The molecular requirements for LAT-mediated differentiation and the role of LAT in limiting pre-B cell expansion

Yu-wen Su[1], Sebastian Herzog[1], Michael Lotz[1], Niklas Feldhahn[2], Markus Müschen[2] and Hassan Jumaa[1]

[1] Institute for Biology III, Albert-Ludwigs University of Freiburg and Max Planck Institute for Immunobiology, Freiburg, Germany
[2] Laboratory for Molecular Stem Cell Biology, Heinrich-Heine-Universität of Düsseldorf, Düsseldorf, Germany

Successful recombination of the heavy-chain locus in developing B cells results in the expression of the pre-BCR, which induces the proliferation and expansion of pre-B cells. To avoid uncontrolled proliferation, pre-BCR signals transmitted via the adaptor protein SLP-65 (SH2-domain-containing leukocyte protein of 65 kDa) lead to the down-regulation of pre-BCR expression and to pre-B cell differentiation. Here, we show that, similarly to SLP-65, the adaptor protein LAT (linker for activation of T cells) limits pre-B cell proliferation and reduces the potential of a tumorgenic pre-B cell line to develop leukemia in immune-deficient mice. We further show that the four distal tyrosines are required for LAT activity in pre-B cells. Mutation at Y136 completely abolishes LAT activity, whereas single point-mutations at Y175, Y195 or Y235 impair, but do not block, LAT-induced pre-B cell differentiation. As LAT is also expressed in human pre-B cells, our results suggest that LAT cooperates with SLP-65 to promote the differentiation and control the proliferation of both murine and human pre-B cells.

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1 Introduction

B lymphocyte development is a highly regulated process that can be divided into distinct stages according to the expression of various surface markers and the recombination status of the heavy-chain (HC) and light-chain (LC) genes [1, 2]. Productive recombination results in μHC expression that, together with the surrogate-LC and the signaling components Ig-α and Ig-β, forms the pre-BCR, which is then expressed on the cell surface [3]. Pre-BCR expression represents a critical checkpoint during B cell development, as signals from the pre-BCR are essential for survival, selection and expansion of large pre-B cells [4]. Pre-BCR engagement results in the activation of protein tyrosine kinases of the Src, Syk and Tec families that mediate the phosphorylation of substrate proteins including Ig-α/Ig-β.

One major substrate of Syk is the adaptor protein SLP-65 (SH2-domain-containing leukocyte protein of 65 kDa), which is also known as BLNK (B cell linker) or BASH (B cell adaptor containing SH2 domain) [5–7]. Phosphorylated SLP-65 is a central modulator of the Ca^{2+} response that couples Syk and the Tec-family kinase Btk to phospholipase C (PLC)-γ2 [8–10]. After phosphorylation by Syk and Btk, PLC-γ2 is activated and generates the second messengers diacylglycerol (DAG) and inositol 1,4,5-tris-phosphate (IP_3). IP_3 leads to Ca^{2+} release from intracellular stores, a process that regulates transcription factors and cellular responses. SLP-65$^{-/-}$ mice show a partial block of early B cell development at the pre-B cell stage [11–14]. Moreover, pre-B cells from SLP-65$^{-/-}$ mice fail to down-regulate the pre-BCR and also show increased proliferation *in vitro* [15]. The partial block of B cell development in SLP-65$^{-/-}$ mice indicates the existence of alternative pre-BCR signaling pathways that rescue B cell differentiation. Recently, we demonstrated that the adaptor protein LAT (linker for activation of T cells) is involved in pre-BCR signaling. LAT transmits pre-BCR signals in a SLP-65-independent manner and partially rescues pre-B cell development in SLP-65$^{-/-}$ mice [16].

LAT and the adaptor protein SLP-76 play a central role in T cell activation and T cell development [17–20], as mice deficient for LAT or SLP-76 show an early block of T cell development and an absence of peripheral T cells

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Abbreviations: HC: Heavy chain **LAT:** Linker for activation of T cells **LC:** Light chain **PLC:** Phospholipase C **SLP-65:** SH2-domain-containing leukocyte protein of 65 kDa **wt:** Wild-type

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[21–24]. Upon TCR engagement, LAT is phosphorylated on several tyrosines by the Syk-family kinase Zap-70 and associates subsequently with multiple signaling components including SLP-76, Gads, Grb2, PLC-γ, Cbl, Vav or PI3K [25–27]. $LAT^{-/-}$ Jurkat T cells showed decreased tyrosine phosphorylation of SLP-76, Vav and PLC-γ upon TCR engagement. Moreover, these cells are defective in Ca^{2+} mobilization, activation of the mitogen-activated protein kinase (MAPK) pathway and of the transcriptional factors AP-1 and NF-AT [28].

The four distal tyrosines at positions 136, 175, 195 and 235 are known to be essential for LAT activity because knock-in mice with point-mutations at these four residues show a block of T cell development that is identical to that in $LAT^{-/-}$ mice [29]. The essential role of LAT Y136, known as PLC-γ1-binding site, was demonstrated in LAT^{Y136F} mutant mice [30, 31]. T cell development in young LAT^{Y136F} mice is blocked at the double-negative stage like it is in $LAT^{-/-}$ mice. However, lymphoproliferation and autoimmune disease develop in adult mice. Autoreactive $CD4^+$ Th2 cells from these mice show a strongly reduced tyrosine phosphorylation of LAT and PLC-γ1, and no Ca^{2+} response upon TCR engagement, indicating that binding to PLC-γ plays a key role for LAT activity. Interestingly, a similar phenotype of lymphoproliferation and autoimmunity was observed in $LAT^{Y175/195/235F}$ mutant mice, in which the development of autoreactive $\gamma\delta$ T cells is increased [32].

These studies demonstrate the essential role of LAT in the regulation of positive and negative selection during T cell development, control of the $CD4^+$:$CD8^+$ T cell ratio and T cell homeostasis between $\alpha\beta/\gamma\delta$ T cells. The four distal tyrosines of LAT represent the key residues for these processes in T cells. However, it is not clear whether these tyrosines are also essential in pre-BCR-mediated signaling. Here, we used SLP-65/$LAT^{-/-}$ pre-B cells and LAT–GFP fusion proteins to elucidate the function of each distal tyrosine in pre-BCR signaling, pre-B cell proliferation and differentiation.

2 Results

2.1 The role of the distal tyrosines in LAT-mediated pre-B cell differentiation

To understand the role of the four distal tyrosines of LAT in pre-BCR-mediated signaling, we mutated each of these tyrosines to phenylalanine in a retroviral vector encoding a murine LAT–GFP fusion protein (Fig. 1A). The resulting constructs were transduced into the *Oct* pre-B cell line that lacks both SLP-65 and LAT [16]. The expression levels of the different LAT–GFP fusion proteins were similar as suggested by the comparable fluorescence intensity of GFP (Fig. 1B). To compare the expression of the LAT–GFP fusions with LAT expression in wild-type (wt) pre-B cells, we performed a western-blot analysis of transduced *Oct* pre-B cells (Fig. 1C). The results indicate that the average level of retrovirally expressed LAT–GFP fusions is lower than LAT expression in wt pre-B cells (Fig. 1C). Furthermore, the results confirmed the comparable expression levels of the different LAT–GFP fusions. To assess the effect of pre-BCR engagement on Tyr-phosphorylation of the LAT–GFP fusions, we treated the respective *Oct* pre-B cells with anti-μ. This treatment led to increased Tyr-phosphorylation of each LAT mutant (Fig. 1D). However, mutation at Y136 and Y175 seemed to result in a reduction of pre-BCR-induced Tyr-phosphorylation of LAT (Fig. 1D).

To compare the effects of the Tyr-mutations on LAT activity, we measured pre-BCR-induced Ca^{2+} mobilization, down-regulation of surface pre-BCR expression and differentiation for all mutants. Compared with wt LAT, the Y136F mutant completely lost the capacity to mobilize Ca^{2+} upon pre-BCR engagement (Fig. 2A). In contrast, no difference in Ca^{2+} response was detected between pre-B cells expressing the Y175F mutant or wt LAT. On the other hand, both the Y195F and the Y235F mutant induced a modest Ca^{2+} response (Fig. 2A). When double mutations for Y175/Y195 and for Y175/Y235 were generated, no difference was detected in Ca^{2+} response between Y175/235F and Y235F mutants; however, the Ca^{2+} response in Y175/195F double-mutants was reduced compared with either Y175F or Y195F single-mutants (Fig. 2A). These results indicate that Y175 contributes more to the Ca^{2+} response induced by Y195 than to that induced by Y235.

We also tested whether expression of LAT mutants in *Oct* pre-B cells leads to down-regulation of surface pre-BCR [16]. Similar to the Ca^{2+} response, the Y136F mutant completely lost the capacity to down-regulate the pre-BCR, whereas the Y175F mutant induced a moderate pre-BCR down-regulation. Mutation of Y195 or Y235 resulted in a severe defect of LAT-induced pre-BCR down-regulation. This down-regulation was nearly lost when the LAT double-mutants Y175/Y195F and Y175/Y235F were tested (Fig. 2B). We further tested the effect of mutated LAT on pre-B cell differentiation by removing IL-7 from the culture and determining the differentiated B cells by surface κLC staining. The proportion of κLC^+ cells in the Y136F mutant was similar to untransduced control or GFP-expressing cells, which show a basal differentiation ratio of 0.4–0.7% (Fig. 2C). The differentiation capacity was reduced to 9–10% in the other single tyrosine mutants. However, transductants with the Y175F mutant reproducibly displayed a slightly better differentiation capacity than the Y195F and Y235F mutants

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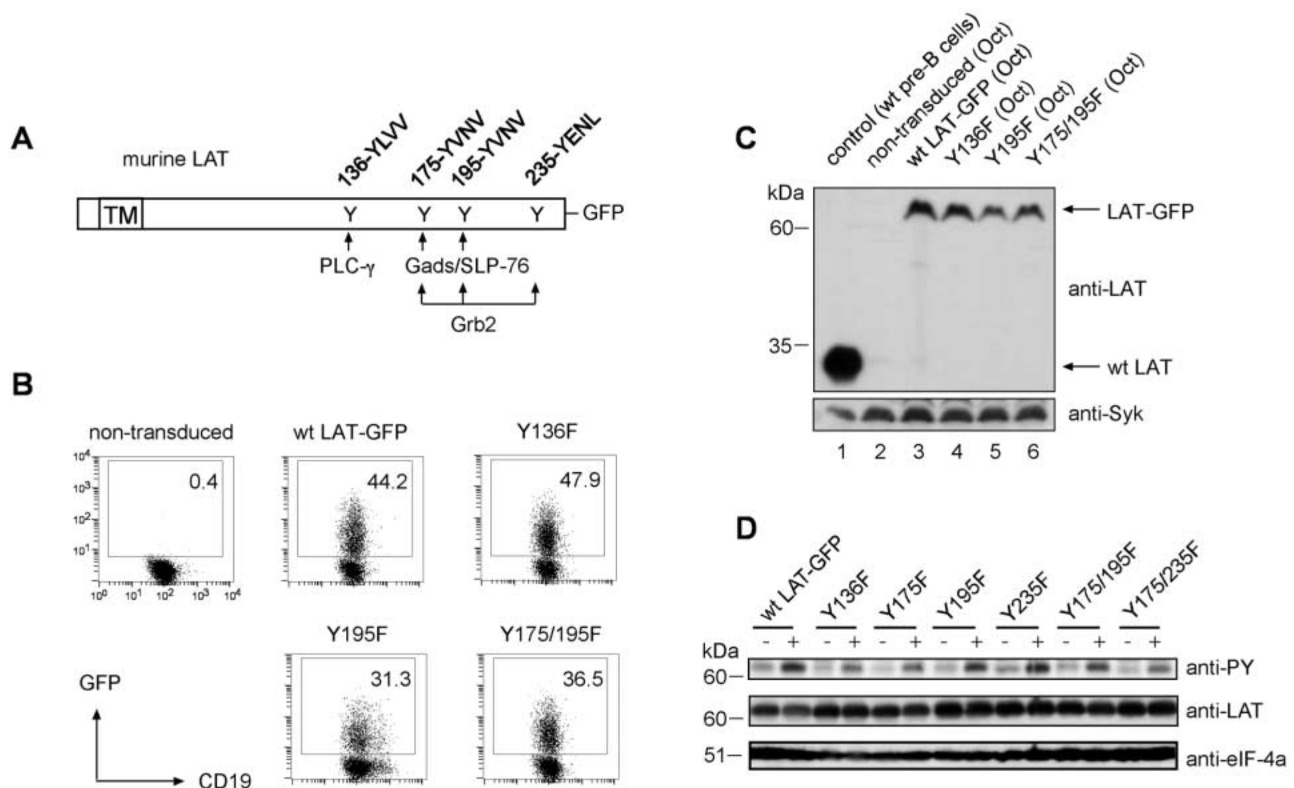

Fig. 1. Expression of LAT mutants in murine pre-B cells. (A) Scheme of the adaptor protein LAT. Tyrosine residues at positions 136, 175, 195 and 235 and the protein-binding motifs for the associated molecules are indicated. Retroviral vectors expressing GFP fusion proteins of wt or Tyr-mutated LAT were used. Mutations at each of the four distal tyrosines, or combined mutations at Y175/195 and Y175/235, were tested. TM: transmembrane region. (B) GFP versus CD19 FACS profiles of *Oct* pre-B cells transduced with the indicated constructs. The numbers in quadrants indicate the percentage of transduced (GFP-expressing) cells. (C) Western-blot analysis of *Oct* pre-B cells transduced with the indicated constructs. Total cellular lysates (TCL) of the cells shown in (B) were used in lanes 2–6. In lane 1, a TCL of bone-marrow-derived pre-B cells was used as a control for LAT expression. The membrane was first developed with anti-LAT and subsequently with anti-Syk for a loading control. The positions of endogenous wt LAT (lane 1) and LAT–GFP fusions (lanes 2–6) are indicated. (D) Western-blot analysis of total lysates from LAT–GFP-transduced pre-B cells. *Oct* pre-B cells reconstituted with wt or mutated LAT–GFP fusions were left unstimulated (–) or were stimulated (+) with anti-μ at 37°C for 2 min. Blots were developed with anti-phosphotyrosine (upper panel), anti-LAT (middle panel) or anti-eIF-4a (lower panel). Each lane contained the lysate from 8×10^5 cells.

did. The Y175/195F and Y175/235F double-mutants showed a further reduction (3–4%), but not a complete loss of LAT-mediated pre-B cell differentiation (Fig. 2C).

2.2 LAT expression in pre-B cells prevents their expansion *in vivo*

To investigate the role of LAT and its Tyr-mutants in pre-B cell differentiation *in vivo*, we injected *Oct* pre-B cells expressing different LAT–GFP fusion proteins into RAG2/$\gamma c^{-/-}$ mice [33]. Injection of *Oct* pre-B cells into RAG2/$\gamma c^{-/-}$ mice leads to splenomegaly and leukemia, whereas expression of SLP-65 in *Oct* cells eliminated their tumorgenic potential [34]. We tested whether, like SLP-65, expression of LAT in *Oct* pre-B cells controls pre-B cell proliferation *in vivo* and inhibits splenomegaly and leukemia in RAG2/$\gamma c^{-/-}$ mice. *Oct* pre-B cells were transduced with retroviral vectors expressing either GFP alone or different LAT–GFP fusion proteins (see Fig. 1B).

GFP-expressing or LAT–GFP-expressing cells were selected *in vitro* by cultivating the transduced cells in the presence of puromycin for two weeks (Fig. 3A). The selected *Oct* pre-B cells were injected into RAG2/$\gamma c^{-/-}$ mice, which were analyzed one month after injection. The spleen size and GFP expression in the splenocytes were compared between mice injected with GFP alone or different LAT–GFP mutants (Fig. 3B, C). In contrast to *Oct* pre-B cells expressing only GFP, wt-LAT-expressing pre-B cells did not cause splenomegaly or other signs of leukemia (Fig. 3C). However, injection of pre-B cells

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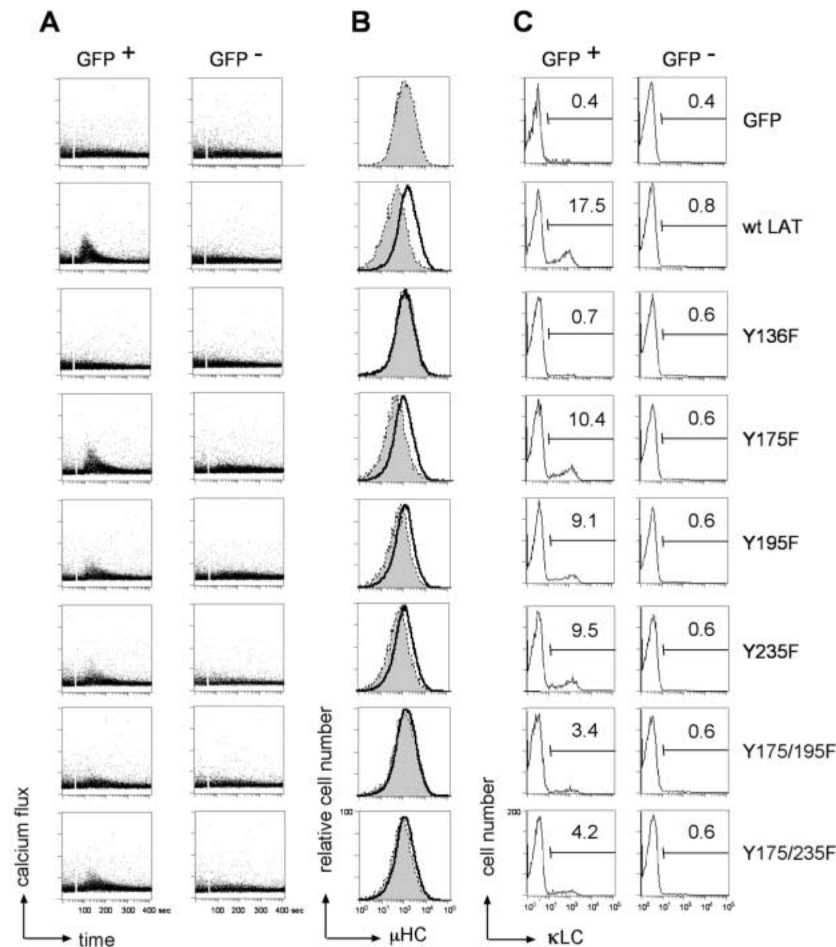

Fig. 2. Functional analysis of LAT mutants in pre-B cells. (A) Ca^{2+} mobilization in pre-B cells transduced with LAT mutants. Cells were loaded with Indo-1 24 h after transduction and a Ca^{2+} response was induced by treatment with anti-μ. The data show the Ca^{2+} response for the GFP^+ (left panel) and GFP^- (right panel) populations. (B) Down-regulation of pre-BCR in LAT mutants. Cells were stained, 24 h after transduction, with anti-μ to determine the surface pre-BCR expression. The histograms show a comparison of pre-BCR expression between the GFP^+ (dotted line filled with gray color) and GFP^- (black line) populations. (C) Pre-B cell differentiation in LAT mutants. After 4 days of culture in medium lacking IL-7, cells were stained with anti-κ to determine BCR expression and differentiation. Again, κ expression was compared between GFP^+ (left panel) and GFP^- (right panel) populations. Numbers refer to the percentages of differentiated cells relative to total cells in each histogram.

expressing the Y136F LAT mutant led to splenomegaly in most (four out of six) RAG2/$\gamma c^{-/-}$ mice (Fig. 3C). In parallel, none of the mice (out of five tested) injected with *Oct* pre-B cells expressing the LAT Y195F showed splenomegaly whereas mice injected with *Oct* pre-B cells expressing the Y175/195F double-mutant showed splenomegaly in some cases (two out of six). These results confirm the *in vitro* data suggesting a residual function of the LAT Y195F mutant and a severely defective function of the LAT Y175/195F mutant (Fig. 2). Together, our experiments indicate that LAT acts as an intrinsic tumor suppressor that inhibits leukemia presumably by inducing pre-B cell differentiation. Moreover, the results emphasize Y136 as the key tyrosine residue for LAT activity.

2.3 LAT expression in human pre-B cells

To show that LAT expression is not restricted to murine pre-B cells, we employed a semi-quantitative RT-PCR to analyze LAT expression during human B cell development. Human pre-B cells ($CD10^+CD19^+\mu^+$), B1 B cells ($CD19^+CD5^+$), mature B cells including naive B cells ($CD19^+CD27^-$), memory B cells ($CD19^+CD27^+$) and plasma cells ($CD19^+CD138^+$) were isolated from the bone marrow or peripheral blood of healthy donors [35]. The results show that LAT expression is restricted to human pre-B cells and is not detectable in B1 B, mature B or plasma cells (Fig. 4). A similar pattern of LAT expression was described in murine B cells [16, 36]. In contrast to the restricted expression of LAT to pre-B cells,

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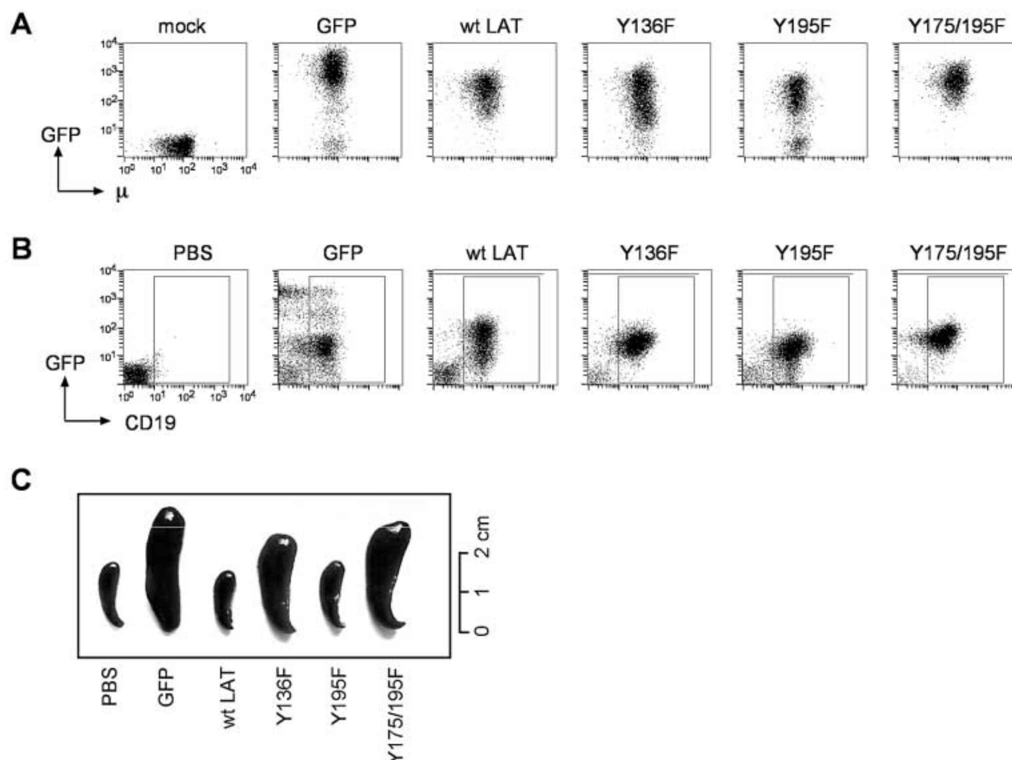

Fig. 3. Intrinsic LAT activity controls the proliferation of tumorgenic pre-B cells. (A) GFP versus μ FACS analysis of pre-B cells transduced with the indicated GFP or LAT–GFP fusion constructs and selected for GFP expression *in vitro* before injection. (B) GFP versus CD19 FACS analysis of splenocytes from recipient RAG2/$\gamma c^{-/-}$ mice 4 weeks after injection. Rectangles show the reconstituted $CD19^{+}$ B cells in the RAG2/$\gamma c^{-/-}$ mice. (C) Splenomegaly in RAG2/$\gamma c^{-/-}$ mice injected with *Oct* pre-B cells. The spleens of the mice injected with the indicated pre-B cells are shown. Mice injected with PBS served as a control. Representative data of several independent experiments are shown.

SLP-65 is expressed throughout B cell development (Fig. 4).

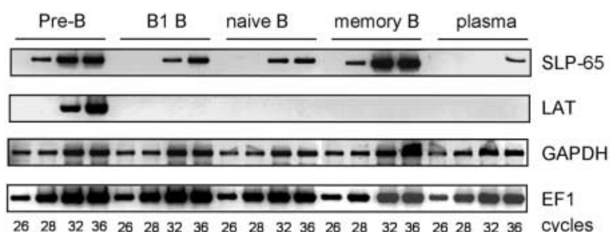

Fig. 4. LAT is expressed in human pre-B cells. Semi-quantitative RT-PCR analysis showing that LAT is expressed in pre-B cells, but not in B1 B, naive B, memory B or plasma cells. Cells were isolated from healthy donors. Different PCR cycles (26, 28, 32 and 36) were used for the amplification as indicated at the bottom. The expression of housekeeping gene products GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and EF1 (elongation factor 1) was used as a control.

3 Discussion

In this study, we examined the function of the four distal tyrosines of LAT in pre-B cells by the analysis of Ca^{2+} mobilization, down-regulation of surface pre-BCR and pre-B cell differentiation *in vitro*. Interestingly, all four LAT mutants displayed impaired functions but to various extents. Mutation at Y136 led to a complete loss of LAT function, confirming previous data that demonstrated the unique role of the motif YLVV for PLC-γ binding, Ca^{2+} mobilization and LAT function [30, 31].

Previous studies showed that phosphorylation of Y175 and Y195 in the YVNV motifs was involved in the interaction with Gads SH2 domain [37], which is constitutively associated with SLP-76 [38, 39]. The presence of a Gads-binding motif enables SLP-76 to recruit a Tec-family kinase to the LAT–PLC-γ complex. In addition, binding to Gads–SLP-76 stabilizes LAT–PLC-γ complexes because the SH3 domain of PLC-γ can bind to the proline-rich region of SLP-76 [40]. Despite the similar protein-binding motif, Y175 and Y195 have

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different effects in LAT-mediated signaling in pre-B cells. Mutating Y195, but not Y175, causes a severe reduction in Ca^{2+} response, pre-BCR down-regulation and differentiation. However, the results of the Y175/195F and Y175/235F double-mutants suggest that Y175 is required for efficient pre-BCR down-regulation and pre-B cell differentiation. Moreover, our results indicate that Y235, which together with Y175 and Y195 is involved in Grb2 binding [37], is crucial for LAT function in pre-B cells as mutating this tyrosine impairs Ca^{2+} mobilization, pre-BCR down-regulation and differentiation.

The ability of LAT mutants to induce Ca^{2+} mobilization is an absolute requirement for LAT-mediated differentiation in pre-B cells. However, the amplitude of the Ca^{2+} response did not always reflect the differentiation capacity, For instance, wt LAT and the Y175F mutant showed a comparable Ca^{2+} response, whereas the differentiation capacity with the Y175F mutant was reduced compared with wt LAT. Similar results were obtained with Y235F and Y175/235F mutants, where the Y235F mutant showed a better differentiation capacity than the Y175/235F double-mutant did, although both constructs induced a comparable Ca^{2+} response.

The transfer experiments into RAG2/$\gamma c^{-/-}$ mice, together with the *in vitro* analyses, show that LAT activity, similarly to SLP-65 activity, improves the differentiation of pre-B cells and reduces the capacity of tumorgenic pre-B cells to induce leukemia *in vivo*. Therefore, LAT expression may not only enable pre-B cell differentiation but also reduce the incidence of pre-B cell leukemia in SLP-65 deficient mice. In agreement with this, SLP-65/LAT double-deficient mice show a high incidence of pre-B cell leukemia, similarly to SLP-65/Btk$^{-/-}$ mice [41], demonstrating the importance of LAT for the regulation of pre-B cell differentiation and proliferation. Our results suggest that LAT involvement in pre-BCR signaling is not restricted to murine pre-B cells, as human pre-B cells also express LAT; this suggests a role for LAT in human pre-B cell differentiation.

Our previous and current results suggest the existence of two independent signaling complexes that are organized by SLP-65 and LAT, respectively. Effective transmission of the differentiation signals derived from the pre-BCR requires both complexes (Fig. 5). In the absence of SLP-65 or LAT, pre-B cell differentiation is only partially blocked, as the alternative complex enables residual pre-

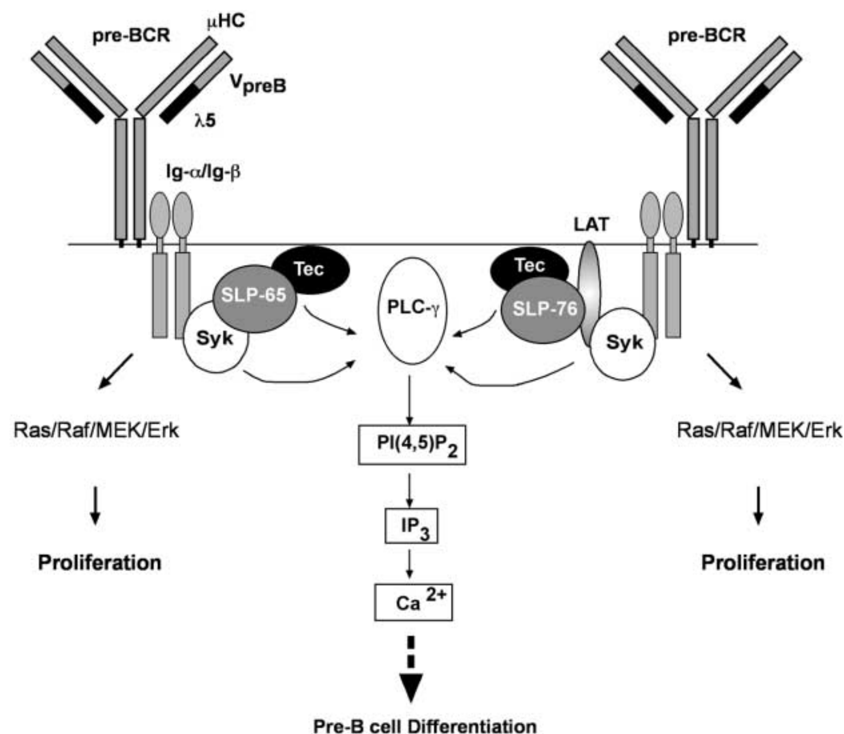

Fig. 5. Two independent complexes for the differentiation signals of the pre-BCR. SLP-65 organizes a complex in which Syk and Btk (a Tec-family kinase) activate PLC-γ2, resulting in the generation of second messengers (*e.g.* Ca^{2+}) and subsequent cellular responses. In an alternative complex, LAT is the central protein in a pre-BCR complex that activates PLC-γ2 and leads to differentiation. Since PLC-γ1 and PLC-γ2 are expressed in pre-B cells, the LAT-organized complex may activate both. Moreover, Syk or ZAP-70 may phosphorylate and activate LAT, as both kinases are expressed in pre-B cells. Independently of SLP-65 and LAT, proliferation signals derived from the pre-BCR may activate the MAPK Erk.

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B cell differentiation. Our results also suggest that LAT and SLP-65 are not involved in the proliferation signals derived from the pre-BCR (Fig. 5). Instead, SLP-65 and LAT prevent the proliferation of pre-B cells by activating their differentiation program. Thus, investigating the role of LAT in pre-BCR signaling may not only elucidate the mechanisms underlying pre-B cell differentiation but also improve our understanding of the development of leukemia.

4 Materials and methods

4.1 Mice

All mice were described previously [11, 16, 24]. Animal experiments were performed in compliance with the guidelines of the German law and the MPI for Immunology.

4.2 Retroviral constructs and transduction

Retroviral transductions and retroviral vector pMOWS-LAT-GFP were described previously [7]. The QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) was utilized for LAT point-mutation according to the manufacturer's instructions. Transfection was performed with Phoenix™ retroviral producer cell line using GeneJuice™ (Novagen) according to the manufacturer's instructions. For transduction, *Oct* pre-B cells derived from the bone marrow of SLP-65$^{-/-}$ mice were mixed with retroviral supernatants and centrifuged at 1,800 rpm at 37°C for 3 h. Transduction efficiency, measured one day later, was between 20 and 50%. The Ca^{2+} response and down-regulation of pre-BCR were analyzed 24 h post transduction, and the differentiation assay was performed 4 days post-transduction.

4.3 Cell culture

Pre-B cells were cultured in Iscove's medium containing 10% inactive FCS (Vitromex), 100 U/ml penicillin, 100 U/ml streptomycin (Gibco BRL), 5×10^{-5} M β-mercaptoethanol and IL-7. The IL-7 was obtained using supernatant of J558L cells stably transfected with the vector encoding murine IL-7. Cell lines Nalm6 and 697 were maintained in RPMI-1640 medium supplemented as for Iscove's medium.

4.4 Flow cytometry

Aliquots of splenocytes or transduced cells (10^6) were stained for FACS analysis (FACSCalibur, Becton Dickinson) using PE–anti-CD19 (BD Bioscience PharMingen), Cy5–anti-IgM (μ-chain-specific, Southern Biotechnology), biotin–anti-κ (Southern Biotechnology) and streptavidin–Cy5 (Dianova) antibodies.

4.5 Measurement of Ca^{2+} release

Cells (5×10^6) were incubated with 5 μg/ml of Indo-1 AM (Molecular Probes) and 0.5 μg/ml of pluronic F-127 (Molecular Probes) in Iscove's medium supplemented with 1% FCS (Vitromex) at 37°C for 45 min. The cell pellets were resuspended in Iscove's medium plus 1% FCS and kept on ice for fluorimetric analysis. The Ca^{2+} response was induced by adding 20 μg/ml of goat anti-mouse-IgM (μ-chain-specific, Southern Biotechnology).

4.6 Adoptive transfer into RAG2/γc$^{-/-}$ mice

Oct pre-B cells retrovirally transduced with wt LAT or various tyrosine mutations were selected with medium containing puromycin at 1 μg/ml over two weeks. Then single-cell suspensions were adjusted to 1×10^7/ml in sterile PBS and 2×10^6 cells were injected into 8–10-week-old RAG2/γc$^{-/-}$ mice via the tail vein. Four weeks after injection, mice were sacrificed and splenocytes were analyzed by FACS.

4.7 Semi-quantitative RT-PCR analysis

Enrichment of CD19$^+$VpreB$^+$ pre-B cells was done as described earlier [42]. B1 B cells were collected from human umbilical cord blood samples of three healthy donors by depletion of T cells using anti-CD3 beads (Miltenyi Biotec) following enrichment of CD19$^+$ cells using anti-CD19 immunomagnetic beads (Miltenyi Biotec). Memory B cells were isolated from human buffy coats of 12 healthy donors by double-depletion of T cells by anti-CD3 immunomagnetic beads (Dynal, Oslo) and anti-CD3 beads (Miltenyi Biotec) following enrichment of CD27$^+$ cells using anti-CD27 immunomagnetic beads (Miltenyi Biotec). Plasma cells were isolated from the remaining cells using anti-CD138 beads (Miltenyi Biotec). CD3$^-$CD27$^-$CD138$^-$ cells were used for enrichment of naive B cells using anti-CD19 beads (Miltenyi Biotec).

RNA was extracted from the purified B cell subsets and RT-PCR was performed for 26, 28, 32 and 36 cycles as previously described [35]. The cDNA amounts were normalized by OD measurements and amplification of a specific fragment of the *GAPDH* gene using 5′-TTAGCACCCCTGGC-CAAGG-3′ as a forward and 5′-CTTACTCCTTGGAGGC-CATG-3′ as a reverse primer and of the *EF1* gene using 5′-CATGTGTGTTGAGAGCTTC-3′ as a forward and 5′-GAAAACCAAAGTGGTCCAC-3′ as a reverse primer. For the detection of *SLP-65*, 5′-TGGACAGTTATTCGTGTCTCTT-3′ as a forward and 5′-GTGAACTGCTTTCTGTGGGA-3′ as a reverse primer were used; for the detection of *LAT*, 5′-ACATCCTCAGATAGTTTGTAT-3′ was used as a forward and 5′-AACGTTCACGTAATCATCAAT-3′ as a reverse primer.

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4.8 Western-blot, cell stimulation and immuno-purification

Cells were collected and washed twice with PBS. Cell pellets were lysed in ice-cold lysis buffer [50 mM Tris-HCl (pH 7.4); 1% n-Octyl-β-D-glucopyranosid (Applichem), 137.5 mM NaCl, 1% glycerol, 1 mM Na-orthovanadate, 0.5 mM EDTA (pH 8.0), supplemented with protease inhibitor cocktail (Sigma)], and the whole cellular lysates were subjected to 10% SDS-PAGE and blotted onto PVDF membranes (Millipore). Murine LAT and Syk were detected by anti-LAT (M-19; Santa Cruz) and anti-Syk (N-19; Santa Cruz), respectively. Primary antibodies were revealed by HRPO-labeled anti-goat or anti-rabbit antibody (Pierce) and developed with an ECL system (Amersham Pharmacia). For stimulation, cells were incubated with 20 μg/ml of anti-mouse- or anti-human-IgM (μ-chain-specific; Southern Biotechnology) at 37°C for 2 min. Stimulation was stopped by adding ice-cold PBS and the cell pellets were quickly spin down, following lysis in ice-cold lysis buffer as described above. For immuno-purification the whole cellular lysates were incubated with anti-phosphotyrosine-coupled agarose beads (4G10; Upstate) at 4°C overnight. Purified proteins were washed with PBS or lysis buffer, subjected to 10% SDS-PAGE and analyzed by anti-human-LAT (fl-233; Santa Cruz) immunoblotting as described above.

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Correspondence: Hassan Jumaa, Max Planck Institute for Immunobiology, Stübeweg 51, 79108 Freiburg, Germany
Fax: +49-761-5108-423
e-mail: jumaa@immunbio.mpg.de

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