Knock-In Mutation of the Distal Four Tyrosines of Linker for Activation of T Cells Blocks Murine T Cell Development

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Abstract

The integral membrane adapter protein linker for activation of T cells (LAT) performs a critical function in T cell antigen receptor (TCR) signal transduction by coupling the TCR to downstream signaling pathways. After TCR engagement, LAT is tyrosine phosphorylated by ZAP-70 creating docking sites for multiple src homology 2–containing effector proteins. In the Jurkat T cell line, the distal four tyrosines of LAT bind PLCγ-1, Grb2, and Gads. Mutation of these four tyrosine residues to phenylalanine (4YF) blocked TCR-mediated calcium mobilization, Erk activation, and nuclear factor (NF)-AT activation. In this study, we examined whether these four tyrosine residues were essential for T cell development by generating LAT "knock-in" mutant mice that express the 4YF mutant protein under the control of endogenous LAT regulatory sequences. Significantly, the phenotype of 4YF knock-in mice was identical to $LAT^{-/-}$ (null) mice; thymocyte development was arrested at the immature $CD4^{-}CD8^{-}$ stage and no mature T cells were present. Knock-in mice expressing wild-type LAT protein, generated by a similar strategy, displayed a normal T cell developmental profile. These results demonstrate that the distal four tyrosine residues of LAT are essential for preTCR signaling and T cell development in vivo.

Key words: thymocyte • development • signal transduction • adapter • gene targeting

Introduction

Engagement of the TCR initiates a signaling cascade that results in proliferation, cytokine production, and effector responses. After TCR cross-linking, the invariant chains of the TCR are rapidly tyrosine phosphorylated creating docking sites for the recruitment and activation of src homology 2 (SH2) domain–containing protein tyrosine kinases (PTKs) such as ZAP-70. These PTKs activate other downstream effector molecules thereby triggering signaling pathways that lead to calcium mobilization and mitogen-activated protein (MAP) kinase activation (1, 2).

The adapter protein linker for activation of T cells (LAT)* is one of the predominant tyrosine phosphorylated proteins observed after T cell activation (3). LAT contains two juxtamembrane cysteine residues that serve as palmitoylation sites, targeting the protein to glycolipid-enriched microdomains in the cell membrane (4). After TCR engagement, LAT is tyrosine phosphorylated by ZAP-70, creating docking sites for multiple downstream SH2-containing effector proteins. Proteins that associate directly or indirectly with LAT include: PLC-γ1, the p85 subunit of PI3-kinase, Grb2, Grap, SLP-76, Vav, Itk, Cbl, and Gads (3, 5–7). T cell lines deficient in LAT undergo proximal activation steps such as TCR-ζ chain phosphorylation and recruitment and phosphorylation of ZAP-70, but exhibit markedly decreased tyrosine phosphorylation of PLC-γ1, calcium mobilization, Ras and Erk activation, and transcriptional activation of AP-1 and nuclear factor (NF)-AT after TCR cross-linking, suggesting that in the absence of LAT, the TCR is uncoupled from downstream signaling pathways (8, 9). Transfection of wild-type human LAT cDNA into these cell lines reconstituted normal activation responses to TCR stimulation. Recently, these LAT-defi-

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*Abbreviations used in this paper: CIC, clonotype independent complex; LAT, linker for activation of T cells; Rag, recombinase-activating gene.

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cient cell lines were also used as recipients for transfection with mutant LAT cDNAs to examine the importance of various LAT tyrosine residues (5). The results of this study demonstrated distinct and overlapping functions of the distal four tyrosines. The LAT mutant lacking all four distal tyrosines failed to bind Grb2, Gads, and PLC-γ1, and LAT-deficient cells reconstituted with this mutant failed to respond to TCR cross-linking (5).

The generation of LAT-deficient mice has also revealed a critical role for this protein during T cell development. $LAT^{-/-}$ mice contain no mature T cells, including lymphoid αβ T cells, γδ T cells, and intestinal intraepithelial lymphocytes (10). Moreover, thymocyte development is blocked at the immature $CD4^-CD8^-$ stage, suggesting that LAT is required for preTCR signaling. T cell development requires the transduction of multiple signals at different stages mediated by both the preTCR and the TCR (1, 11). Since the reconstitution experiments in Jurkat cells specifically address signal transduction through the mature TCR, it is possible that other sequences in LAT may be necessary for T cell development, particularly since the structure and downstream signaling components of the preTCR may not be identical to those of the mature TCR. In this study, we asked whether the same four tyrosines required for T cell activation in Jurkat cells are also required for T cell development. To this end, we replaced the wild-type LAT gene in mice with a LAT gene mutated in the four distal tyrosines by homologous recombination. Significantly, the phenotype of knock-in mice that express the mutated LAT protein was identical to that of $LAT^{-/-}$ (null) mice; thymocyte development was arrested at the immature $CD4^-CD8^-$ stage and no mature T cells were present. Knock-in mice that express wild-type LAT protein, generated by a similar strategy, displayed a normal T cell developmental profile. These results demonstrate that the distal four tyrosine residues of LAT are essential for preTCR signaling and T cell development in vivo.

Materials and Methods

Generation of LAT Knock-In Mice. The LAT knock-in targeting construct depicted in Fig. 1 B contained the following DNA elements: a 5′ mouse LAT genomic flank (from an original BglII site to the EcoRI site in exon 5), LAT cDNA containing exons 5–12, the PGK-neomycin phosphotransferase gene flanked by *loxP* sites (from pGKneoLoxP, Washington University Embryonic Stem Cell Core, St. Louis, MO), a 1.8-kb 3′ mouse genomic LAT flank (BamHI to EcoRV), and PGK-thymidine kinase. Site-directed mutagenesis was performed on LAT cDNA containing exons 5–12 (Quik-Change Site-Directed Mutagenesis kit; Stratagene). Mutations were confirmed by DNA sequencing.

The targeting construct was linearized and transfected into 129 R1 ES cells by electroporation. DNA from G418 and gancyclovir double-resistant clones was analyzed for homologous recombination by PCR. A three-primer PCR was used for screening ES cell clones including a 5′ primer from LAT intron 8 (5′-GCTAATAACTGTACTGCAATGGC-3′), a 5′ primer from the NEO gene (5′-GCATCGCCTTCTATCGCCTTC-3′), and a 3′ primer from LAT genomic DNA that is downstream from the 3′ targeting flank (5′-ATCACCTGCTGCTGGCAGTTC-3′). Positive clones were confirmed for homologous recombination by Southern blot analysis (3′ end) and PCR (3′ and 5′ ends). Positive clones were injected into C57BL/6 blastocysts using standard procedures (10). Chimeric mice were mated to EIIa CRE transgenic mice (12) that had been backcrossed onto a C57BL/6 background for 10 generations. Progeny containing recombinant LAT alleles in which the PGK-NEO cassette had been deleted by CRE-mediated recombination were intercrossed to obtain homozygous mutant mice.

Cell Preparation and Flow Cytometry. Single cell suspensions were prepared from thymi and lymph nodes. Flow cytometry was performed using a FACScan™ and CELLQuest™ software (Becton Dickinson). Antibodies to mouse antigens were purchased from BD PharMingen. LAT intracellular staining was performed essentially as described previously (13) with the exception that detergent permeabilization was for 4 min in 0.1% Triton X-100.

Anti-CD3 Injection. Mice of ~6 wk of age were injected intraperitoneally with 100 μg of purified anti-CD3 (monoclonal antibody 145-2C11 in PBS). 1 wk later the mice were killed and thymocytes were analyzed by flow cytometry.

Western Blot Analysis. Thymocytes were lysed in Brij97 lysis buffer (1% Brij97, 25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and 1× complete protease inhibitor; Roche). Cleared lysates were analyzed by gel electrophoresis and Western blot analysis was used with rabbit anti-LAT serum (3) or goat antiactin serum (Santa Cruz Biotechnology, Inc.).

Results and Discussion

Generation of LAT Knock-In Mice. Out of nine potential tyrosine phosphorylation sites in human LAT, the distal four tyrosine residues have been shown to bind signaling molecules critical for T cell activation in Jurkat T cells (5). Y136, Y175, Y195, and Y235 are the mouse counterparts of those tyrosines and are shown in Fig. 1 A with the proteins that bind the analogous sites in stimulated Jurkat T cells. To determine the importance of these tyrosine residues for T cell development in mice, we wanted to substitute a mutant form of LAT for wild-type LAT while still maintaining the normal level and timing of LAT expression. Consequently, we used a "knock-in" gene targeting approach, whereby the mouse wild-type LAT gene was replaced by a coding sequence containing tyrosine to phenylalanine mutations of Y136, Y175, Y195, and Y235. The targeting construct carrying the mutant LAT gene is shown in Fig. 1 B. The endogenous LAT gene was replaced with a minigene composed of genomic DNA containing exons 1–5 (up to and including an EcoRI site) and LAT cDNA encoding exons 5–12 (from the EcoRI site). Use of the minigene allowed for easier site-specific mutation of the four LAT tyrosine residues, which are encoded in exons 7, 9, 10, and 11. In addition, the PGK-NEO gene was flanked by *loxP* sites, which enabled its subsequent excision in the mouse germline by crossing to EIIa-CRE recombinase transgenic mice (14). This knock-in strategy should result in expression of a mutant LAT protein at normal levels with normal timing during ontogeny since the minigene is placed in the proper genomic context by homologous re-

combination. However, since the minigene does not contain all of the genomic elements of the wild-type gene (e.g., the large eighth intron is deleted), we thought it important to generate in parallel a construct analogous to the one carrying the four Y→F mutations but encoding tyrosines at positions 136, 175, 195, and 235 (i.e., wild-type LAT).

Mice homozygous for the wild-type and mutant (LAT protein with distal four tyrosines mutated to phenylalanines [4YF]) LAT knock-in alleles were generated from two independent targeted ES cell clones each. The duplicate lines exhibited identical phenotypes, indicating that it is unlikely that genetic alterations in addition to the homologous recombination event at the LAT locus contributed to the phenotypes observed (data not shown).The presence of knock-in alleles and the excision of PGK-NEO were detected by PCR of tail DNA, and LAT coding sequences were confirmed by reverse transcription PCR of thymocyte mRNA (data not shown). We designated homozygous LAT wild-type knock-in mice with PGK-NEO excised as $LAT^{WT/WT}$ and homozygous LAT mutant knock-in mice with PGK-NEO excised as $LAT^{4YF/4YF}$. Mice containing the wild-type LAT genomic sequences were designated $LAT^{+/+}$, and mice homozygous for the LAT null mutation (described previously; reference 10) were designated $LAT^{-/-}$.

Characterization of $LAT^{WT/WT}$ Mice. $LAT^{WT/WT}$ mice were analyzed first to determine if LAT expressed from the recombinant, knock-in gene could support T cell development. Analysis of LAT protein levels in total thymocytes by intracellular staining (Fig. 2 A) and by Western blot analysis (Fig. 2 B) revealed that $LAT^{WT/WT}$ mice contained LAT protein at levels that were slightly lower than those in $LAT^{+/+}$ mice but higher than those in $LAT^{+/-}$ (heterozygous) mice. Significantly, the LAT protein levels in $LAT^{+/-}$ mice are sufficient to completely support thymocyte development (10).

Total thymocyte numbers were normal in $LAT^{WT/WT}$ mice (Fig. 3, and data not shown), and the percentages of $CD4^-CD8^-$, $CD4^+CD8^+$, and mature $CD4^+$ and $CD8^+$ thymocytes were comparable in $LAT^{WT/WT}$ and $LAT^{+/+}$ mice (Fig. 3). In addition, surface levels of activation/differentiation markers including CD3ϵ, CD5, CD69, CD24, and CD25 were essentially the same on thymocytes from $LAT^{WT/WT}$ and $LAT^{+/+}$ mice (Fig. 3, and data not shown). Likewise, the number of peripheral CD4 and CD8 T cells were normal in $LAT^{WT/WT}$ mice and these cells expressed similar levels of CD3ϵ, CD5, CD44, and CD62L to the corresponding cells from $LAT^{+/+}$ mice (Fig. 3, and data not shown). B and NK cell numbers were also were normal in $LAT^{WT/WT}$ mice (data not shown). Collectively, these results indicated that the knock-in strategy could be

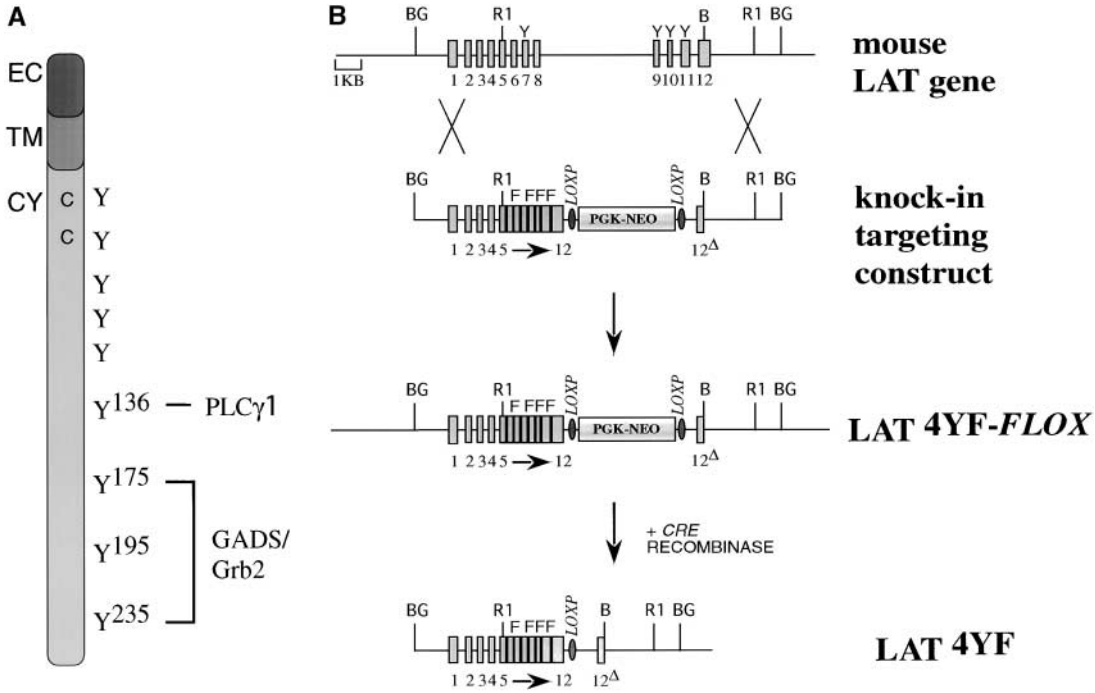

Figure 1. (A) Schematic representation of the mouse LAT molecule. Domains include EC (extracellular), TM (transmembrane), and CY (cytoplasmic). C denotes cysteine residues that are sites of palmitoylation and Y denotes tyrosine residues that are potential phosphorylation sites. Mutation of Y132 (human counterpart of Y136 in mouse LAT) results in a loss of binding of PLC-γ1 in Jurkat T cells and mutation of Y171, Y191, and Y226 (human counterparts of Y175, Y195, and Y235 in mouse) results in a loss of Gads and Grb-2 binding (5). (B) Strategy for 4YF LAT knock-in mutation. The knock-in targeting construct contains a LAT minigene (LAT genomic DNA 5′ to the EcoRI site in exon 5 and LAT cDNA 3′ to the EcoRI site), the PGK-NEO gene flanked by *loxP* sites, and the PGK-TK gene (not shown). The $LAT^{4YF\text{-}FLOX}$ gene is generated by homologous recombination of the knock-in targeting construct and the wild-type mouse LAT gene. After exposure to CRE recombinase, the LAT^{4YF} allele (bottom) is produced. The LAT^{WT} allele was made in an analogous fashion but does not contain the four Y→F mutations.

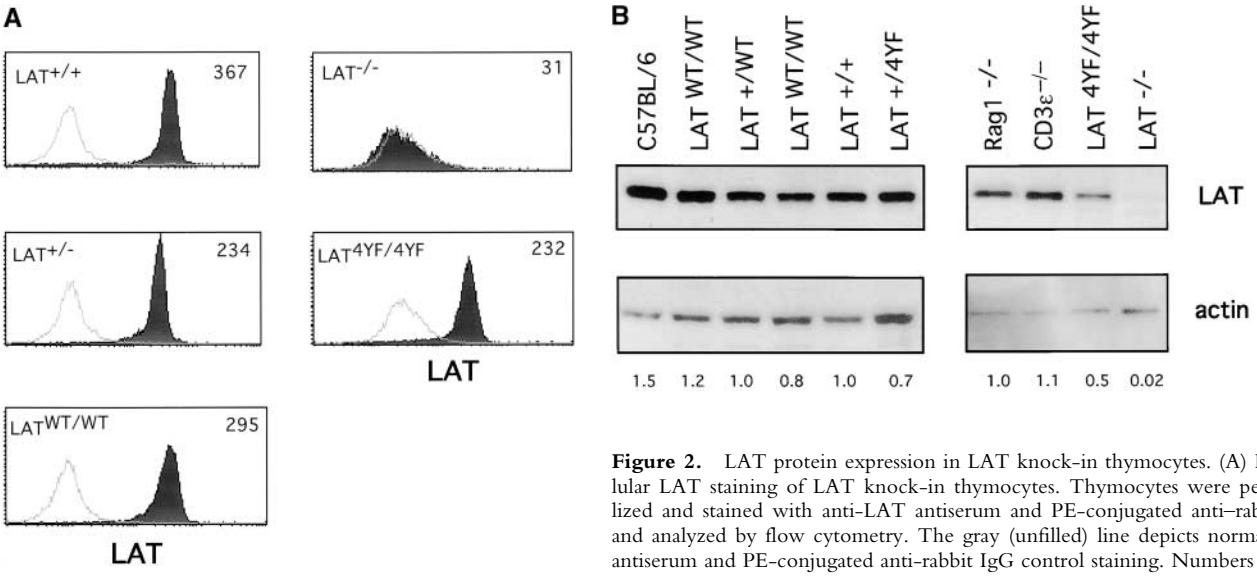

Figure 2. LAT protein expression in LAT knock-in thymocytes. (A) Intracellular LAT staining of LAT knock-in thymocytes. Thymocytes were permeabilized and stained with anti-LAT antiserum and PE-conjugated anti–rabbit IgG and analyzed by flow cytometry. The gray (unfilled) line depicts normal rabbit antiserum and PE-conjugated anti-rabbit IgG control staining. Numbers indicate mean fluorescence intensity. (B) Western blot analysis of LAT knock-in thymocytes. Total thymocyte detergent soluble lysates were analyzed by Western blot analysis using anti-LAT rabbit antiserum. The blot was stripped and reprobed for actin. Values beneath the blot indicate the densitometric ratio of LAT protein to actin protein.

employed to analyze the effect of specific LAT tyrosines on T cell development.

Characterization of LAT$^{4YF/4YF}$ Mice. LAT$^{4YF/4YF}$ mice exhibited a phenotype dramatically different from that of LAT$^{WT/WT}$ mice resembling that of LAT$^{-/-}$ mice, although LAT$^{4YF/4YF}$ thymocytes contained similar LAT protein levels to LAT$^{+/-}$ and LAT$^{WT/WT}$ mice (Fig. 2 A). We also compared LAT protein levels from LAT$^{4YF/4YF}$ thymocytes to those from recombinase-activating gene (Rag) 1$^{-/-}$ and CD3ε$^{-/-}$ thymocytes by Western blot analysis, since these mice all have thymi of similar size that contain similar thymocyte subpopulations. Some experiment to experiment variability was observed; however, the protein levels in LAT$^{4YF/4YF}$ thymocytes were consistently between 0.5–1.0 times the level of LAT present in thymocytes from Rag1$^{-/-}$ and CD3ε$^{-/-}$ mice (Fig. 2 B).

We reported previously that T cell, but not B cell, mast cell, NK cell, or platelet development is blocked in LAT$^{-/-}$ mice (10). Therefore, we focused our analysis of LAT$^{4YF/4YF}$ mice on T cell development. Thymi from LAT$^{4YF/4YF}$

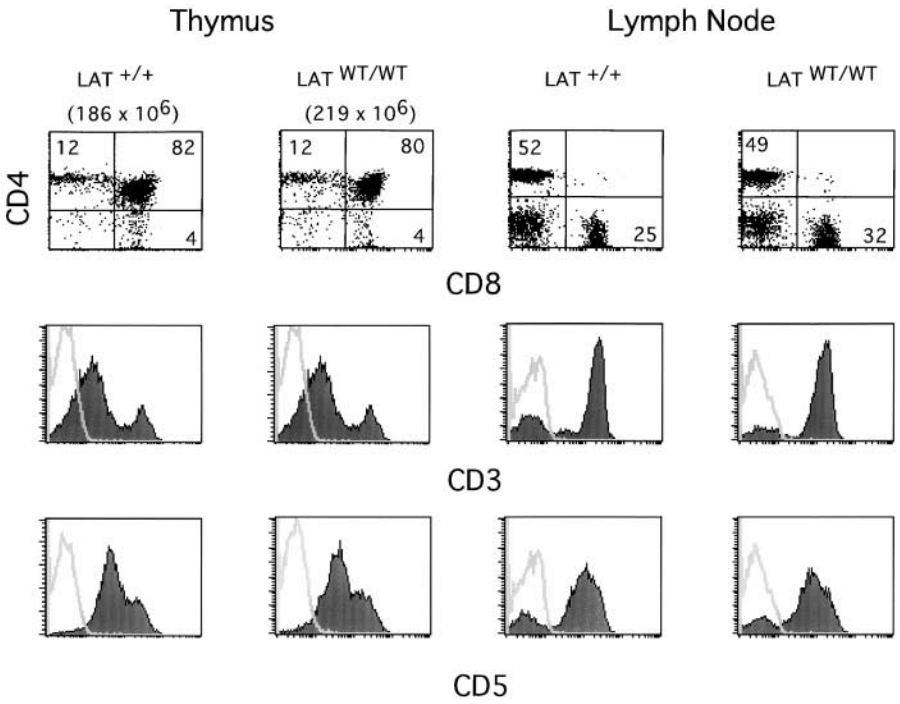

Figure 3. Flow cytometric analysis of LAT$^{WT/WT}$ thymocytes and lymph node cells. Single cell suspensions from thymus and lymph node were surface stained and analyzed by flow cytometry. Two color plots of CD4 and CD8 are shown as are one color histograms for CD3ε and CD5. Gray lines indicate isotype-matched negative control antibody staining. Thymocyte numbers are given in parentheses.

mice were small, typically about one-tenth the size of thymi from $LAT^{+/+}$ mice (similar to the size of $LAT^{-/-}$ thymi; Fig. 4 A, and data not shown). Moreover, the average thymocyte size, as measured by forward and side scatter, was larger in $LAT^{4YF/4YF}$ mice than in $LAT^{+/+}$ or $LAT^{WT/WT}$ mice suggesting that these cells were immature (Fig. 4 A). Consistent with this finding, all of the thymocytes from $LAT^{4YF/4YF}$ mice were $CD4^{-}CD8^{-}$. CD3ε (Fig. 4 A) and TCR-β (data not shown) were nearly undetectable on the surface of $LAT^{4YF/4YF}$ thymocytes; however, intracellular TCR-β staining was detected in $LAT^{4YF/4YF}$ and $LAT^{-/-}$ thymocytes indicating that the

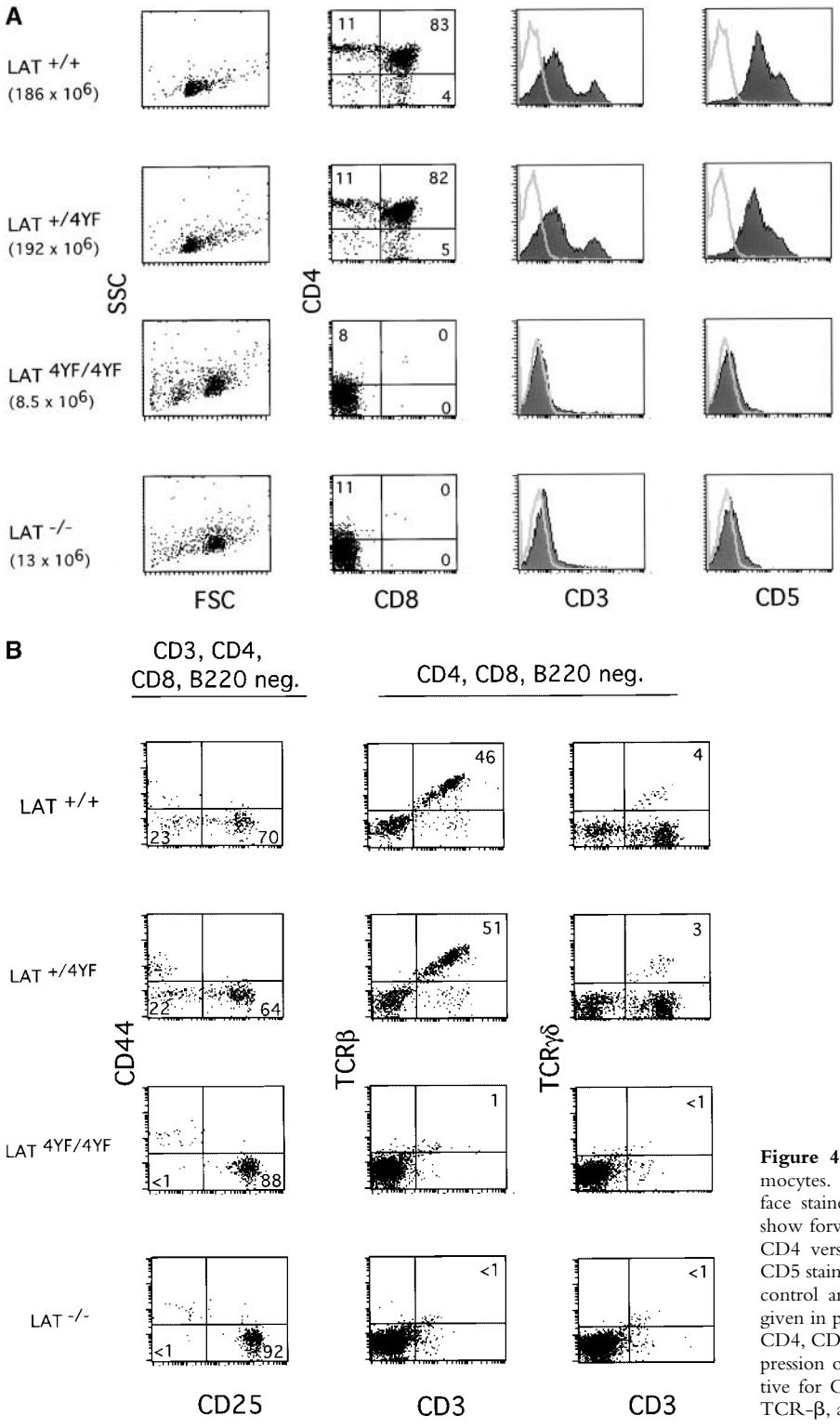

Figure 4. Flow cytometric analysis of $LAT^{4YF/4YF}$ thymocytes. (A) Single cell thymocyte suspensions were surface stained and analyzed by flow cytometry. Dot plots show forward scatter versus side scatter measurements and CD4 versus CD8 staining. Histograms show CD3ε and CD5 staining. Gray lines indicate isotype-matched negative control antibody staining. Total thymocyte numbers are given in parentheses. (B) Thymocytes gated as negative for CD4, CD8, CD3ε, and B220 were analyzed for surface expression of CD44 and CD25. Thymocytes gated as negative for CD4, CD8, and B220 were analyzed for CD3ε, TCR-β, and TCR-γδ surface expression.

TCR-β gene is rearranged and expressed (data not shown). To determine more specifically where the block in T cell development occurred in $LAT^{4YF/4YF}$ mice, thymocytes were stained for CD25 and CD44. Immature $CD4^-CD8^-$ thymocytes progress through the four developmental stages before differentiating to the $CD4^+CD8^+$ stage: $CD25^-CD44^+ \rightarrow CD25^+CD44^+ \rightarrow CD25^+CD44^- \rightarrow CD25^-CD44^-$ (15). As shown in Fig. 4 B, T cell development was blocked at the $CD25^+CD44^-$ stage in $LAT^{4YF/4YF}$ mice. Significantly, T cell development is also blocked at this stage in $LAT^{-/-}$ mice (10) and in $Rag^{-/-}$ (16, 17), $CD3\epsilon^{-/-}$ (18, 19), and $SLP76^{-/-}$ mice (20), consistent with the idea that preTCR signals are required for the development of $CD4^-CD8^-CD25^+CD44^-$ thymocytes to the $CD4^-CD8^-CD25^-CD44^-$ and subsequently, to the $CD4^+CD8^+$ stage.

Clonotype independent complexes (CICs), which contain CD3 subunits paired with the chaperone calnexin, can be expressed on the cell surface of $CD4^-CD8^-CD25^+$ $CD44^-$ thymocytes (21). CICs have been most readily demonstrated on the surface of cells that lack expression of clonotypic subunits, such as from $Rag^{-/-}$ mice. Signal transduction through the preTCR or through CICs can be triggered by injection of anti-CD3ε which induces proliferation of $CD4^-CD8^-$ thymocytes and their maturation to the $CD4^+CD8^+$ stage (22). To determine if $LAT^{4YF/4YF}$ thymocytes could transduce signals though the preTCR or CICs, mice were injected with anti-CD3ε. In $LAT^{-/-}$ mice, this treatment does not result in thymocyte maturation or proliferation (Fig. 5, and reference 10), presumably because signal transduction downstream of the preTCR or CICs is blocked. Injection of anti-CD3ε also did not result in thymocyte maturation or proliferation in $LAT^{4YF/4YF}$ mice (Fig. 5), indicating that even under these strong stimulation conditions the 4YF mutant LAT protein is unable to function in preTCR or CIC signal transduction.

Finally, all of the mature T cell subsets that were absent in $LAT^{-/-}$ mice were also absent in $LAT^{4YF/4YF}$ mice. These include $CD4^-CD8^-$ TCR-$\alpha\beta^+$ thymocytes ($NK1.1^+$ T cells; Fig. 4 B, and reference 23), thymic and peripheral γδ TCR^+ T cells (Fig. 4 A, and data not shown), peripheral αβ TCR^+CD4^+ and $CD8^+$ T cells, and intestinal intraepithelial T lymphocytes (data not shown).

These results confirm a critical role for LAT during T cell development and extend our previous data obtained by standard knockout technology to identify LAT tyrosine residues essential for thymocyte maturation. In Jurkat cells, mutation of Y132 (the human counterpart of Y136 in mouse LAT) abrogates association of PLC-γ1 with LAT whereas mutation of Y171, Y191, and Y226 (the human

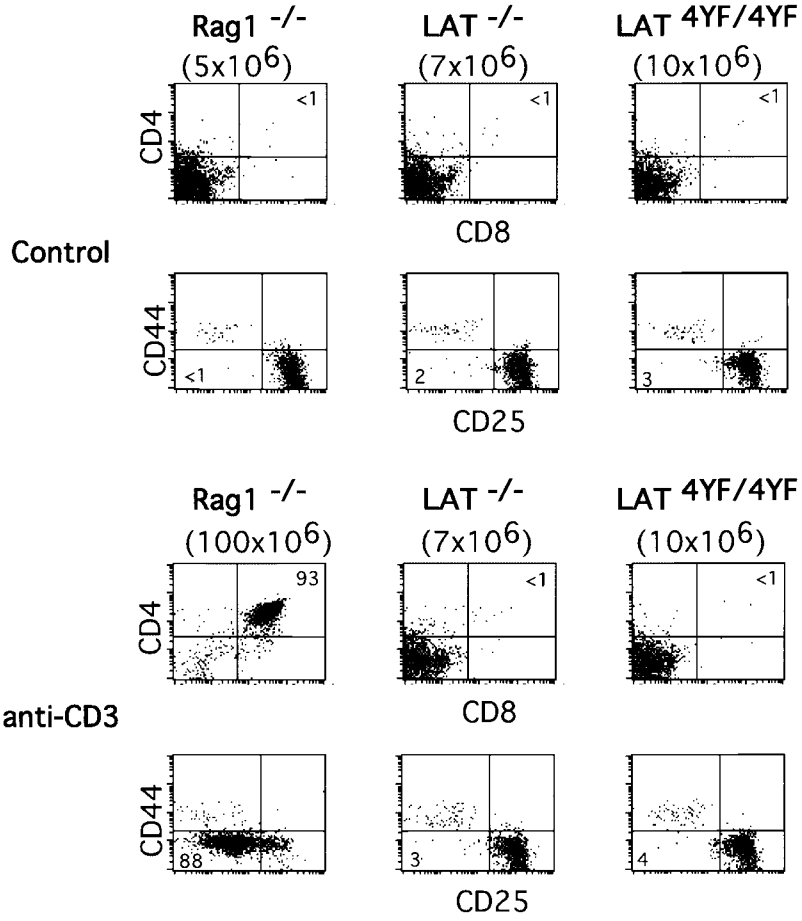

Figure 5. Defective preTCR signaling in $LAT^{4YF/4YF}$ thymocytes. 6-wk-old $Rag1^{-/-}$, $LAT^{-/-}$, or $LAT^{4YF/4YF}$ mice received a single intraperitoneal injection of anti-CD3ε (145-2C11, lower panels) or PBS (control, upper panels). 1 wk later, total thymocytes were analyzed by flow cytometry. Two color plots of CD4 versus CD8 and CD44 versus CD25 (gated on CD4, CD8, CD3ε, and B220-negative cells) are depicted. Thymocyte numbers are shown in parentheses.

counterparts of Y175, Y195, and Y235) abrogates association of Grb-2 and Gads with LAT. If these results from Jurkat cells can be extrapolated to immature thymocytes, then associations with PLC-γ1, Grb-2, and Gads (and other proteins) could be critical for LAT function in preTCR signaling. By analogy to TCR signaling in mature T cells, several downstream pathways are likely to be affected by the loss of these associations, in particular, pathways dependent on calcium mobilization and Ras-MAPK activation. Gads may also activate downstream signaling pathways via its association with SLP-76 (for a review, see reference 24). SLP-76 also associates with Vav and may therefore regulate activation of Rac/cdc42 and actin reorganization (for a review, see reference 25). Interestingly, the phenotype of SLP-76$^{-/-}$ mice with respect to T cell development is similar to that of LAT$^{-/-}$ and LAT$^{4YF/4YF}$ mice (20). Given the importance of these interactions for signal transduction, it might be predicted that 4YF LAT would act as an inert or null protein. Consistent with this notion, LAT$^{+/4YF}$ mice display normal thymus size and normal T cell development indicating that the 4YF LAT protein does not exert a dominant negative effect in the presence of the wild-type protein.

In addition to the necessity of the four distal tyrosines for T cell development, recent experiments suggest that they are also sufficient for T cell development (unpublished data). A retrovirus expressing a mutant LAT protein containing only the four distal tyrosines (i.e., having the first five tyrosines mutated to phenylalanines) was used to infect LAT$^{-/-}$ bone marrow cells and these cells were adoptively transferred to irradiated LAT$^{-/-}$ mice. This mutant form of LAT was fully capable of restoring T cell development when introduced into LAT$^{-/-}$ mice, indicating that of the nine potential tyrosine phosphorylation sites in LAT, the distal four were sufficient for T cell development.

We had previously shown that LAT$^{+/-}$ mice exhibit normal T cell development suggesting that thymocyte maturation and T cell function are not sensitive to modest alterations in LAT expression. Consistent with this idea, LAT protein levels in LAT$^{WT/WT}$ thymocytes were slightly lower than in wild-type levels but thymocyte development was unaffected. It remains to be determined if T cell development is altered by overexpression of LAT. Mast cell and platelet activation are also affected in LAT$^{-/-}$ mice (26, 27). Although not specifically addressed in this study, the availability of the knock-in mice will allow us to test the ability of the 4YF mutant protein to function in these lineages.

Importantly, the observation that LAT$^{WT/WT}$ mice exhibit normal T cell development validates the knock-in strategy for the study of LAT function in T cell development. These results imply that it should be possible to generate additional LAT knock-in mice that contain single or multiple Y→F mutations in LAT using a similar approach. These studies should enable the systematic assessment of the contribution of specific LAT tyrosines and LAT-coupled signal transduction pathways to T cell development.

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