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Essential Role of LAT in T Cell Development

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Summary

The linker molecule LAT is a substrate of the tyrosine kinases activated following TCR engagement. Phosphorylated LAT binds many critical signaling molecules. The central role of this molecule in TCR-mediated signaling has been demonstrated by experiments in a LAT-deficient cell line. To probe the role of LAT in T cell development, the *LAT* gene was disrupted by targeting. LAT-deficient mice appeared healthy. Flow cytometric analysis revealed normal B cell populations but the absence of any mature peripheral T cells. Intrathymic development was blocked within the $CD4^-CD8^-$ stage. No gross abnormality of NK or platelet function was observed. LAT is thus critical to both T cell activation and development.

Introduction

Immunoreceptors for antigen and immunoglobulin expressed on several lineages of hematopoietic cells share many structural and functional features. T cell and B cell antigen receptors and the Fc receptors for IgE and IgG are comprised of multiple subunits (Ravetch and Kinet, 1991; Weiss and Littman, 1994; Chan and Shaw, 1996; Wange and Samelson, 1996; Buhl and Cambier, 1997). Some of these components are dedicated to ligand binding, while other subunits with long cytosolic tails contain immunoreceptor tyrosine-based activation motifs (ITAMs) that are subject to rapid phosphorylation. Members of the Src family of protein tyrosine kinases (PTK) directly interact with these receptors or coreceptors and are primarily responsible for ITAM phosphorylation. Phosphorylation of the two tyrosines within an ITAM creates the binding sites for the tandem SH2 domains of Syk family PTKs, Syk, or ZAP-70. Syk and ZAP-70, when bound to phosphorylated ITAMs, are further activated by Src family kinases. Thus, the hallmark of the activated immunoreceptor is its association with active PTKs.

Though the effector functions of these various hematopoietic cells may differ widely, the various immunoreceptors couple to similar signaling pathways. Tyrosine phosphorylation of phospholipase-Cγ isoforms is observed after activation of these receptors. Activation of phospholipase-Cγ isoforms leads via phosphoinositide cleavage to calcium elevation and PKC activation. Engagement of these receptors also leads to activation of small G proteins such as Ras and Rac. The details of how the immunoreceptors couple to these signaling pathways are now under intense investigation. For these different receptor systems, a central question is how proteins in the signaling cascades are brought from the cytosol and elsewhere in the membrane to the activated receptors. A number of cytosolic linker or adaptor molecules have been described that directly bind additional linker proteins and effectors. These include the ubiquitous Grb2, which binds SOS, SLP-76 and Cbl, and the Grb2-related molecules Grap and Gads, which have similar binding properties. The linker molecule SLP-76 performs its function by binding Vav, SLAP, and other proteins (Liu et al., 1999; Peterson et al., 1998; Rudd, 1999; Samelson, 1999). In T cells these complexes have been shown to bind the integral membrane adaptor molecule LAT (Weber et al., 1998; Zhang et al., 1998a). How these LAT-associated protein complexes comprised of enzymes and adaptor molecules are brought to immunoreceptors is now an important area of study.

LAT is a 36–38 kDa palmitoylated, integral membrane adaptor protein expressed in T cells, mast cells, NK cells, and megakaryocytes (Weber et al., 1998; Zhang et al., 1998a, 1998b; Facchetti et al., 1999). It is not found in B cells or other cell types. Limited biochemical evidence shows that a 36–38 kDa protein, which is likely to be LAT, is phosphorylated on tyrosine residues in mast cells upon FcεRI engagement and in NK cells following target interaction (Turner et al., 1995; Galandrini et al., 1996; Valiante et al., 1996; Hendricks-Taylor et al., 1997). The function of LAT is best characterized in T cells. Upon TCR activation, LAT is phosphorylated on multiple tyrosines, most likely by the ZAP-70 PTK. LAT then associates with PLC-γ1, Cbl, Vav, PI-3 kinase, SLP-76, Grb2, and other proteins directly or indirectly. Mutation of two critical LAT tyrosines to phenylalanine abolishes the binding to these molecules. Overexpression of this mutant LAT molecule in Jurkat cells inhibits AP-1 and NF-AT-directed transcriptional activation mediated through the TCR, suggesting its important role in T cell activation (Zhang et al., 1998a). As further confirmation of the function of LAT, in LAT-deficient Jurkat cells the tyrosine phosphorylation of PLC-γ1, Vav, and SLP-76 is reduced. Ca^{2+} mobilization, activation of MAPK, and activation of AP-1 and NF-AT transcription factors are also defective in these cells. Reintroduction of LAT can

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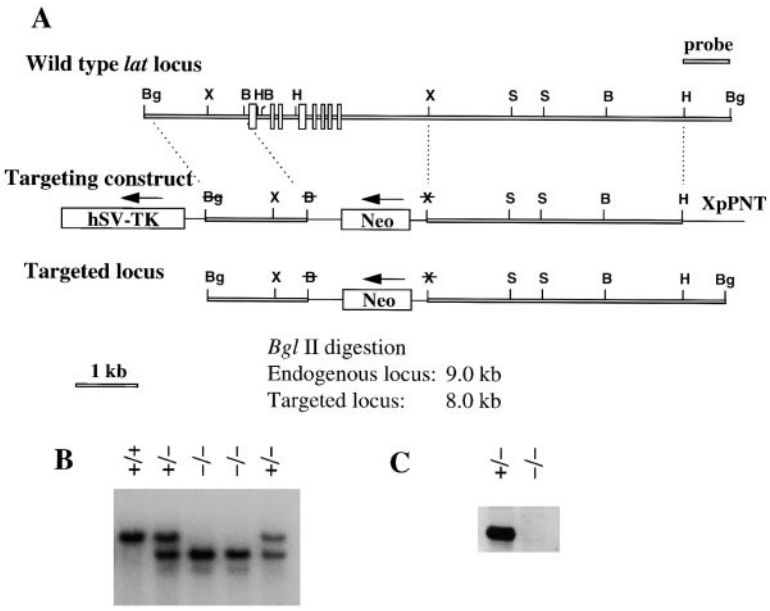

Figure 1. Generation of LAT-Deficient Mice

(A) The genomic structure of murine *LAT* gene, the XpPNT targeting construct, and the predicted structure of the targeted locus after homologous recombination. The precise intron–exon structure of the entire *LAT* gene has not been determined. The open boxes indicate the exons of LAT from amino acid 1–169. The first box contains the beginning of the LAT coding sequence. Exons containing 5′ untranslated sequences have not been defined. Neo, neomycin resistance gene; TK, thymidine kinase; Bg, BglII; X, XbaI; B, BamHI; H, HindIII; S, SacI. The restriction sites that are struck through are the sites that were abolished during subcloning.

(B) Southern analysis of genomic DNA from littermates of LAT heterozygous (+/−) mating. Genomic DNA was isolated from tails of littermates and digested with BglII. DNA fragments were separated by electrophoresis, transferred to nylon membrane, and analyzed using the probe as indicated in (A).

(C) Analysis of LAT expression in thymocytes from $LAT^{+/-}$ and $LAT^{-/-}$ mice. Lysates from 1.5×10^6 cells solubilized with Brij lysis buffer were analyzed by SDS-PAGE and immunoblotting with anti-LAT antiserum.

restore all these signaling defects (Finco et al., 1998). These results, in total, demonstrate that LAT is indispensable for T cell activation via the TCR.

Targeted gene disruption has been widely used to study the in vivo role of enzymes and adaptor molecules involved in signaling in hematopoietic cells (Pfeffer and Mak, 1994; Bolen and Brugge, 1997; Killeen et al., 1998). A system that has been particularly amenable to such experimentation is the analysis of T cell development. This process is characterized by multiple, sequential steps that can be easily defined by the expression of particular cell surface proteins. The majority of T cells develop in the thymus through an ordered maturation process, giving rise to mature T cells with antigen-specific TCRs (Fehling and von Boehmer, 1997). The earliest immature T cells lack expression of CD4 or CD8 coreceptors and are thus known as double negative (DN). DN thymocytes express the TCR signaling subunits, TCRζ, and the CD3 chains, but remain negative for TCR until rearrangement of the clonotypic TCR (γ, δ, β) genes commences. Successful rearrangement of the TCRγ and TCRδ genes results in the expression of γδTCR surface complexes. $\gamma\delta TCR^+$ thymocytes remain DN and exit the thymus, homing to specific sites within the periphery. Alternatively, rearrangement and expression of the TCRβ gene directs DN thymocytes to the αβ (CD4/CD8) lineage. TCRβ chain associates with the invariant surrogate TCRα chain to form a pre-TCR complex. Signaling through the pre-TCR drives the differentiation of DN cells to the $CD4^+CD8^+$ (double positive [DP]) stage, where rearrangement of TCRα then leads to expression of the mature form of the αβTCR. DP, $\alpha\beta TCR^+$ thymocytes undergo a number of TCR-mediated selection events and ultimately differentiate into either $CD4^+$ or $CD8^+$ mature single-positive (SP) T cells.

To elucidate the role of LAT in the cells in which it is expressed, we generated mice deficient in LAT expression by targeted disruption. In contrast to SLP-76- or Syk-deficient mice, no bleeding disorder was noted in $LAT^{-/-}$ (Cheng et al., 1995; Pivniouk et al., 1998; Clements et al., 1999). NK cell cytotoxic activity was normal. The most striking effect of LAT deficiency was in the T cell compartment. Mature αβ and γδ T cells were absent in $LAT^{-/-}$ mice. Thymocyte development in LAT-deficient mice showed an early arrest in the DN population, and most thymocytes were blocked at the $CD25^+CD44^-$ stage. These results indicate that the requirement for LAT expression is most important in the T cell lineage. In this respect the LAT null mice are as severely impaired as those mice lacking both Lck and Fyn, both ZAP-70 and Syk, or SLP-76 (Groves et al., 1996; van Oers et al., 1996; Cheng et al., 1997; Clements et al., 1998; Pivniouk et al., 1998).

Results

Generation of LAT-Deficient Mice

To generate LAT-deficient mice by targeted gene disruption, the genomic fragment (from the first BamHI site to the second XbaI) that contains exons 1–8 of the *LAT* gene (encoding amino acids 1–169 region of murine LAT) was replaced by a neomycin phosphotransferase gene (*neo*) in embryonic stem (ES) cells. Southern blot analysis (using the probe as indicated in Figure 1A) confirmed the homologous recombination event in ten ES cell lines. Targeted ES cells were injected into blastocysts to establish chimeric mice. Heterozygous mice ($LAT^{+/-}$) were inbred to obtain mice homozygous for the disrupted *LAT* gene ($LAT^{-/-}$). Transmission of the targeted allele was verified by Southern blot analysis of tail DNA from littermates (Figure 1B). To confirm the absence of LAT protein in $LAT^{-/-}$ mice, we analyzed lysates of thymocytes by immunoblotting with anti-LAT antiserum. As shown in Figure 1C, murine LAT protein migrates as a 36 kDa band instead of as a 36–38 kDa doublet observed

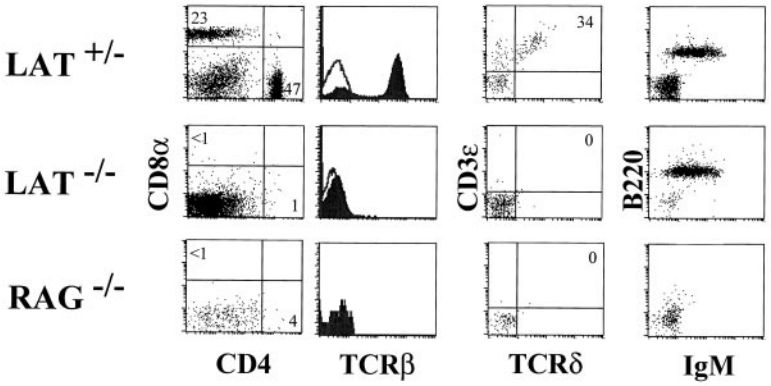

Figure 2. Flow Cytometric Analysis of Lymph Node Cells from LAT$^{+/-}$, LAT$^{-/-}$, and RAG-2$^{-/-}$ Mice

Lymph node cells from 3- to 4-week-old adult LAT$^{+/-}$, LAT$^{-/-}$, and RAG-2$^{-/-}$ mice were prepared, surface stained, and analyzed by standard flow cytometry. Two-color plots show staining of total lymph node cells with antibodies against CD4 and CD8α (column 1) or IgM and B220 (column 4). The two-color plot in column 3 shows CD3 and TCRδ expression on gated (CD4$^-$CD8$^-$B220$^-$) lymph node cells. Single-color plot (column 2) shows staining of total lymph node cells with anti-TCRβ (shaded) or control antibody (unshaded). Numbers in quadrants indicate percentage of gated cells within the quadrant.

in the human Jurkat T cell line. Significantly, LAT protein was detectable in thymocytes from LAT$^{+/-}$ but not LAT$^{-/-}$ mice.

Absence of Mature T Cells in LAT-Deficient Mice

LAT$^{-/-}$ mice appeared healthy and fertile under specific pathogen-free conditions. The frequency of LAT$^{-/-}$ littermates generated from breeding LAT$^{+/-}$ mice was the expected 25% of offspring. Unlike SLP-76$^{-/-}$ or Syk$^{-/-}$ newborns, which develop subcutaneous bleeding, LAT-deficient pups were viable and had no obvious signs of bleeding. In addition, platelet morphology, platelet numbers, and bleeding times were grossly normal in LAT$^{-/-}$ mice (data not shown).

Spleens and lymph nodes from LAT$^{-/-}$ mice were normal in size and cellularity. However, flow cytometric analysis revealed that T cells (i.e., cells expressing CD3, CD4, CD8, αβTCR, or γδTCR) were absent in LAT$^{-/-}$ mice (Figure 2). However, LAT$^{-/-}$ mice contained normal numbers of B cells, and B cell development appeared unaffected as assessed by expression of B220 and sIgM (Figure 2), indicating that LAT is required for maturation of T cells but not B cells. These results were not unexpected based on our previous findings that LAT protein and mRNA are not present in normal B cells, B cell neoplasia, or in transformed B cell lines (Zhang et al. 1998a; Facchetti et al., 1999).

Thymocyte Development in LAT-Deficient Mice

The thymi from mice heterozygous for the targeted gene (LAT$^{+/-}$) did not show any significant difference in size and cellularity compared with LAT$^{+/+}$ mice. However, the thymi from LAT$^{-/-}$ mice were very small, comparable to the thymi from RAG-2$^{-/-}$ mice. Thymocyte numbers in LAT$^{-/-}$ mice were consistently 10–20 times less than that of LAT$^{+/-}$ and LAT$^{+/+}$ mice, ranging from 0.5–1.2 × 10^7 cells for each mouse. Since there were no mature T cells in the spleens or lymph nodes of LAT$^{-/-}$ mice, we next examined at which stage the LAT$^{-/-}$ thymocytes were developmentally arrested. As shown in Figure 3A, thymocytes in LAT$^{-/-}$ mice were exclusively CD4$^-$CD8$^-$ (DN). CD4$^+$CD8$^+$ (DP) and CD4$^+$ or CD8$^+$ (SP) thymocytes were undetectable, indicating that LAT$^{-/-}$ thymocytes fail to develop beyond the DN stage. We also

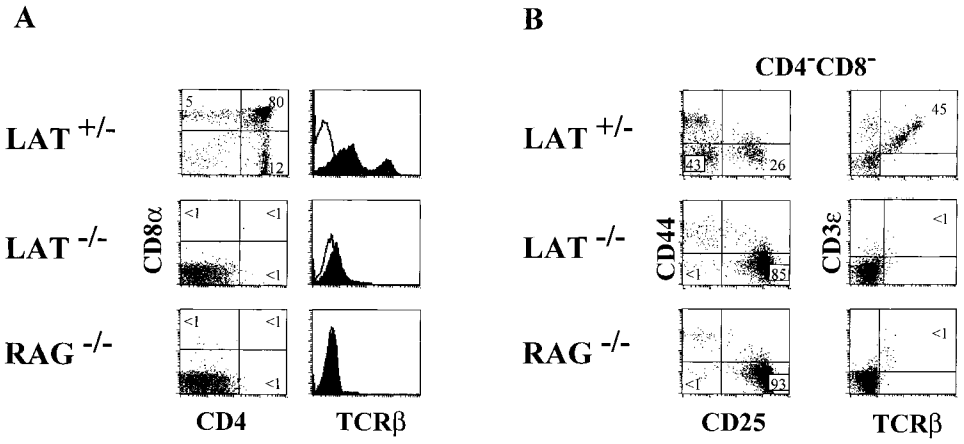

Figure 3. Thymocyte Development in LAT-Deficient Mice

(A) Total thymocytes from LAT$^{+/-}$, LAT$^{-/-}$, and RAG-2$^{-/-}$ mice were analyzed for expression of CD4, CD8, and CD3ε by flow cytometry.
(B) Column 1: thymocytes from LAT$^{+/-}$, LAT$^{-/-}$, and RAG-2$^{-/-}$ mice were stained with anti-CD44-FITC, anti-CD25-PE, and a mixture of Quantum red–conjugated anti-CD3, anti-CD4, anti-CD8α, and anti-B220 antibodies. Analysis of CD44 and CD25 expression was performed on gated CD3$^-$CD4$^-$CD8$^-$B220$^-$ cells. Column 2: thymocytes were stained with anti-TCRβ-FITC, anti-CD3ε, and a mixture of Quantum red–conjugated anti-CD4, anti-CD8α, and anti-B220 antibodies. Analysis of TCRβ and CD3ε expression was performed on gated CD4$^-$CD8$^-$B220$^-$ cells.

examined αβTCR surface expression by staining thymocytes with antibodies specific for TCRβ chains. Thymocytes from LAT$^{+/-}$ mice, like those from LAT$^{+/+}$ mice (data not shown), were composed of a small population of cells expressing high levels of αβTCR (predominantly SP cells) and a large population of cells expressing low-to-medium levels of αβTCR (predominantly DP cells). In contrast, LAT$^{-/-}$ mice lacked cells with moderate or high levels of αβTCR (Figure 3A).

To further define the stage at which thymocyte development was blocked in LAT$^{-/-}$ mice, we analyzed DN subpopulations. DN cells can be subdivided into four discrete subsets by surface expression of CD44 and CD25 (Godfrey and Zlotnik, 1993). Maturation within the DN compartment occurs in the sequence CD25$^-$CD44$^+$→CD25$^+$CD44$^+$→CD25$^+$CD44$^-$→CD25$^-$CD44$^-$. The TCRβ chain is first expressed in CD25$^+$CD44$^+$ thymocytes upon successful rearrangement of TCRβ gene. Association of the TCRβ chain with the surrogate α chain (pre-Tα), TCRζ, and the CD3 subunits results in the expression of the pre-TCR complex at the CD25$^+$CD44$^-$ state. Signaling through the pre-TCR at the CD25$^+$CD44$^-$ stage drives the differentiation of these cells to the CD25$^-$CD44$^-$ stage. As shown in Figure 3B, thymocyte development was arrested at the CD25$^+$CD44$^-$ stage in LAT$^{-/-}$ mice. Comparison of thymocytes from LAT$^{-/-}$ and RAG$^{-/-}$ mice stained with anti-CD44 and anti-CD25 antibodies revealed that thymocyte development was arrested at the identical stage in both mice (Figure 3B). In control (LAT$^{+/-}$) mice, DN thymocytes contain a subpopulation of mature αβTCR$^+$ cells, some of which also express the NK1.1 surface marker (Bendelac et al., 1997). Although DN αβTCR$^+$ thymocytes were present in LAT$^{+/-}$ mice, these cells were undetectable in LAT$^{-/-}$ mice (Figure 3B).

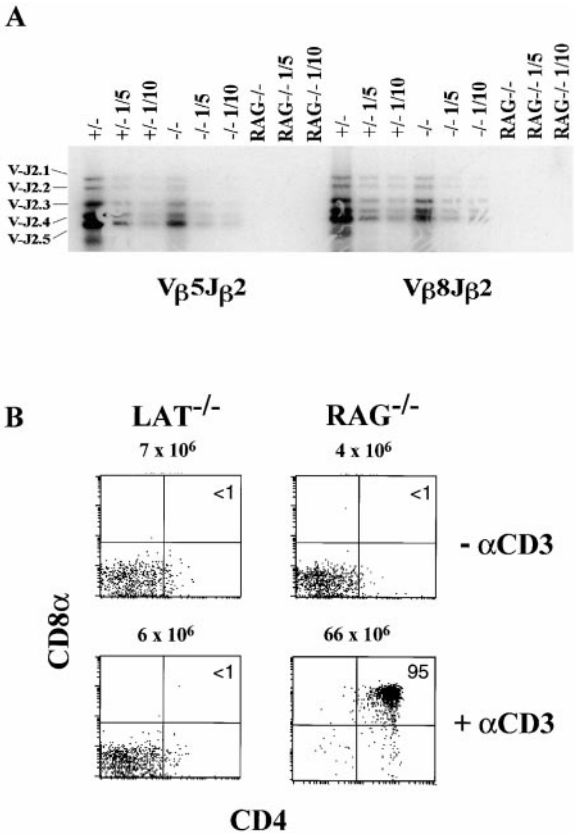

Figure 4. Defective Pre-TCR Signaling in LAT$^{-/-}$ Thymocytes

(A) TCRβ gene rearrangement in LAT$^{-/-}$ mice. Genomic DNA preparations from LAT$^{+/-}$, LAT$^{-/-}$, and RAG-2$^{-/-}$ thymocytes were used as templates to perform PCR reactions using Vβ5, Vβ8, Vβ13, and Dβ2-specific primers as the 5′ primers and Jβ2-specific primer as the 3′ primer. Southern blot analysis was done using a Jβ2-specific probe. Only the results of Vβ5 and Vβ8 are shown. The fractions indicate the dilution of genomic DNA used as template in the PCR. (B) Failure to rescue the progression of CD4$^-$CD8$^-$ thymocytes in LAT$^{-/-}$ mice by injection of anti-CD3ε antibody. Eight-week-old adult RAG-2$^{-/-}$ and LAT$^{-/-}$ mice were injected intraperitoneally with 100 μg of anti-CD3 antibody (mAb 145–2C11). Four days later, total thymocytes were isolated, enumerated, and analyzed by flow cytometry.

Defective Pre-TCR Signaling in LAT-Deficient Mice

To further characterize the nature of the developmental arrest in LAT-deficient thymocytes, we examined whether TCR gene rearrangement was affected by the absence of LAT. DNA-based PCR and Southern blot analysis were used to detect V(D)J rearrangement events in thymocytes. As shown in Figure 4A, using Vβ5- or Vβ8-specific primers with a Jβ2-specific primer in PCR amplification, we can detect TCRβ gene rearrangements in LAT$^{-/-}$ thymocytes. These rearrangements were not observed in RAG-2-deficient mice as previously reported (Shinkai et al., 1992). The same results were obtained with Vβ13- or Dβ2-specific primers (data not shown). These results indicate that LAT-deficient thymocytes are capable of expressing pre-TCR surface complexes. Indeed, the low level of TCRβ surface expression on DN thymocytes from LAT$^{-/-}$ mice is consistent with pre-TCR surface expression (Figure 3A).

Cross-linking of CD3 complexes on DN thymocytes by incubation with anti-CD3 antibody can induce maturation of thymocytes from the DN to the DP stage. Anti-CD3 induction occurs even in the absence of TCRβ expression in thymocytes from RAG$^{-/-}$ mice, as these cells express low surface levels of CD3 chains in the absence of clonotypic subunits (Wiest et al., 1997). We next examined whether treatment of LAT$^{-/-}$ mice with the anti-CD3ε antibody 2C11 could bypass the developmental arrest in LAT$^{-/-}$ thymocytes. 2C11 was injected intraperitoneally into young adult LAT$^{-/-}$ and RAG-2$^{-/-}$ mice. As shown in Figure 4B, anti-CD3 mediated cross-linking-induced cell proliferation and the appearance of CD4$^+$CD8$^+$ cells in RAG-2$^{-/-}$ mice. In contrast, CD3 engagement in LAT$^{-/-}$ mice failed to induce either cell proliferation or DN-to-DP progression. The failure of anti-CD3 antibody to induce the proliferation or maturation of thymocytes indicates that the pre-TCR signaling pathway is defective in LAT$^{-/-}$ mice.

γδ T Cell and iIEL Development Are Arrested in LAT-Deficient Mice

To assess γδ T cell development in LAT$^{-/-}$ mice, we examined thymocytes from fetal day 15, as γδ T cells are known to arise in defined waves during fetal ontogeny

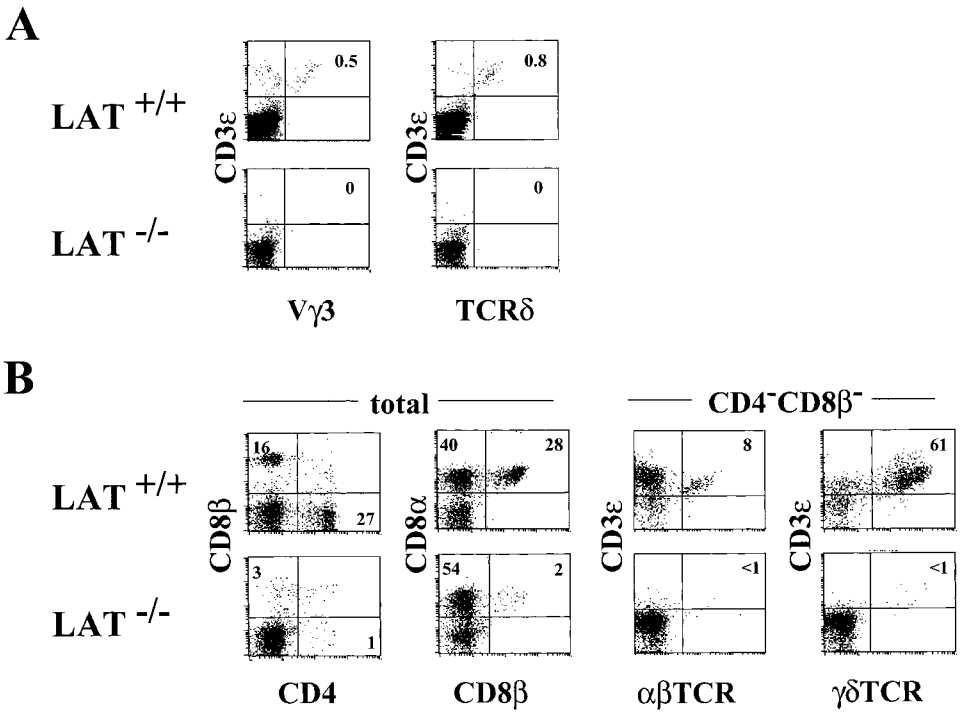

Figure 5. Analysis of γδ T Cells and Intestinal Intraepithelial Lymphocytes in LAT-Deficient Mice

(A) Development of γδ T cells in LAT$^{-/-}$ mice. Thymocytes were isolated from fetal day 15 thymus and stained with anti-CD3ε-FITC and anti-Vγ3-PE or with anti-CD3ε-PE and anti-TCRδ-FITC.

(B) Intestinal intraepithelial lymphocytes (iIELs) from LAT$^{+/+}$ and LAT$^{-/-}$ mice were prepared as described in Experimental Procedures and analyzed for the expression of CD4, CD8α, and CD8β (columns 1 and 2). Expression of γδTCR, αβTCR, and CD3ε was also analyzed on gated CD4$^-$CD8β$^-$ cells (columns 3 and 4).

(Havran and Allison, 1988). Analysis of fetal day 15 thymocytes, by staining for Vγ3 (the predominant TCR at fetal day 15), and pan-TCR γδ mAb GL3 demonstrated that γδTCR$^+$ cells were absent in LAT$^{-/-}$ mice (Figure 5A). γδTCR$^+$ cells were also undetectable in the peripheral lymphoid organs of LAT$^{-/-}$ mice (Figure 2). Significantly, as with αβT cells, the absence of γδ T cells in LAT$^{-/-}$ mice could not be attributed to failure to rearrange the TCRγ or TCRδ genes, as TCRγ and TCRδ gene rearrangements were detectable in thymocytes from LAT$^{-/-}$ mice (data not shown).

We then examined intestinal intraepithelial lymphocyte (iIEL) populations in LAT$^{-/-}$ mice. In normal (LAT$^{+/+}$) mice, iIEL subpopulations include CD4$^+$ or CD8αβ$^+$ T cells that express αβTCR and resemble other peripheral αβTCR$^+$ lymphocytes. In addition, the iIEL compartment includes αβTCR$^+$ and γδTCR$^+$ cells that are either CD4$^-$CD8$^-$ or that coexpress CD8αα homodimers. These latter cells are thought to develop extrathymically, and they are not subject to conventional repertoire selection processes (Guy-Grand and Vassalli, 1993; Poussier and Julius, 1994). Cell surface analysis demonstrated that, as observed in the spleen and lymph nodes, CD4$^+$ and CD8α$^+$β$^+$ T cells were markedly reduced in LAT$^{-/-}$ mice (Figure 5B). In contrast, cells expressing CD8αα homodimers were present in normal numbers in LAT$^{-/-}$ mice. However, none of the cells isolated from LAT$^{-/-}$ mice expressed surface αβTCR or γδTCR complexes (Figure 5B, right panel). Thus, LAT$^{-/-}$ mice were found to be devoid of all subsets of mature TCR$^+$ lymphocytes.

Development and Function of NK Cells in LAT$^{-/-}$ Mice

Our previous finding that *LAT* mRNA is expressed in an NK-like cell line (YT) led us to assess NK cell development and function in the LAT$^{-/-}$ mice. To examine the development of NK cells, we isolated splenocytes from LAT +/+, +/−, and −/− mice and analyzed them by FACS for the expression of the IL2 receptor β chain (CD122) and the NK cell marker DX5. As shown in Figure 6A, cells expressing both IL2 receptor β chain and DX5 were present in LAT$^{-/-}$ mice. These results suggest that NK development is not compromised in the LAT$^{-/-}$ mice. To assess NK function, we isolated total splenocytes from LAT +/+ and −/− mice and further enriched for NK cells. The purity of these NK cell populations is >80%. To confirm the LAT expression in murine NK cells, we further enriched NK cells from LAT$^{+/+}$ mice to a purity of >99% by FACS depletion of any residual CD3$^+$ cells. Figure 6B shows that while NK cells do express LAT, they do so at a level approximately one tenth that of T cells. To assess the cytolytic function of LAT$^{-/-}$ NK cells, we tested the ability of the LAT$^{+/+}$ and LAT$^{-/-}$ NK cell populations to lyse the mouse T cell lymphoma YAC-1 and the human B cell line 721.221 (Figure 6C). No difference was seen in the ability of the

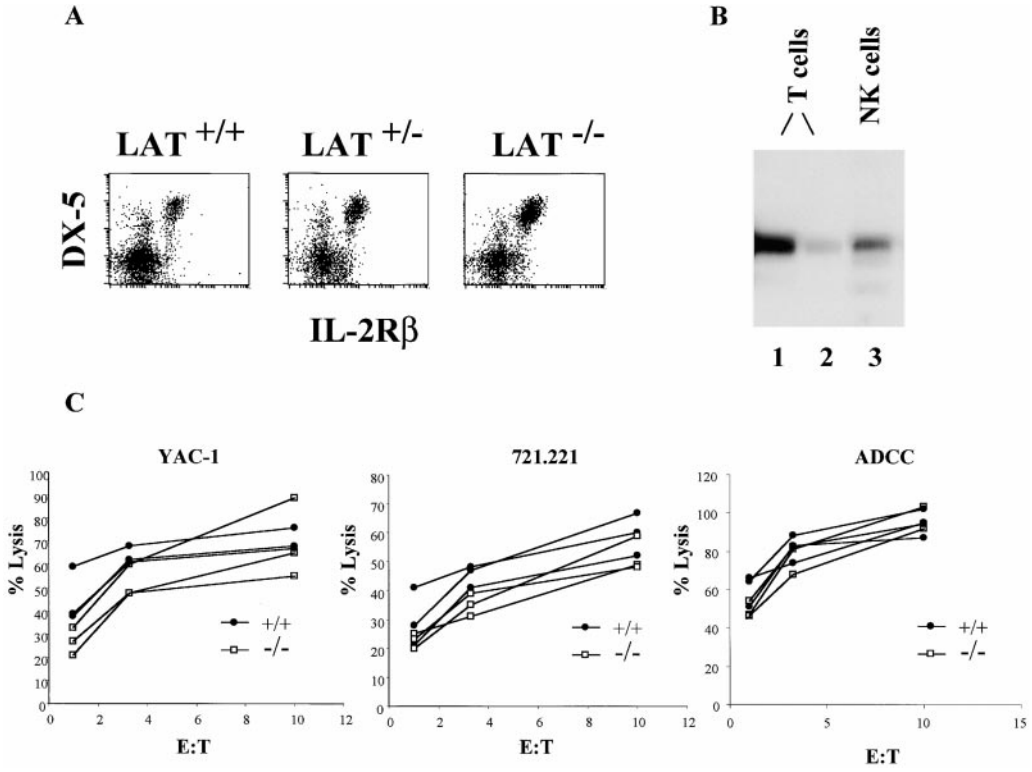

Figure 6. Analysis of NK Cells from LAT-Deficient Mice

(A) The development of NK cells is not affected by LAT deficiency. Splenocytes were surface stained with anti-IL-2Rβ-FITC and the NK marker DX-5-PE.

(B) LAT expression in NK cells. NK cells and T cells were purified from $LAT^{+/+}$ mice. NK cells were further purified to >99% purity by FACS depletion of $CD3^+$ cells. These cells were directly solubilized in sample buffer and analyzed by immunoblotting with anti-LAT antiserum. Lane 1, 10^5 T cells; lane 2, 2×10^4 T cells; lane 3, 5×10^5 NK cells.

(C) NK cytolytic activity is not affected by lack of LAT. The cytolytic activity was analyzed with ^{51}Cr release assays using the mouse T lymphoma cell line YAC-1 and the human B cell line 721.221. Antibody-dependent cellular cytotoxicity was measured after binding the mAb L243 to 721.221 cells.

$LAT^{-/-}$ and $LAT^{+/+}$ NK cells to lyse these targets across several E:T ratios. Similarly, addition of a monoclonal antibody to coat the 721.221 targets in order to produce antibody-dependent cellular cytotoxicity (ADCC) also revealed no difference between the NK cells from the $LAT^{-/-}$ and $LAT^{+/+}$ mice.

Discussion

Disruption of LAT expression might be expected to have a deleterious effect on development and function in the T cell, NK, and platelet lineages. Instead, in our initial analysis, we observed a wide range of results. Platelets and NK cells seemed not to be affected. In contrast, disruption of the *LAT* gene resulted in a profound block in T cell development. Thymocytes in $LAT^{-/-}$ mice were arrested at the DN, $CD25^+CD44^-$ stage, and there were no mature αβT cells in the lymph nodes and spleens of these mice. γδ T cell development was also blocked in $LAT^{-/-}$ mice as was the maturation of "thymus-independent" iIELs. Together, these results demonstrate that LAT is essential for the development of all T cell subsets.

A number of previous studies detail the effects of the disruption of genes involved either in the surface expression of the pre-TCR/TCR or in T cell signaling pathways. The block in T cell development observed in LAT-deficient mice shares many features with those seen with $RAG^{-/-}$ (Mombaerts et al., 1992; Shinkai et al., 1992), $CD3\epsilon^{-/-}$ (Malissen et al., 1995; DeJarnette et al., 1998), $Lck^{-/-} \times Fyn^{-/-}$, (Groves et al., 1996; van Oers et al., 1996), $ZAP\text{-}70^{-/-} \times Syk^{-/-}$ (Cheng et al., 1997), and $SLP\text{-}76^{-/-}$ (Clements, et al., 1998; Pivniouk et al., 1998) mice. Thymocytes in all these mice do not develop beyond the $CD25^+CD44^-$ subset of the $CD4^-$ $CD8^-$ stage. The block at this point of differentiation reflects the conclusion that expression of a pre-TCR complex, coupled to an intracellular signaling pathway resembling that utilized by the mature TCR, is required for transition past this step. In particular, it is thought that signaling via the pre-TCR is required for induction of CD4 and CD8 expression and TCRα gene rearrangement (Fehling and von Boehmer, 1997). In $RAG^{-/-}$ and $CD3\epsilon^{-/-}$ mice, the developmental arrest can be attributed to structural defects resulting in failure to express the pre-TCR. Experimentally, it is possible to drive differentiation past this block in $RAG^{-/-}$ mice by cross-linking the very low level of CD3 chains present on the surface of these early thymocytes with antibodies (Levelt et al., 1993). However, such a response to CD3 engagement did not occur in the LAT-deficient thymocytes, despite the fact

that these cells should express intact pre-TCR surface complexes. These results indicate that LAT is critical for coupling the pre-TCR to the intracellular signaling pathways that mediate this developmental response.

Disruption of the individual PTKs involved in pre-TCR/TCR signaling does not result in a block in intrathymic development as dramatic as does disruption of LAT. In the absence of Lck, thymocyte numbers are reduced and most cells are blocked at the DN stage; however, some thymocytes can develop to the DP stage (Molina et al., 1992). Thymocyte development is much less affected in Fyn$^{-/-}$ mice, and only a mild defect in signaling in thymic SP cells can be detected (Appleby et al., 1992; Stein et al., 1992). Mice lacking both Lck and Fyn exhibit the same phenotype as LAT-deficient animals, namely, a complete block at the DN, CD25^{+}CD44^{-} stage (Groves et al., 1996; van Oers et al., 1996). This indicates that the Src kinases Lck and Fyn have a redundant but obligatory function in pre-TCR signaling. From analysis of T cell signaling in T cell lines, this function is likely to be the phosphorylation of the ITAMs associated with the pre-TCR and of the Syk family PTKs activated with the pre-TCR. Independent disruption of *ZAP-70* or *Syk* genes also do not result in as severe a phenotype as is observed following deletion of LAT. Loss of ZAP-70 blocks thymocyte development between the DP and SP stages (Negishi et al., 1995). Differentiation past the DN stage in these mice is due to the presence of the related kinase Syk. The deletion of Syk affects only a subset of γδ T cells (Mallick-Wood et al., 1996). The deletion of both Syk and ZAP-70 leads to a complete developmental block at the DN stage (Cheng et al., 1997). Thus, these two PTKs also have a redundant but necessary role in signaling through the pre-TCR and progression past the DN stage.

In T cell lines, TCR engagement leads to LAT tyrosine phosphorylation and binding of LAT to multiple proteins and protein complexes. Grb2 binding to LAT is of major importance, since Grb2 is associated with SOS, Cbl, and the complex of SLP-76, Vav, SLAP, and p62. These Grb2-associated proteins, at the least, control activation of Ras and Rac family GTPases. In addition, LAT is associated with PI-3 kinase and PLC-γ1, which is required for Ca^{2+} and PKC activation. Loss of LAT thus has the potential to impinge on the multiple signaling pathways that are likely to be involved in intrathymic TCR-mediated signaling leading to differentiation.

Ras activation has been shown to be involved in pre-TCR signaling and T cell development. The activated form of Ras can induce the differentiation of DN cells to DP cells in RAG-2$^{-/-}$ mice (Swat et al., 1996). The likely absence of Grb2-SOS recruitment in the LAT-deficient cells suggests that such required Ras activation is affected. The loss of some Vav recruitment likely explains why Vav phosphorylation is reduced in LAT-deficient cells. However, Vav$^{-/-}$ mice show normal development of thymocytes from DN to DP and only have a partial block between the DP and the SP cells (Fischer et al., 1995; Tarakhovsky et al., 1995; Zhang et al., 1995). The loss of SLP-76 recruitment to LAT via Grb2 may be more important. Mice lacking this molecule show the same intrathymic developmental block observed in the LAT-deficient mice (Clements et al., 1998; Pivniouk et al., 1998). In these mice ligation of CD3 molecules with antibody also fails to induce differentiation to the DP stage. The absence of SLP-76 in Jurkat cells has a very dramatic effect on signaling, showing a defect in normal TCR-mediated PLC-γ1 and Ras activation. These deficiencies may be mediated not just by failure of Vav recruitment, but also by the absence of other proteins to which SLP-76 binds (SLAP and p62). The fact that disruption of either LAT or SLP-76 alone is sufficient to block T cell development indicates that these proteins are nonredundant in T cells.

SLP-76$^{-/-}$ mice also demonstrate decreased viability and increased hemorrhage. Platelet numbers are reduced in SLP-76$^{-/-}$ mice, and they do not respond normally to stimulation with collagen (Pivniouk et al., 1998; Clements et al., 1999). LAT is expressed in megakaryocytes and platelets (Facchetti et al., 1999), and thus it was a surprise that LAT$^{-/-}$ newborns were viable and healthy without obvious problems with hemorrhage. Recent studies on platelets derived from LAT-deficient mice demonstrate that engagement of the collagen receptor (GPVI) by collagen-related peptide fails to activate phospholipase C (J.-M. Pasquet, S. P. Watson, W. Z., and L. E. S., unpublished data). In this regard LAT and FcRγ-chain-deficient mice share defects in this pathway without evidence of the bleeding defect observed in SLP-76- and Syk-deficient mice (Cheng et al., 1995; Poole et al., 1997; Pivniouk et al., 1998; Clements et al., 1999).

NK cells play an important role in the nonadaptive immune response to microbial infections and tumors. NK cells can lyse target cells directly by natural killing or through Fc receptors and are a primary source of IFN-γ production. The issue of LAT function in NK cells is of considerable interest because a 36 kDa phosphoprotein has also been found in association with PLC-γ1 and Grb2 in NK cells following FcR engagement (Galandrini et al., 1996) and after interaction with tumor target cells (Valiante et al., 1996). Valiante and colleagues showed that the 36 kDa protein is no longer associated with Grb2 or PLC-γ1 when NK inhibitory receptors are engaged by MHC class I molecules on target cells. They concluded that the phosphorylation of the 36 kDa protein is critical for NK activation and that the tyrosine phosphatase recruited by the inhibitory receptors dephosphorylates the 36 kDa protein (Burshtyn et al., 1996).

Our data in this paper show that LAT is indeed present in NK cells isolated from mice, though in lower amounts than in T cells. Similar results have been observed in human NK cells (Facchetti et al., 1999). LAT appears to participate in signaling in NK cells because cross-linking of Fc receptors on human NK cells induces LAT phosphorylation (Jevremovic et al., 1999; C. C. S., D. N. B, and E. O. L., unpublished data). However, as shown in this paper, expression of LAT in mouse NK cells is not required for natural cytotoxicity or ADCC. This is a surprising result because of the overlap in molecules involved in activation of T and NK cells, and because NK-mediated cytolysis is dependent on Ca^{2+} mobilization, which is LAT-dependent in T cells. To elucidate a role for LAT in NK cells, one may need to take into account the clonal heterogeneity of the receptors expressed by

NK cells and possible differences in the signaling pathways of human and mouse NK cells.

The effect of LAT deficiency thus varies in some of the cell types in which it is expressed. In platelets and NK cells, further studies must define the function of LAT. It appears that LAT also has a role in mast cell function, as our preliminary studies with bone marrow–derived mast cells from LAT-deficient mice demonstrate a defect in FcεRI-mediated secretion (S. Saitoh, R. Arudchandran, J. Rivera, W. Z., and L. E. S., unpublished data). The next phase in further investigation of LAT and thymocyte development will be reconstitution of the $LAT^{-/-}$ mice using transgenics expressing different LAT mutants. These studies will attempt to determine how various signaling pathways couple to and are dependent on LAT.

Experimental Procedures

Vector Construction

A 9.0 Kb BglII fraction from a LAT genomic Bac clone was cloned into a BamHI site of pBluescript KS to generate pBKS/LAT plasmid. A 1.6 Kb fragment from SpeI to BamHI was excised from pBKS/LAT, blunt-ended with DNA polymerase Klenow fragment, and cloned into a blunted BamHI/EcoRI site of XpPNT vector as the 5′ targeting flank. A 3.5 kb XbaI–HindIII fragment from pBKS/LAT was cloned into pBluescript XbaI–HindIII sites. The resulting plasmid was digested with XbaI and ligated with an annealed pair of two oligonucleotides with the sequences CTAGTGAGCTCGAGGTACC and CTAGGGTACCTCGAGCTCA to generate an additional XhoI site. This plasmid was then digested with XhoI and the 3.5 Kb XhoI fragment was used as the 3′-end targeting flank and cloned into the XhoI site of XpPNT containing the 5′ targeting flank. The targeting construct was linearized with NotI. The linearized DNA fragment was gel purified and used to transfect 2×10^7 ES cells by electroporation. After transfection, ES cells were selected in the presence of G418 and gancyclovir. Of 216 resistant clones, 10 had undergone homologous recombination as assessed by Southern blot analysis. One targeted clone was injected into C57BL/6 mice. LAT-deficient mice ($LAT^{-/-}$) were obtained by interbreeding LAT heterozygous mice ($LAT^{+/-}$).

NK Cell Separation and Cytolytic Killing Assay

NK cells from $LAT^{+/+}$ and $LAT^{-/-}$ mice were generated as previously described (Karlhofer and Yokoyama, 1991). Briefly, spleen cell suspension was passed over a nylon wool column at 37°C and the pass-through fractions were cultured in the presence of IL-2 for 3 days. The nonadherent cells were then removed and the remaining cells were cultured for 3–6 days. The purity of the NK populations was determined on day 6 of culture using the monoclonal antibody 145–2C11 (anti-CD3ε) directly conjugated to phycoerythrin (PharMingen). The $LAT^{+/+}$ and $LAT^{+/-}$ contained 5%–20% $CD3^+$ cells, and there were no $CD3^+$ cells in the $LAT^{-/-}$ populations. Cytotoxic assays were performed on day 9 by measuring specific release from ^{51}Cr-labeled target cells. To measure ADCC, ^{51}Cr-labeled 721.221 cells were preincubated with 1 μg/ml L243 (anti-HLA-DR) as described (Rojo et al., 1997).

Cell Preparation and Flow Cytometry

Thymus, spleen, and lymph nodes were excised from mice and single cell suspensions were prepared. Intestinal intraepithelial lymphocytes (iIELs) were prepared from the small intestine as previously described (Shores et al., 1997). Standard flow cytometry was performed as previously described (Shores et al., 1997) using a Becton Dickinson FACScan and Cell Quest software (Becton Dickinson Immunoocytometry Systems). Commercial antibodies to mouse antigens used include anti-CD3ε-FITC, anti-IgM-FITC, anti-CD44-FITC, anti-αβTCR-FITC, anti-γδ-FITC, anti-CD8β-FITC, anti-Vγ3-FITC, anti-CD4-PE, anti CD25-PE (Pharmingen), anti-CD8α-Quantum red, anti-B220-Quantum red, anti-CD4-Quantum red, and anti-CD3-Quantum red (Sigma).

TCR Gene Rearrangement

TCR gene rearrangements were detected by PCR and Southern blot analysis using the following primers and probes as described (Anderson et al., 1992). For PCR reaction, Vβ5-specific primer (GATT AAGTTACAGAAAGCCAGTAGC) or Vβ8-specific primer (GTTCTCCT TATTTCCTTTCTGTGCAGAG) was used as the 5′ end primer and Jβ2-specific primer was used (TGAGAGCTGTCTCCTACTATCGATT) as the 3′ end primer. A Jβ2-specific probe (TTTCCCTCCCGGAGAT TCCCTAA) was in Southern blot analysis.

Western Blot Analysis

Thymocytes (10^7) from $LAT^{+/-}$ and $LAT^{-/-}$ mice were lysed in 50 ml lysis buffer (1% Brij97; 25 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA) on ice for 10 min. After centrifugation, the supernatant was then mixed with 2× SDS sample buffer. To detect LAT expression in mouse NK cells, enriched NK cells were further purified by removing residual $CD3^+$ cells from a $LAT^{+/+}$ culture on day 6. Cells (10^5) were lysed directly into 4× SDS sample buffer. The genomic DNA present in the samples was sheared by repeated pipetting. These samples were analyzed by SDS-PAGE and Western blotting with anti-LAT antiserum.

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