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

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Materials and Methods Figs. S1 to S6

Table S1

17 December 2001; accepted 23 April 2002

A LAT Mutation That Inhibits T Cell Development Yet Induces Lymphoproliferation

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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

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(LATY136F$^{m/m}$) was about half that in LAT$^{+/+}$ cells (fig. S1) (*8*). A similar reduction in LAT expression was observed in LAT$^{+/-}$ (heterozygous knockout) and LAT$^{WT/WT}$ knock-in mice (expressing the comparable wild-type knock-in version of LAT), both of which exhibit no impairment in T cell development or function (*6*, *10*).

Analysis of thymocytes from 2-week-old LATY136F$^{m/m}$ mice revealed a severe but incomplete block in T cell development (Fig. 1A). Thymi from LATY136F$^{m/m}$ mice were small (5 to 10% of LAT$^{+/+}$ thymocyte numbers) and contained a high percentage (but not increased numbers) of immature CD4$^-$CD8$^-$ cells relative to LAT$^{+/+}$ littermates. In addition, the number and percentage of intermediate-stage CD4$^+$CD8$^+$ thymocytes and mature CD4$^+$ and CD8$^+$ thymocytes and splenocytes were markedly reduced in LATY136F$^{m/m}$ mice (Fig. 1A and fig. S2) (*8*). Examination of immature CD4$^-$CD8$^-$ thymocyte subsets in LATY136F$^{m/m}$ mice revealed that T cell development was blocked at the CD25$^+$CD44$^{lo/-}$ stage (Fig. 1A). Because pre-TCR signals are required for transition of CD25$^+$CD44$^{lo/-}$ cells to the CD25$^-$CD44$^-$ stage (*11*), these results suggest that the LATY136F mutation compromises signaling by the pre-TCR complex.

In older LATY136F$^{m/m}$ mice, an entirely different phenotype was observed. At about 4 weeks of age, LATY136F$^{m/m}$ mice began to exhibit lymphadenopathy and splenomegaly (fig. S3). Lymph nodes and spleens contained primarily CD4$^+$ T cells and elevated numbers of B cells, macrophages, and eosinophils (Fig. 1B) (*9*). Thymi from older LATY136F$^{m/m}$ mice were small and also contained a high percentage of CD4$^+$ T cells and B cells, which suggests they had been infiltrated by cells from perithymic lymph nodes. Histological analysis revealed multiorgan infiltration of CD4$^+$ T cells, eosinophils, B cells, and macrophages (fig. S3) (*9*). Most LATY136F$^{m/m}$ mice died before the age of 6 months.

In view of the small thymus size and the paucity of mature CD4$^+$ and CD8$^+$ thymocytes and T cells in young LATY136F$^{m/m}$ mice, the discovery of large numbers of CD4$^+$ T cells in the peripheral lymphoid organs of older mice suggested that these cells had arisen by proliferative expansion. Indeed, CD4$^+$ T cells from LATY136F$^{m/m}$ mice were larger than those from LAT$^{+/+}$ mice and exhibited a phenotype indicative of prior activation, CD62L^{lo}-CD45RB^{lo}CD44^{hi} (fig. S2) (*8*). LATY136F$^{m/m}$ mice contained a higher percentage of proliferating T cells than LAT$^{+/+}$ mice (Fig. 1B). The TCR repertoire of peripheral CD4$^+$ T cells was diverse (as measured by TCRVβ usage), indicating polyclonal expansion (*9*). CD4$^+$ T cells from LATY136F$^{m/m}$ mice were also resistant to TCR-mediated cell death (Fig. 1B and fig. S4), suggesting another possible mechanism for cell expansion.

T cells from LATY136F$^{m/m}$ mice expressed very low levels of surface TCR (Fig. 1B). TCR expression increased, although not to normal levels, after several days of in vitro culture (*9*). Despite their low TCR expression, LATY136F$^{m/m}$ T cells were CD5^{hi} (fig. S2) (*8*), which is characteristic of cells expressing TCRs that react with high avidity to self ligands (*12*). Together with the abnormality in thymocyte development (Fig. 1A) and data presented in the accompanying report (*13*), these results suggest that thymocyte selection may be impaired in LATY136F$^{m/m}$ mice, resulting in survival of autoreactive T cells.

CD4$^+$ T cells from LATY136F$^{m/m}$ mice proliferated poorly in vitro in response to activation by antibody to CD3 (anti-CD3), even in the presence of the costimulatory antibody anti-CD28 (Fig. 2A). In contrast, their proliferative response to a combination of phorbol ester [phorbol 12-myristate 13-acetate (PMA)] and calcium ionophore (ionomycin) (Fig. 2B) or to anti-CD3 plus anti-CD28 antibodies in the presence of ionomycin (Fig. 2A) was similar to that of LAT$^{+/+}$ T cells. In fact, ionomycin was sufficient to induce proliferation of T cells from LATY136F$^{m/m}$ mice in the absence of additional stimuli (Fig. 2A). T cells from LATY136F$^{m/m}$ mice also proliferated in response to interleukin-2 (IL-2) in the absence of TCR stimulation (Fig. 2A). Thus, T cells in LATY136F$^{m/m}$ mice are activated in vivo but appear to be defective in calcium signaling and in their ability to produce IL-2 in response to TCR engagement.

To investigate the molecular basis for the developmental and functional abnormalities in LATY136F$^{m/m}$ mice, we performed a biochemical analysis of TCR signaling responses. CD4$^+$ lymph node T cells were purified and stimulated with anti-CD3 and anti-CD4 antibodies (*8*). Proximal activation events that do not depend on LAT phosphorylation, such as tyrosine phosphorylation of TCRζ and ZAP-70, were unaffected in LATY136F$^{m/m}$ T cells (Fig. 3A). In addition, tyrosine phosphorylation of the adaptor protein SLP-76, which binds to LAT tyrosines other than Y136 through its association with the adaptor Gads, was unimpaired (*9*). However, tyrosine phosphorylation of LAT and of PLC-γ1 were markedly reduced in LATY136F$^{m/m}$ T cells after antibody stimulation (Fig. 3A). CD4$^+$ lymph node T cells and thymocytes from LATY136F$^{m/m}$ mice failed to mobilize calcium in response to CD3 plus CD4 or CD3 plus CD28 cross-linking, even when incubated with high concentrations of stimulating antibodies (Fig. 3B) (*9*). TCR-induced calcium flux was also abrogated or reduced in CD4$^+$CD8$^+$ thymocytes (Fig. 3B), which express levels of TCR similar to those from LAT$^{+/+}$ mice (*9*). TCR cross-linking also failed to activate the calcineurin-dependent transcription factors NF-ATc1 and NF-ATc2 (fig. S5) or to induce IL-2 production (Fig. 2B). IL-2 has been shown to increase FasL expression after TCR engagement and to sensitize T

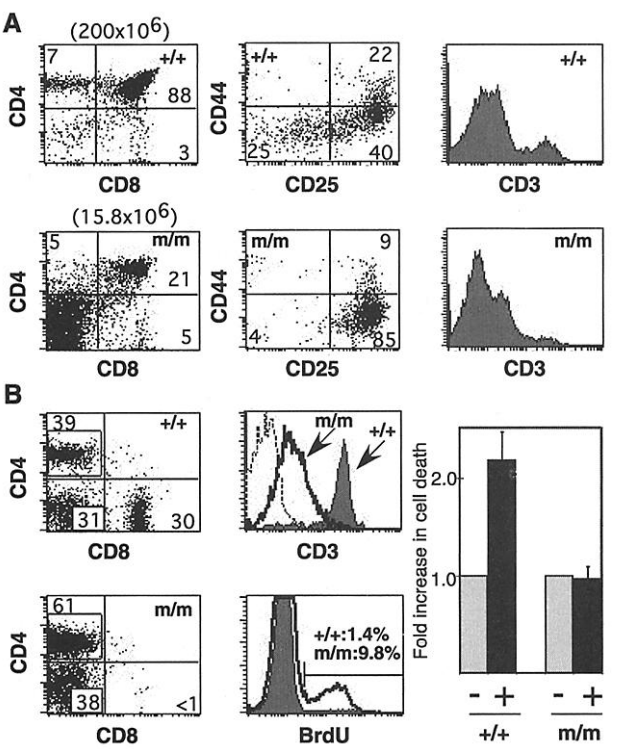

Fig. 1. T cell development in LATY136F$^{m/m}$ mice. **(A)** Phenotype of thymocytes from 2-week-old LATY136F$^{m/m}$ mice. Thymocytes were enumerated (total thymocyte numbers are in parentheses) and analyzed by flow cytometry. CD44 versus CD25 staining is from gated CD3$^-$CD4$^-$CD8$^-$B220$^-$ thymocytes. **(B)** Phenotype of lymph node (LN) cells from adult mice. CD4 versus CD8 plots show staining of total LN cells from 11-week-old mice. The CD3 histogram is from gated CD4$^+$ cells. Shaded histograms are from LAT$^{+/+}$ mice and unshaded histograms are from LATY136F$^{m/m}$ mice. Dashed line indicates an isotype-matched antibody negative control. The bromodeoxyuridine (BrdU) histogram represents incorporation by CD4$^+$ LN cells in vivo (*8*). To measure TCR-mediated cell death, we stimulated purified CD4$^+$ LN T cells from LATY136F$^{m/m}$ mice for 48 hours with PMA plus ionomycin in the presence of IL-2. Cultures were rested in medium containing IL-2 for 3 days and then restimulated with anti-CD3 or medium alone (*8*). The fold increase in annexin V–positive cells relative to medium alone is shown. No restimulation, –; anti-CD3 restimulation, +. Means and standard error of the mean from three separate experiments are shown.

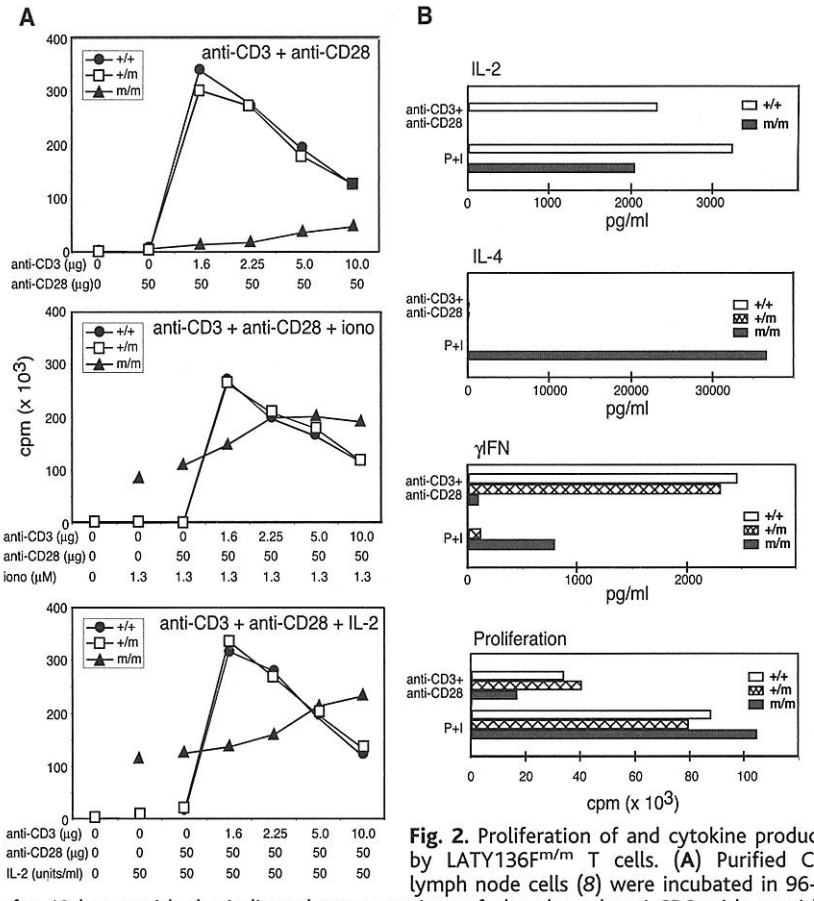

Fig. 2. Proliferation of and cytokine production by LATY136F$^{m/m}$ T cells. (**A**) Purified CD4$^+$ lymph node cells (*8*) were incubated in 96-well plates for 48 hours with the indicated concentrations of plate-bound anti-CD3 with or without plate-bound anti-CD28 (50 μg). We added ionomycin (iono) and IL-2 to selected cultures as indicated. Cells were then pulsed for 16 hours with [^{3}H]thymidine and were harvested for counting. (**B**) CD4$^+$ T cells were treated as described in (A), except that the cells were stimulated with either anti-CD3 (10 μg) and anti-CD28 (50 μg) or PMA (5 ng/ml) plus ionomycin (1.3 μM). Cytokines were measured in cell supernatants by standard enzyme-linked immunosorbent assay after 18 hours (IL-2) or 48 hours (IL-4 and IFN-γ) of incubation. Proliferation was measured as described in (A). Results are representative of four individual experiments.

cells to Fas-mediated killing (*14*, *15*). LATY136F$^{m/m}$ T cells expressed lower surface levels of Fas than T cells from LAT$^{+/+}$ mice before and after activation in vitro and failed to induce FasL expression in response to TCR engagement (fig. S4). Consistent with this, cell death mediated by Fas antibody was defective in LATY136F$^{m/m}$ T cells (fig. S4).

Surprisingly, and in contrast to the results obtained in T cell lines (*3*–*5*), Erk activation in response to CD3 plus CD4 cross-linking was normal or only slightly reduced in CD4$^+$ T cells (Fig. 3A) and in CD4$^+$CD8$^+$ thymocytes (*9*) from LATY136F$^{m/m}$ mice. In addition, stimulation of CD4$^+$CD8$^+$ thymocytes from LATY136F$^{m/m}$ mice with anti-CD3 induced expression of CD5 (*9*), a response that has been shown to be Ras-dependent (*16*, *17*). These results indicate that the LATY136F knock-in mutation uncouples TCR signaling from PLC-γ1-calcium–dependent signaling but does not block activation of the Ras-Erk–dependent pathway in vivo.

The phenotype of LATY136F$^{m/m}$ mice bears a striking resemblance to that of mice lacking NF-ATc1 and NF-ATc2, which suggests that it may be attributed, at least in part, to failure to activate these transcription factors (*18*). CD4$^+$ T cells in NF-ATc1$^{-/-}$ NF-ATc2$^{-/-}$ mice are activated, T helper 2 (T_H2) biased, and secrete large amounts of IL-4 (*18*). To determine whether CD4$^+$ T cells from LATY136F$^{m/m}$ mice exhibited a T_H2 cytokine bias, we assayed stimulated cells for production of IL-4 and interferon-γ (IFN-γ). T cells from LATY136F$^{m/m}$ mice produced very low levels of cytokines when stimulated with anti-CD3 plus anti-CD28 antibodies (Fig. 2B). However, when TCR signaling was bypassed by stimulation

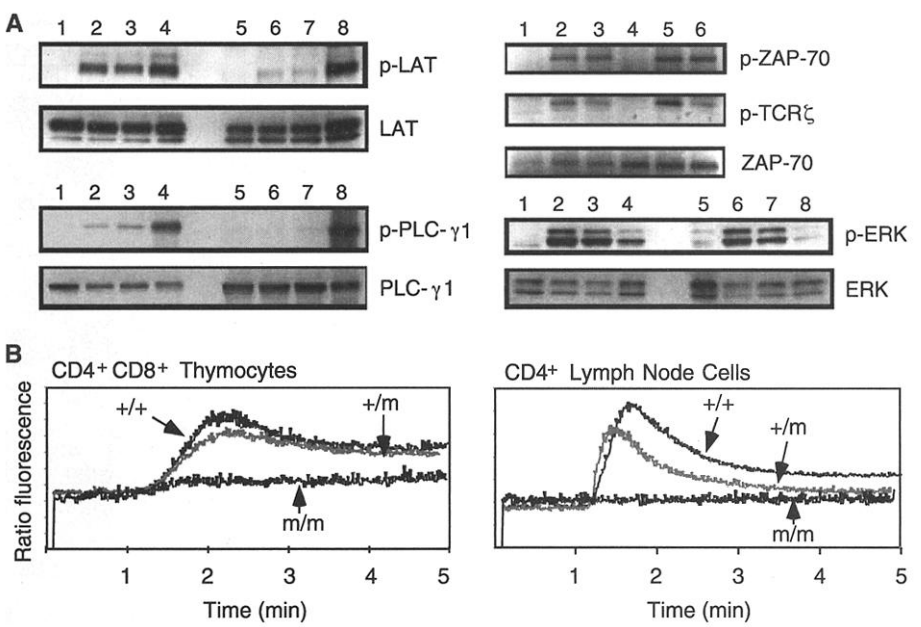

Fig. 3. Decreased TCR-induced LAT and PLC-γ1 tyrosine phosphorylation and calcium flux but normal Erk activation in LATY136F$^{m/m}$ T cells. (**A**) Purified CD4$^+$ lymph node T cells from LAT$^{+/+}$ (+/+) or LATY136F$^{m/m}$ (m/m) mice were stimulated with anti-CD3 and anti-CD4 or pervanadate. We determined levels of phospho (p)-LAT, p-PLC-γ1, p-ZAP-70, p-TCRζ, and p-Erk by immunoprecipitation and Western blotting (*8*). For LAT, PLC-γ1, and Erk: lanes 1 to 4, +/+; lanes 5 to 8, m/m. For ZAP-70: lanes 1 to 3, +/+; lanes 4 to 6, m/m. For LAT and PLC-γ1: lanes 1 and 5, unstimulated; lanes 2 and 6, anti-CD3 + anti-CD4 (1 min); lanes 3 and 7, anti-CD3 + anti-CD4 (5 min); lanes 4 and 8, pervanadate stimulated. For ZAP-70: lanes 1 and 4, unstimulated; lanes 2 and 5, anti-CD3 + anti-CD4 (2 min); lanes 3 and 6, anti-CD3 + anti-CD4 (5 min). For Erk: lanes 1 and 5, unstimulated; lanes 2 and 6, anti-CD3 + anti-CD4 (1 min); lanes 3 and 7, anti-CD3 + anti-CD4 (2 min); lanes 4 and 8, anti-CD3 + anti-CD4 (5 min). Results are representative of three independent experiments. (**B**) Thymocytes from 2-week-old mice (left) and lymph nodes from adult mice (right) were analyzed for calcium flux over time (*8*). At 30 s, biotinylated anti-CD3 and anti-CD4 antibodies were added; at 60 s, streptavidin was added. Data shown are for gated CD4$^+$CD8$^+$ thymocytes (left) and CD4$^+$ lymph node cells (right). Wild-type (+/+), LATY136F heterozygous knock-in (+/m), and LATY136F$^{m/m}$ (m/m) mice are shown. Results are representative of three independent experiments.

with PMA and ionomycin, CD4$^+$ T cells from LATY136F$^{m/m}$ mice produced large amounts of IL-4 relative to CD4$^+$ T cells from heterozygous knock-in or LAT$^{+/+}$ mice (Fig. 2B). Consistent with the T_H2 cytokine profile, serum concentrations of immunoglobulins G1, E, and M were elevated (fig. S6) and the multiorgan infiltrates in LATY136F$^{m/m}$ mice included large numbers of eosinophils (*9*). Lastly, LATY136F$^{m/m}$ mice also had elevated serum levels of DNA and nuclear antigen autoantibodies (fig. S6).

The in vivo effects of a defined point mutation in LAT reveal a role for the PLCγ1–calcium signaling pathway in early T cell development. Calcium signaling is important for setting the threshold for negative selection in the thymus (*19*, *20*). Thus, in LATY136F$^{m/m}$ mice, thymocytes that normally would be eliminated by negative selection may survive and populate the periphery. These cells may differ from normal T cells in their requirement for PLC-γ1 for Ras-Erk activation. In this regard, it is notable that PLC-γ1–DAG independent activation of Ras has been described in peripheral blood–derived T cells (*21*) and that the LAT132YF mutant protein retains the ability to recruit Grb2 together with the Ras guanine nucleotide exchange factor Sos after TCR stimulation (*3–5*). Alternatively, the low levels of catalytically active PLC-γ1 generated in LAT136YF$^{m/m}$ T cells may be sufficient to activate Ras-GRP or protein kinase C, resulting in Ras-Erk activation. The profound disturbance in T cell homeostasis caused by the selective loss of PLC-γ1–mediated signaling but not Erk signaling in LATY136F$^{m/m}$ mice further demonstrates a critical role for LAT in integrating signaling downstream of the TCR.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/296/5575/2040/DC1

Materials and Methods

Figs. S1 to S6

17 December 2001; accepted 23 April 2002

Cerebellum Activation Associated with Performance Change but Not Motor Learning

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The issue of whether the cerebellum contributes to motor skill learning is controversial, principally because of the difficulty of separating the effects of motor learning from changes in performance. We performed a functional magnetic resonance imaging investigation during an implicit, motor sequence-learning task that was designed to separate these two processes. During the sequence-encoding phase, human participants performed a concurrent distractor task that served to suppress the performance changes associated with learning. Upon removal of the distractor, participants showed evidence of having learned. No cerebellar activation was associated with the learning phase, despite extensive involvement of other cortical and subcortical regions. There was, however, significant cerebellar activation during the expression of learning; thus, the cerebellum does not contribute to learning of the motor skill itself but is engaged primarily in the modification of performance.

Despite extensive research, the role of the cerebellum in learning motor skills remains controversial (*1*, *2*). The concept of the cerebellum as a learning machine comes from the theoretical work of Marr (*3*) and Albus (*4*) and has been supported by data showing that it is essential for adaptive modification of reflex behavior (*5*, *6*) and is activated during motor learning (*7–9*). However, learning invariably leads to changes in motor performance, which in itself can activate the cerebellum (*10*, *11*). Previous efforts to deal with the issue of learning versus performance have required complex behavioral manipulations, such as subtracting an estimate of the performance effect (*9*). Here, we present a learning paradigm in which learning and performance change are effectively dissociated, using a modification of the serial reaction time task (*12*, *13*). Typically, participants learn the sequence embedded in the serial reaction time task within a few hundred trials. However, when asked to perform the task concomitantly with certain distractor tasks, they show no evidence of sequence learning (*14*). When retested upon removal of this distractor, it is evident that participants did actually learn the sequence during the initial training. Therefore, the distractor task served only to suppress performance change but did not prevent learning, allowing the determination of the underlying neural substrates for sequence learning separately from performance.

We obtained high-field (4 T) functional magnetic resonance imaging (fMRI) images of the cerebellum (*15*, *16*) in participants performing the modified serial reaction time task. Participants (n = 6) pressed one of four buttons with the right hand when instructed by a visual display. Trials were presented in blocks in which the lights were illuminated either randomly or on the basis of a 12-element repeating sequence. During the encoding phase, participants performed the sequence task concurrently with a distractor task, whereas for the expression phase the distractor was absent. Finally, participants were probed for their awareness of the existence of the sequence with a questionnaire and then asked to perform a free recall task.

During the encoding phase, there were no

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