response.

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