(*17*), a mouse pre–B cell line (*18*), human Burkitt lymphoma lines (*19*, *20*), B cells of mismatch repair–deficient mice (*21*, *20*), and an XRCC2/3-deficient chicken B-cell lymphoma line (*23*), although mutated bases in SHM of V genes in human and mouse B cells in vivo did not shown such a strong bias to G/C base pairs (*24*, *25*). This difference in nucleotide preference could be attributed to relative abundance of error-prone DNA polymerases (*26*) or specific DNA repair proteins. In addition to point mutations, 18 deletion and two duplication events were observed, consistent with the features of SHM of immunoglobulin (*1*) (Fig. 2A).

In summary, AID-induced hypermutation in NIH 3T3 cells has common properties with SHM of immunoglobulin genes; it shows strict dependence on AID, dependency on transcription of the target gene, and mutations biased to specific motifs. In addition, AID induced occasional deletions and duplications, along with a high mutation frequency. These properties are also shared by hypermutation in the Sμ region in B cells stimulated with lipopolysaccharide (*27*). These results suggest that hypermutation in NIH 3T3 cells may use the same molecular machinery as that used in V and Sμ sequences, and they indicate that AID is the only B cell–specific factor required to generate hypermutation experimentally in actively transcribed genes in nonlymphoid cells. Taken together with our previous observation that CSR could be induced by AID in the same cell line, it is likely that all trans-acting factors required for AID to accomplish CSR and hypermutation, are constitutively expressed in nonlymphoid cells, as well as B cells.

The AID enzyme is likely to be involved in the cleavage step of CSR, because AID deficiency abolishes accumulation of γ-H2AX and NBS1, which are involved in DNA repair and recruited to the site of DNA double-strand breakage, at the immunoglobulin $C_H$ locus in CSR-induced spleen B cells (*28*). Because hypermutation can be introduced into the germline Sμ region when CSR takes place in the other $C_H$ allele (*27*, *28*), AID appears to play a similar role in CSR and hypermutation, most likely at the DNA cleavage step itself (*3*, *16*). However, we cannot completely exclude the possibility that AID is involved in the stage after cleavage in SHM (*29*). In that case, the SHM target genes in NIH 3T3 cells would be expected to receive continuous generation of cleavages, even in the absence of AID, and these cleavages would be repaired without inducing mutations. Detection of such cleavages in NIH 3T3 cells is a critical test for the latter model. In summary, widespread expression of all the components other than AID that are required for CSR and SHM provides an important clue for analyzing the molecular mechanisms for the two genetic events; the target and cofactors of AID are also widespread.


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**Supporting Online Material**

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Materials and Methods


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# Induction of T Helper Type 2 Immunity by a Point Mutation in the LAT Adaptor


**Enrique Aguado,[1] Sylvie Richelme,[1] Selene Nuñez-Cruz,[1] Arkadiusz Miazek,[1] Anne-Marie Mura,[1] Mireille Richelme,[1] Xiao-Jun Guo,[1*] Danielle Sainty,[2] Hai-Tao He,[1] Bernard Malissen,[1†] Marie Malissen[1]**



**The transmembrane protein LAT (linker for activation of T cells) couples the T cell receptor (TCR) to downstream signaling effectors. Mice homozygous for a mutation of a single LAT tyrosine residue showed impeded T cell development. However, later they accumulated polyclonal helper T ($T_H$) cells that chronically produced type 2 cytokines in large amounts. This exaggerated $T_H2$ differentiation caused tissue eosinophilia and massive maturation of plasma cells secreting to immunoglobulins of the E and G1 isotypes. This paradoxical phenotype establishes an unanticipated inhibitory function for LAT that is critical for the differentiation and homeostasis of $T_H$ cells.**


The TCR recognizes peptides bound to major histocompatibility complex (MHC) molecules and relays this information to the T cell through adaptor proteins. The adaptor LAT coordinates the assembly of signaling complexes through multiple tyrosine residues within its intracytoplasmic segment (*1*). Upon TCR-induced phosphorylation, each of these tyrosines manifests some specialization in the signaling proteins it recruits. For instance, mutation of tyrosine 136 (Y136) selectively eliminates binding of phospholipase C–γ1 (PLC-γ1), whereas the simultaneous mutation of Y175 and Y195 results in loss of binding of the Gads adaptor (*2–4*). Mice deficient in LAT ($LAT^{-/-}$) or having a mutation of the four COOH-terminal tyrosine residues revealed that LAT is essential for the


[1]Centre d'Immunologie de Marseille-Luminy, INSERM– and CNRS–Université de la Méditerranée Parc Scientifique de Luminy, 13288 Marseille Cedex 9, France. [2]Department of Biology, Institut Paoli-Calmettes, 232 Boulevard Sainte Marguerite, 13009 Marseille, France.

*On leave from Institut Méditerranéen de Recherche en Nutrition, UMR-INRA, Marseille, France.

†To whom correspondence should be addressed. E-mail: bernardm@ciml.univ-mrs.fr

function of the pre-TCR, a molecular sensor that controls early T cell development and shares common signaling complexes with the TCR (*5*, *6*).

To address the importance of LAT Y136 in vivo and to analyze the consequence of restricting LAT to only a subset of its docking function, we generated knock-in mice with a mutation that replaced tyrosine 136 with phenylalanine (Y136F). Mice homozygous for this mutation, LAT$^{Y136F}$ mice, were born at expected Mendelian frequencies, and T cell expression of LAT was comparable to wild-type T cells [fig. S1 (*7*)]. Although LAT$^{Y136F}$ mice displayed normal peripheral lymphoid organs at birth, their spleen and lymph nodes started to enlarge, so that by 7 weeks of age, spleen cellularity was about 5 times that of wild-type mice (Fig. 1). Despite prominent lymphocytic infiltrations in the lung, liver, and kidney, homozygotes lived to at least 17 weeks of age, and no chronic intestinal inflammation or tumor formation was observed (*8*). The effects of the LAT Y136F mutation were only detectable after breeding mice to homozygosity or to offspring carrying a null allele of the LAT gene.

The thymuses of LAT$^{Y136F}$ mice had about one-tenth the cells of wild-type thymuses and reduced numbers of CD4$^+$CD8$^+$ double-positive (DP) thymocytes (Fig. 2). After reaching a peak in mutant newborn mice, DP cell numbers decreased and were almost undetectable in mice older than 7 weeks (Fig. 2A and fig. S2A). Coincident with this progressive DP cell erosion, discrete populations of CD4 and CD8 single-positive (SP) thymocytes started to dominate the thymus and showed a CD4/CD8 ratio skewed toward CD4 cells (Fig. 2B). TCR expression in LAT$^{Y136F}$ thymocytes was one-half (DP) or one-sixth (SP) that observed in wild-type mice (Fig. 2C). The DP thymocytes found in young mutant mice lacked CD5 molecules at their surface (fig. S2B). CD5 is a negative regulator of TCR signaling, and its expression increases during T cell development in a manner proportional to the intensity of pre-TCR and TCR signaling (*9*–*11*). From these data and the presence of a normal complement of CD4$^-$CD8$^-$ double-negative (DN) cells in LAT$^{Y136F}$ thymuses (*8*), we conclude that the LAT Y136F mutation resulted in a severe but partial impairment of αβ T cell development. Although development of T cells bearing TCR made of γδ chains (γδ T cells) is ablated in LAT$^{-/-}$ mice (*5*), the LAT Y136F mutation did not affect γδ T cell development detectably (fig. S2C).

Given the scarcity of SP thymocytes found in newborn LAT$^{Y136F}$ mice (Fig. 2A and fig. S2A), one would expect very few SP cells in secondary lymphoid organs. However, SP cells appeared in the spleen and lymph nodes of LAT$^{Y136F}$ mice with the same kinetics as in wild-type mice. However, they showed a strong bias for CD4 cells and expanded over time (Fig. 1, B and C). These CD4 cells had a CD25$^-$, CD44$^{hi}$, CD62L$^{lo}$, CD69$^+$ phenotype closely resembling activated and memory T cells (Fig. 1D). They also expressed low levels of TCR on their surfaces, an attribute that may in part account for their inability to proliferate in response to TCR stimulation in vitro (*8*). Despite the absence of CD5 on LAT$^{Y136F}$ DP thymocytes, CD5 molecules were detected on their direct CD4 and CD8 SP progeny (fig. S2B). Analysis of DNA content by propidium iodide staining showed that the CD4 populations from wild-type and LAT$^{Y136F}$ mice contained 3.3 and 7.2% of cells, respectively, in the $G_2$-S-M phases of the cell cycle (*8*). Moreover, when cultured in medium alone, CD4 T cells purified from LAT$^{Y136F}$ mice showed a lower rate of spontaneous apoptosis than wild-type CD4 T cells (*8*). Therefore, the progressive accumulation of CD4 T cells in the periphery of LAT$^{Y136F}$ mice is probably due to both their extended survival and increased proliferation.

When freshly isolated from LAT$^{Y136F}$ mice, CD4 T cells expressed sufficient interleukin-4 (IL-4) and IL-10 transcripts to allow their detection even without ex vivo restimulation (Fig. 3A). Upon activation by phorbol 12-myristate 13-acetate (PMA)–ionomycin IL-5, IL-13, and interferon-γ (IFN-γ) transcripts were also detected (Fig. 3B), and close to 80% of the CD4 T cells expressed very high levels of intracytoplasmic IL-4 (Fig. 3C). In contrast, wild-type CD4 T cells showed only the IL-2 and IFN-γ transcripts expected after activation of primary T cells. Moreover, despite their activated phenotype, CD8 T cells from LAT$^{Y136F}$ mice did not produce any IL-2, IL-4, IL-5, and IFN-γ when stimulated under similar conditions (*12*). Consistent with the notion that the CD4 T cells from LAT$^{Y136F}$ mice were refractory

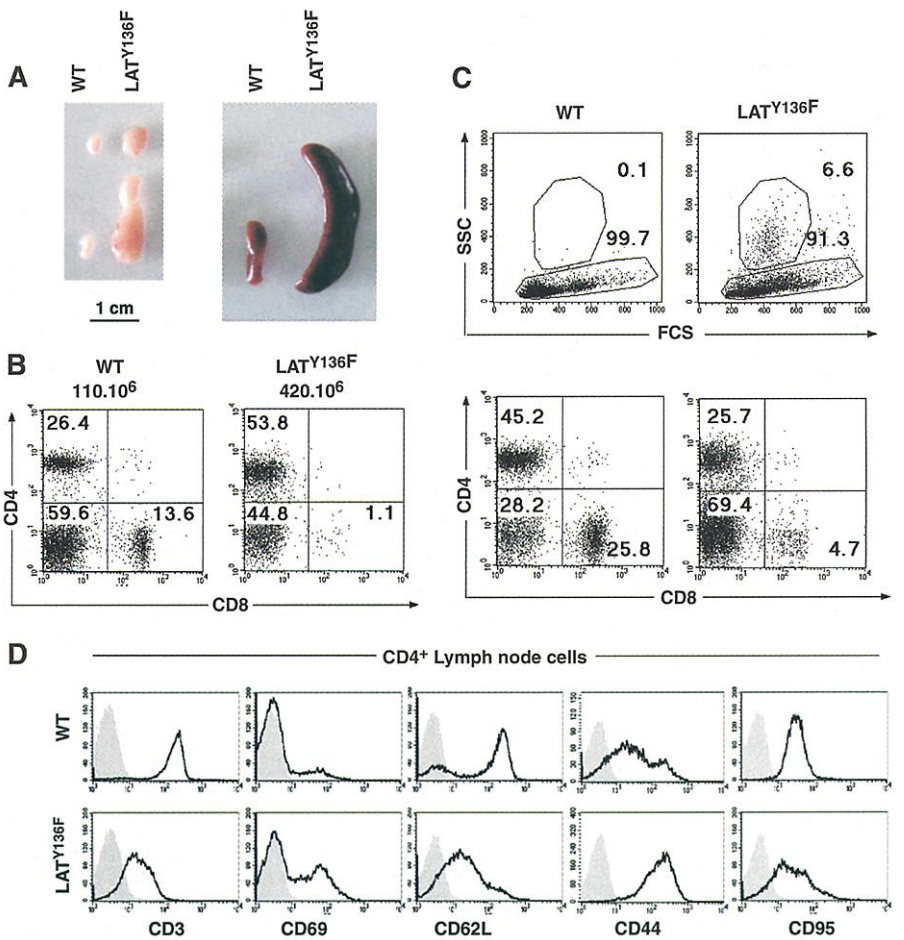


**Fig. 1.** Enlarged secondary lymphoid organs in LAT$^{Y136F}$ mice. (**A**) Spleen (right) and inguinal and mesenteric lymph nodes (left) from 7-week-old wild-type and LAT$^{Y136F}$ mice. (**B**) CD4/CD8 profiles of spleen cells from wild-type and from LAT$^{Y136F}$ mice. (**C**) Light scatter analysis and CD4/CD8 staining profiles of lymph node cells from wild-type and from LAT$^{Y136F}$ mice at 6 weeks of age. The cells with an intermediate forward scatter (FSC) and a high side scatter (SSC) correspond to eosinophils (see fig. S3). (**D**) Comparison of the levels of CD3, CD69, CD62L, CD44, and CD95 on CD4 T cells from 6-week-old mice expressing wild-type LAT or LAT$^{Y136F}$ molecules. CD4 T cells from LAT$^{Y136F}$ mice express lower surface levels of CD95 relative to wild-type CD4 T cells.

to TCR stimuli, none of them scored as IL-4$^+$ in response to antibody-mediated TCR cross-linking (*8*). Thus, over the first weeks of their life, LAT$^{Y136F}$ mice spontaneously developed a T helper type 2 ($T_H2$) lymphoproliferative disorder. In the case of wild-type CD4 T cells, a $T_H2$ polarization of such magnitude is only achieved after prolonged antigenic stimulation in the presence of IL-4 (*13*).

Analysis of thymic and lymph node cells from LAT$^{Y136F}$ mice older than 4 weeks showed high levels of eosinophils (fig. S3). LAT transcripts were undetectable in these eosinophils, suggesting that the observed eosinophilia resulted from the production of IL-5 by the abnormal CD4 cells present in these mutant mice. Most of the CD4 thymocytes found in LAT$^{Y136F}$ mice older than 4 weeks had a phenotype (CD44$^{hi}$, CD62L$^{lo}$, CD69$^+$, and HSA$^-$) distinct from that expected for genuine CD4 SP thymocytes, but closely resembled that of the abnormal peripheral CD4 cells. Provided that the latter cells effectively recirculated to the thymus, the IL-5 and IL-13 cytokines they produced in situ are likely to be primarily responsible for the progressive disappearance of DP thymocytes and for the thymic eosinophilia (*14*, *15*).

Secondary lymphoid organs of 6-week-old LAT$^{Y136F}$ mice contained 7 to 10 times as many B cells as their wild-type counterparts. Most of the mature B cells found in 6-week-old wild-type littermates had a resting phenotype (B220$^{high}$, MHC class II$^+$, IgM$^+$, IgD$^+$; Fig. 4A). In contrast, among the B cells found in the enlarged secondary lymphoid organs of age-matched LAT$^{Y136F}$ littermates, 25% showed a hyperactivated phenotype (B220$^{high}$, MHC class II $^{high}$, IgD$^-$; Fig. 4A), 50% expressed a phenotype typical of antibody-producing cells (B220$^{low}$, MHC class II$^+$, IgD$^-$, IgM$^-$, CD44$^{high}$), and only 25% had a resting phenotype. Serum IgG1 and IgE concentrations were elevated about 200 times and up to 10,000 times, respectively, compared with wild-type mice (Fig. 4B). In contrast, levels of other immunoglobulin isotypes did not differ from those of wild-type serum. Increased concentrations of κ and λ light chains in the serum of LAT$^{Y136F}$ mice (Fig. 4B), also indicated polyclonal hypergammaglobulinemia E and G1. IgE and IgG1 antibody concentrations peaked at 5 weeks of age (Fig. 4 C), the values of which exceeded those reported for mice deprived of NFATc2 and NFATc3 transcription factors (*16*). Given that B cells do not express LAT proteins (*1*) and considering that isotype switching to IgE and IgG1 depends on IL-4 and IL-13 (*14*), overproduction of IgE and IgG1 noted in LAT$^{Y136F}$ mice is probably secondary to the presence of an abnormally high frequency of $T_H2$ effectors.

To establish that CD4 cells were responsible for the disorders documented in LAT$^{Y136F}$ mice, we first used a mutation that prevents the development of all mature T cells [CD3-ε$^{Δ5}$ (*17*)]. Introduction of the LAT Y136F mutation had no effect on the B cells and eosinophils present in CD3-ε$^{Δ5}$ mice (fig. S5). Moreover, the DN cells found in CD3-ε$^{Δ5}$ × LAT$^{Y136F}$ mice were identical to those of CD3-ε$^{Δ5}$ mice, indicating that the effects of the LAT Y136F mutation become manifest only at the DN to DP transition. By breeding the LAT$^{Y136F}$ mice to $\beta_2$-microglobulin–deficient mice, we observed that the residual CD8 T cells found in LAT$^{Y136F}$ mice were dispensable for the development of the disorders (*8*). In contrast, in mice deficient for both MHC class I and class II molecules (MHC KO), the absence of CD4 T cells protected them from the pathological effects of the LAT Y136F mutation, and their small complement of DP cells remained stable over time (fig. S5). Because noxious CD4 T cells cannot develop in MHC KO mice and interfere with developing thymocytes, this established that the LAT Y136F mutation has a direct impact on the DN to DP transition and that the CD4 T cells were responsible for the erosion of the DP cell compartment observed in LAT$^{Y136F}$ mice.

The absence of CD4 T cells in LAT$^{Y136F}$ × MHC KO mice suggests that their development requires a selective process involving MHC class II molecules. Given that the LAT$^{Y136F}$ CD4 T cells do not respond to TCR cross-linking, we converted them into T cell hybridomas. All the hybridomas derived from LAT$^{Y136F}$ CD4 T cells reacted with syngeneic MHC class II mole-

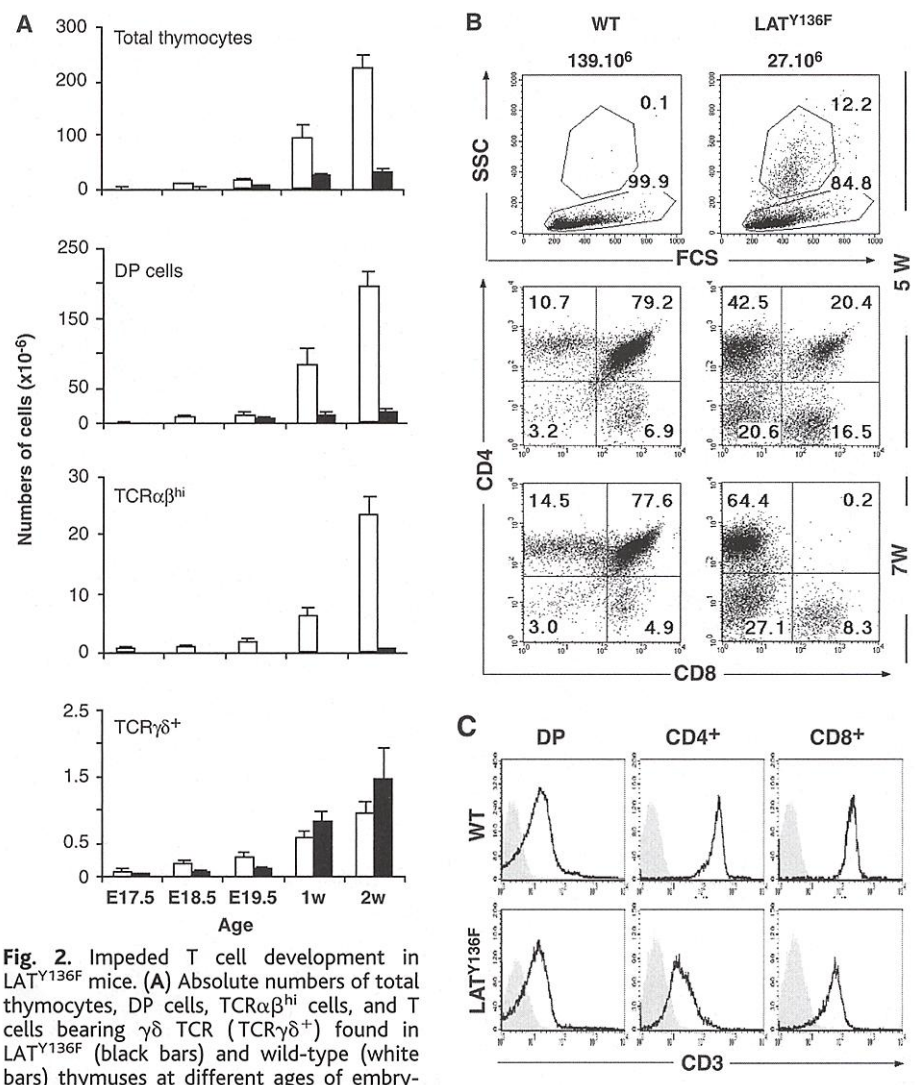


**Fig. 2.** Impeded T cell development in LAT$^{Y136F}$ mice. (**A**) Absolute numbers of total thymocytes, DP cells, TCRαβ$^{hi}$ cells, and T cells bearing γδ TCR (TCRγδ$^+$) found in LAT$^{Y136F}$ (black bars) and wild-type (white bars) thymuses at different ages of embryonic life and at 1 and 2 weeks of age. (**B**) Light scatter analysis and CD4/CD8 staining profiles of total thymocytes from wild-type and from LAT$^{Y136F}$ mice at 5 and 7 weeks of age. The cells with an intermediate forward scatter and a high side scatter correspond to eosinophils (see fig. S3). The percentage of cells within each gate is indicated. Also shown is the total number of thymocytes (averaged from six experiments). (**C**) Comparison of the levels of CD3-ε, a TCR transduction subunit, on DP, CD4 SP, and CD8 SP thymocytes from 6-week-old mice expressing wild-type LAT or LAT Y136F molecules.

cules (table S1), whereas none of those derived from wild-type CD4 T cells showed autoreactivity (*8*). These two series of hybridomas expressed comparable levels of TCR at their surface and used a heterogeneous set of Vα and Vβ segments (*8*). This indicates that the pathogenic CD4 T cells that develop in LAT$^{Y136F}$ mice do not correspond to CD1-d–restricted T cells (*18*). Their unexpected reactivity against self-MHC class II molecules may result from inadequate negative selection and may account for the exaggerated help they provide to B cells in vivo. Alternatively, their TCR may have been appropriately calibrated in relation to the context of LAT$^{Y136F}$ mice, and it is only after introducing them in T hybridomas and artificially increasing both their surface density and output that they started displaying reactivity toward self-MHC class II molecules. Accordingly, the polyclonal B cell activation found in LAT$^{Y136F}$ mice may result from noncognate interactions with aberrant T cells overproducing IL-4 in a chronic manner (*19*, *20*).

Although LAT$^{Y136F}$ mice showed a markedly impeded sequence of T cell development, an early and massive accumulation of $T_H2$ effectors occurred in their periphery. This paradoxical phenotype can be accounted for if residue Y136 activates both a positive and a negative feedback loop. The positive function of residue Y136 may dominate during T cell development, whereas in CD4 T cells, Y136 may dominantly turn off signaling pathways that are activated at the time of positive selection or that keep in check the low-level of TCR signaling that CD4 T cells require for their survival in the periphery (*21*). Once this negative feedback function is relieved, a chronic and low-intensity signaling probably takes place and ultimately leads,

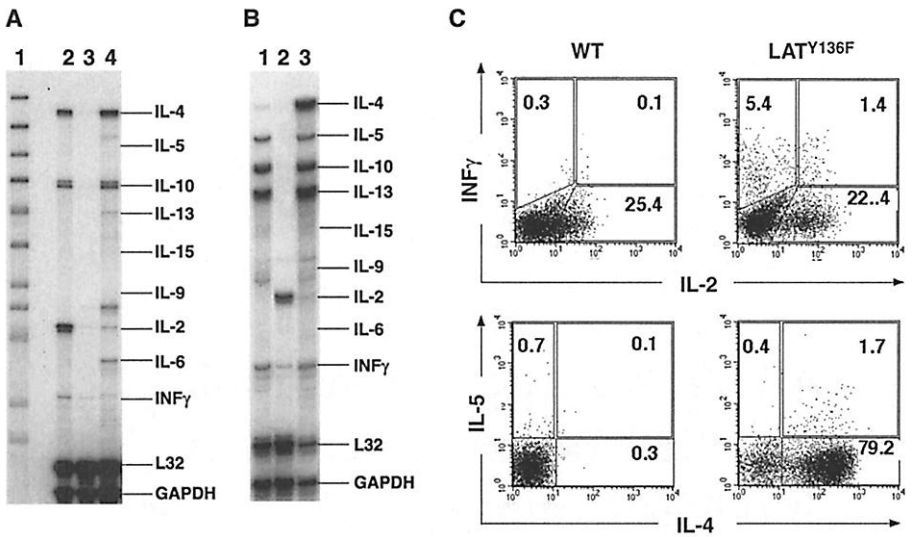


**Fig. 3.** Type-2 cytokine production in CD4 T cells freshly isolated from LAT$^{Y136F}$ peripheral lymphoid organs. (**A**). Analysis of the cytokine transcripts expressed in ex vivo CD4 T cells isolated from the spleens of wild-type mice (lane 3) and LAT$^{Y136F}$ mice (lane 4). Total RNA was analyzed by a multiprobe ribonuclease protection assay using a MCK1 RiboQuant mouse template set. The autoradiogram also shows the MCK1-probe set not treated with RNase (lane 1) and a control sample provided by the supplier (lane 2). The identity of the various protected bands is indicated on the right. (**B**) Analysis of the cytokine transcripts expressed in CD4 T cells isolated from wild-type (lane 2) and LAT$^{Y136F}$ (lane 3) mice after stimulation by PMA/ionomycin for 15 hours. Lane 1 corresponds to wild-type CD4 T cells that were grown under $T_H2$ polarizing conditions. Samples were processed as described in (A). (**C**) IL-2, IL-4, IL-5, and IFN-γ production analyzed in single cells. Ex vivo CD4 T cells purified from wild-type and from LAT$^{Y136F}$ lymph nodes were cultured for 4 hours in the presence of monensin to trap cytokine in the endoplasmic reticulum. During the culture period, cells were stimulated with PMA/ionomycin. At the end of the culture, cells were processed for intracellular staining. Numbers indicate percentages of cells in the respective gates.

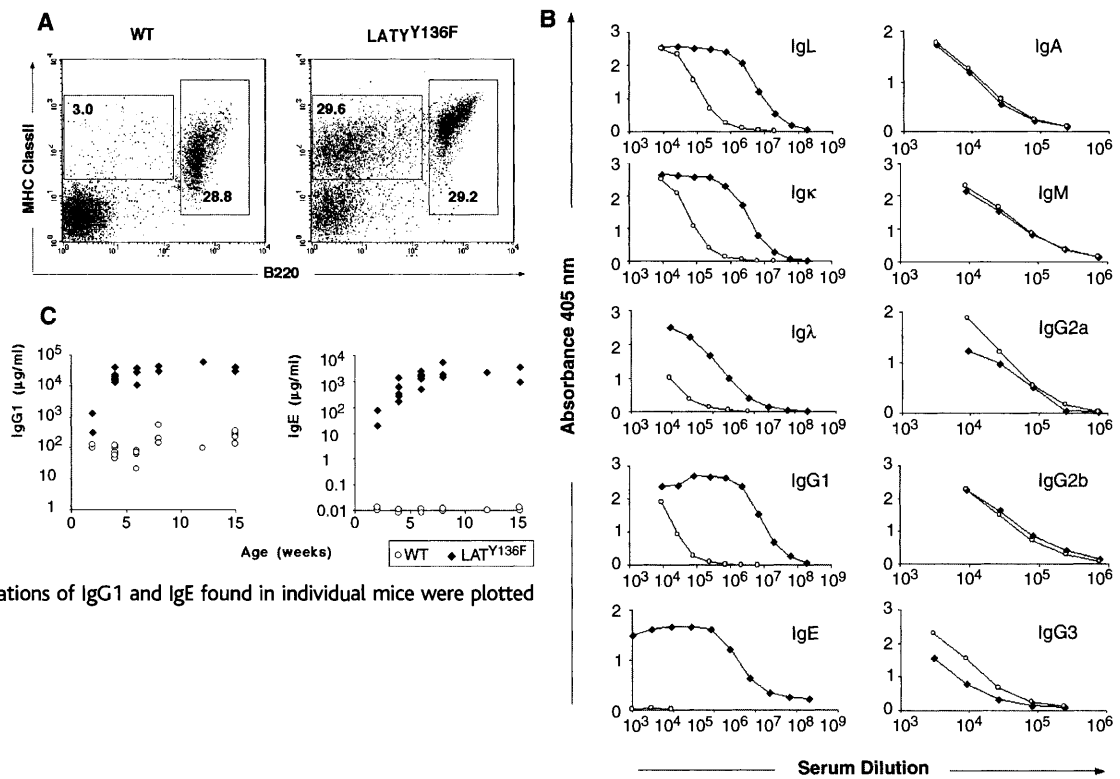


**Fig. 4.** Hyperactivated B lymphocytes and serum levels of IgG1 and IgE antibodies in unimmunized LAT$^{Y136F}$ mice. (**A**) Dot plots show the B220 versus MHC class II profiles of wild-type and LAT$^{Y136F}$ B cells. Boxes define B220$^{low}$ and B220$^{high}$ B cell populations. Data are representative of four mice aged 6 to 7 weeks. (**B**) Serum samples from 6-week-old wild-type and LAT$^{Y136F}$ mice were subjected to a serial dilution three times, and the titers of the specified immunoglobulin isotypes were determined by ELISA. Data are representative of six mice. (**C**) Early appearance of IgG1 and IgE in the serum of LAT$^{Y136F}$ mice. The concentrations of IgG1 and IgE found in individual mice were plotted on a logarithmic scale.

for reasons that have yet to be defined, to the selective differentiation of $T_H2$ effector cells. This dual role is consistent with the pattern of CD5 expression found in $LAT^{Y136F}$ mice. Its lack on the few DP cells that develop in $LAT^{Y136F}$ mice probably allows the pre-TCR to adapt to the lowered signaling potential of the $LAT^{Y136F}$ molecules. Conversely, the high levels of CD5 found on CD4 T cells probably correspond to a failed attempt to desensitize persistent TCR signals. Mutation Y136F does not affect the partition of LAT into glycolipid-enriched microdomains (fig. S6). Therefore, the negative role postulated for the LAT Y136 residue can be reconciled with the recessive nature of the LAT Y136 mutation, provided that in heterozygous mice the aberrant signals delivered by LAT Y136F molecules are blunted by signals from the wild-type LAT molecules that colocalize to glycolipid-enriched microdomains. The $LAT^{Y136F}$ mice differ from other mouse strains with $T_H2$-based diseases (*22*) or T cell lymphoproliferative disorders (*23*), but strikingly resemble mice deprived of NFATc2 and NFATc3 transcription factors (*15*). Therefore, by unleashing signal propagation at two distinct levels of the TCR transduction cassette, the LAT Y136F and NFATc2/c3 mutations presumably triggered a runaway feedback pathway that resulted in similar effects on $T_H2$ polarization. Although the underlying mechanisms by which the LAT Y136F mutation impacts on T cell development and differentiation remain to be defined, our results reveal that restricting LAT to a subset of its docking functions critically affects the differentiation of $T_H$ cells. It is therefore possible that even in a physiological context, the differential phosphorylation of some of the tyrosines found in LAT also influences $T_H$ cell differentiation.


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12. CD8 T cells purified from wild-type and from $LAT^{Y136F}$ lymph nodes were cultured for 4 hours in the presence of nonensin. During the culture period, cells were either stimulated with PMA-ionomycin or left unstimulated. At the end of the culture, the production of IL-2, IL-4, IL-5, and IFN-γ was analyzed at the single-cell level. In contrast to wild-type CD8 T cells that produce IL-2, and to $LAT^{Y136F}$ CD4 T cells that make copious amounts of IL-2, IL-4, and IFN-γ, $LAT^{Y136F}$ CD8 T cells were unable to produce any of the tested cytokines. This suggests that the LAT Y136F mutation has a distinct impact on the function of CD4 and CD8 T cells. The CD8 T cells found in $LAT^{Y136F}$ mice had a $CD25^-$, $CD44^{hi}$, $CD62L^{lo}$, $CD69^+$ phenotype similar to that of activated and memory T cells.
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**Supporting Online Material**

www.sciencemag.org/cgi/content/full/296/5575/2036/DC1

Materials and Methods Figs. S1 to S6

Table S1


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# A LAT Mutation That Inhibits T Cell Development Yet Induces Lymphoproliferation


**Connie L. Sommers,[1] Cheung-Seog Park,[2] Jan Lee,[3] Chiguang Feng,[2] Claudette L. Fuller,[1] Alexander Grinberg,[2] Jay A. Hildebrand,[1] Emanuela Lacaná,[3] Rashmi K. Menon,[1] Elizabeth W. Shores,[3] Lawrence E. Samelson,[1] Paul E. Love[2]***



**Mice homozygous for a single tyrosine mutation in LAT (linker for activation of T cells) exhibited an early block in T cell maturation but later developed a polyclonal lymphoproliferative disorder and signs of autoimmune disease. T cell antigen receptor (TCR)–induced activation of phospholipase C–γ1 (PLC-γ1) and of nuclear factor of activated T cells, calcium influx, interleukin-2 production, and cell death were reduced or abrogated in T cells from LAT mutant mice. In contrast, TCR-induced Erk activation was intact. These results identify a critical role for integrated PLC-γ1 and Ras-Erk signaling through LAT in T cell development and homeostasis.**


LAT is a transmembrane scaffolding protein that, after TCR engagement, becomes tyrosine-phosphorylated and recruits multiple signaling molecules important for T cell activation (*1–5*). The distal four tyrosine residues of LAT (Y132, Y171, Y191, and Y226) are required for its activity, and mutation of all four uncouples the TCR from both the PLC-γ1–calcium and the Ras-Erk signaling pathways, causing a complete block in T cell development (*3–6*). LAT tyrosines bind preferentially to specific effector proteins. Tyrosine-132 selectively binds PLC-γ1, and T cell lines expressing a Y132F ($Tyr^{132}$→Phe) LAT mutant protein exhibit decreased phosphorylation of PLC-γ1, attenuated or absent calcium influx, and reduction of nuclear factor of activated T cells (NF-AT) activity after TCR engagement (*3–5*). This single mutation also blocks Erk phosphorylation in T cell lines (*3–5*), indicating a role for PLC-γ1 in Ras activation in T cells, presumably through Ras-GRP, a diacylglycerol (DAG)-dependent guanine nucleotide exchange factor (*7*).

The importance of individual LAT tyrosines for T cell development and function was further examined by generating a "knock-in" mutation of LAT in mice in which the murine equivalent of human $Tyr^{132}$ ($Tyr^{136}$) was mutated to phenylalanine (LATY136F). Two independently derived embryonic stem cell clones were used to establish homozygous mutant mouse lines that displayed similar phenotypes (*8*). Sequencing of tail DNA confirmed the Y136F mutation and the integrity of the remainder of the coding sequence in the targeted allele (*9*). LAT expression in thymocytes and T cells from homozygous LATY136F mutant mice


[1]Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, [2]Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA. [3]Division of Therapeutic Proteins, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892, USA.

*To whom correspondence should be addressed. E-mail: pel@helix.nih.gov