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Negative Regulation of T Cell Activation and Autoimmunity by the Transmembrane Adaptor Protein LAB

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Summary

LAB (linker for activation of B cells), also known as NTAL (non-T cell activation linker), is a LAT (linker for activation of T cells)-like adaptor protein that is expressed in B, NK, and mast cells. Its role in lymphocytes has not been clearly demonstrated. Here, we showed that aged LAB-deficient (*Lat2*$^{-/-}$) mice developed an autoimmune syndrome. *Lat2*$^{-/-}$ T cells were hyperactivated and produced more cytokines than *Lat2*$^{+/+}$ T cells. Even though LAB was absent in naive T cells, LAB could be detected in activated *Lat2*$^{+/+}$ T cells. LAT-mediated signaling events were enhanced in *Lat2*$^{-/-}$ T cells; however, they were suppressed in T cells that overexpressed LAB. Mice with the *Lat2* gene conditionally deleted from T cells also developed the autoimmune syndrome like *Lat2*$^{-/-}$ mice. Together, these data demonstrated an important role of LAB in limiting autoimmune response and exposed a mechanism regulating T cell activation.

Introduction

In recent years, a number of adaptor proteins have been described to play an important role in regulation of T cell activation (Horejsi et al., 2004; Jordan et al., 2003; Samelson, 2002; Yablonski and Weiss, 2001). While some adaptors, such as LAT (linker for activation of T cells) and SLP-76 (the SH2 domain-containing leukocyte protein), have a positive and essential role in the T cell receptor (TCR)-mediated signaling and thymocyte development (Clements et al., 1998; Finco et al., 1998; Pivniouk et al., 1998; Yablonski et al., 1998; Zhang et al., 1998, 1999), other adaptor proteins can negatively regulate T cell activation. These negative or inhibitory adaptor molecules include membrane-associated adaptors, such as SIT (SH2-interacting transmembrane adaptor protein) and LAX (linker for activation of X cells) (Marie-Cardine et al., 1999; Zhu et al., 2002), and cytoplasmic adaptors, like Cbl (Casitas B-lineage lymphoma) and SAP (SLAM-associated protein) (Joazeiro et al., 1999; Naramura et al., 2002; Veillette, 2004). Mice deficient in some of these negative adaptors develop spontaneous autoimmune diseases, indicating that these proteins are critical in maintaining T cell tolerance or homeostasis.

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LAB (linker of activation of B cells), also known as NTAL (non-T cell activation linker), is an adaptor protein that is expressed in B cells, mast cells, and NK cells, but not in naive T cells (Brdicka et al., 2002; Janssen et al., 2003). Similar to LAT, it has multiple tyrosine residues in its cytoplasmic tail, five of which are within a Grb2 binding motif (YxN). LAB also has a palmitoylation motif and is localized to lipid rafts. Previous studies have clearly demonstrated that LAT is an essential adaptor protein in T cell activation and development. It functions in TCR-mediated MAPK (mitogen-activated protein kinase) activation and calcium flux by binding Grb2, Gads (Grb2-related adaptor downstream of Shc), phospholipase C (PLC)-γ1, and other signaling molecules upon phosphorylation by ZAP-70 (ζ-associated protein-70) tyrosine kinase (Finco et al., 1998; Liu et al., 1999; Zhang et al., 1998, 2000). Recent studies indicate that LAT binding to PLC-γ1 is critical for LAT function in T cell homeostasis (Aguado et al., 2002; Sommers et al., 2002). Mice expressing LAT with a mutation at the PLC-γ1 binding site (Tyr136) develop a severe autoimmune disease resulting from uncontrolled expansion of CD4$^+$ T cells, a consequence of both defective negative selection and lack of T regulatory cells in these mice (Koonpaew et al., 2006; Sommers et al., 2005). Although LAB is similar to LAT and is capable of replacing LAT in thymocyte development (Janssen et al., 2003), LAB does not have the PLC-γ1 binding site and fails to bind PLC-γ1 (Brdicka et al., 2002; Janssen et al., 2003). Transgenic mice expressing LAB in *Lat*$^{-/-}$ T cells develop a Th_2 type autoimmune disease similar to the *Lat*^{Y136F} knockin mice (Janssen et al., 2004), suggesting that LAB functionally resembles the LATY136F mutant.

Upon B cell receptor (BCR) engagement, LAB is phosphorylated and interacts with Grb2. Studies with Grb2-deficient and LAB-deficient DT40 chicken B cells suggest that the Grb2-LAB complex positively regulates BCR-mediated calcium signaling by switching off unidentified inhibitory elements (Stork et al., 2004). However, deletion of the gene encoding LAB, recently renamed *Lat2*, in mice has no substantial effect on B cell development or BCR-mediated signaling (Wang et al., 2005; Zhu et al., 2004). Thus, whether LAB functions in the BCR pathway remains to be determined. LAB is also expressed in bone marrow-derived mast cells. Upon engagement of the high-affinity IgE receptor (FcεRI), LAB is also phosphorylated and associates with Grb2. Mast cells deficient in LAB are hyperresponsive in response to stimulation via the FcεRI. FcεRI-mediated Erk (extracellular-regulated kinase) activation, calcium flux, degranulation, and cytokine production are enhanced in LAB-deficient (*Lat2*$^{-/-}$) mast cells, indicating that LAB negatively regulates mast cell function. LAB also plays a positive role in mast cells. Mast cells deficient in both the *Lat* and *Lat2* genes have a further reduction in FcεRI-mediated signaling and mast cell function in comparison with *Lat*$^{-/-}$ mast cells (Volna et al., 2004; Zhu et al., 2004).

Here, we report that aged LAB-deficient (*Lat2*$^{-/-}$) mice developed a spontaneous autoimmune syndrome,

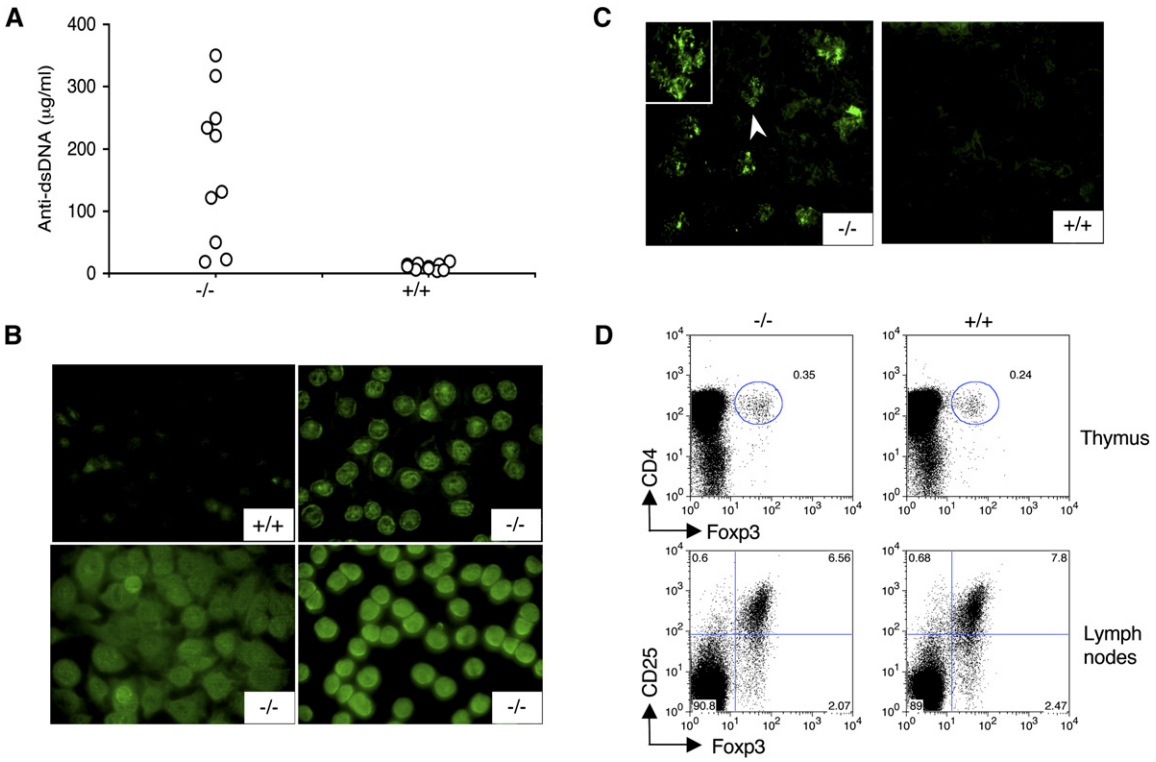

Figure 1. Development of an Autoimmune Syndrome in Aged *Lat2*$^{-/-}$ Mice

(A) The presence of DNA antibodies in aged *Lat2*$^{-/-}$ mice. Sera from 10 6-month-old *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ female mice were assayed for the concentrations of IgG specific to dsDNA by ELISA.

(B) Anti-nuclear antibody assay. Diluted sera from *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice were used to stain slides with fixed HEp-2 cells. The presence of anti-nuclear antibodies was revealed by FITC-conjugated goat anti-mouse IgG. A representative staining with the sera from three *Lat2*$^{-/-}$ mice and one *Lat2*$^{+/+}$ mouse is shown.

(C) Immune complex deposition in the glomeruli of *Lat2*$^{-/-}$ mice. Cryosections of kidneys from three *Lat2*$^{-/-}$ and three *Lat2*$^{+/+}$ mice were stained with FITC-conjugated goat anti-mouse IgG and visualized by fluorescence microscopy.

(D) Expression of Foxp3 in thymocytes and lymph node cells from *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice. Numbers in the FACS plot indicate the percentages of cells in quadrants or gated circles.

evidenced by enlarged spleens and production of autoantibodies. T cells in these mice were hyperactivated and hyperresponsive to anti-CD3 stimulation. They produced higher amounts of cytokines than T cells from *Lat2*$^{+/+}$ mice. Although LAB was not expressed in naive T cells, it was upregulated in *Lat2*$^{+/+}$ T cells after activation via the TCR. TCR-mediated signaling was enhanced in *Lat2*$^{-/-}$ T cells. By using mice in which the *Lat2* gene can be deleted upon expression of the Cre recombinase, we showed that the autoimmune syndrome was caused by abnormal T cells. Our data indicated that LAB negatively regulates T cell activation.

Results

Development of an Autoimmune Syndrome in Aged *Lat2*$^{-/-}$ Mice

Analyses of cells from the bone marrow, thymus, spleen, and lymph nodes by fluorescence-activated cell sorting (FACS) revealed normal development of T and B lymphocytes in LAB-deficient (*Lat2*$^{-/-}$) mice. 5- to 8-week-old *Lat2*$^{-/-}$ mice have no apparent abnormality. The sizes of the spleen, thymus, and lymph nodes from *Lat2*$^{-/-}$ mice were similar to those from *Lat2*$^{+/+}$ mice. However, when old *Lat2*$^{-/-}$ mice and age-matched *Lat2*$^{+/+}$ mice (>6 months old) were analyzed, we found that spleens from *Lat2*$^{-/-}$ mice were enlarged (data not shown). Total numbers of splenocytes for *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice are $(9.5 \pm 2.0) \times 10^7$ and $(4.4 \pm 0.6) \times 10^7$, respectively. The percentages of CD4$^+$, CD8$^+$, and B220$^+$ cells were similar in these mice: 17.7% ± 2.3%, 8.3% ± 1.4%, and 58.0% ± 9.5%, respectively, in *Lat2*$^{-/-}$ mice and 18.5% ± 2.5%, 9.2% ± 1.3%, and 63.0% ± 3.4% in *Lat2*$^{+/+}$ mice.

Because of the apparent splenomegaly in aged *Lat2*$^{-/-}$ mice, we further looked for signs of autoimmune diseases. We first examined whether these mice produced autoantibodies against double-stranded DNA (dsDNA). The concentrations of serum IgG specific for dsDNA were measured by ELISA. At 6 months of age, the concentrations of autoantibodies in *Lat2*$^{-/-}$ mice were substantially higher than in *Lat2*$^{+/+}$ mice (Figure 1A) and were elevated even more in 1-year-old *Lat2*$^{-/-}$ mice (data not shown). We further confirmed the presence of autoantibodies by performing an anti-nuclear antibody (ANA) assay. Sera from 6-month-old *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice were used to stain fixed HEp-2 cells by immunofluorescence microscopy. As shown in Figure 1B, only sera from *Lat2*$^{-/-}$ mice gave clearly positive fluorescence signals. Staining patterns with sera from three *Lat2*$^{-/-}$ mice varied. While some clearly showed nuclear staining, others revealed homogeneous staining positive in both the nucleus and cytoplasm. To determine whether

the increased autoantibody production was associated with immune complex deposition in the glomeruli of *Lat2*$^{-/-}$ mice, cryosections of kidneys were stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and examined by fluorescence microscopy. A strong fluorescence signal was clearly detected in the glomeruli of *Lat2*$^{-/-}$ mice, whereas only background immunofluorescence was seen in the glomeruli of *Lat2*$^{+/+}$ mice (Figure 1C). Together, these data indicated that aged *Lat2*$^{-/-}$ mice develop a spontaneous autoimmune syndrome.

To investigate the cause for autoimmunity in aged *Lat2*$^{-/-}$ mice, we investigated whether T regulatory (Treg) cell development is normal. Treg cells play an important role in suppressing autoreactive T cells (Sakaguchi, 2004; Shevach et al., 2001). They are CD4$^+$CD25$^+$ and express Foxp3, a transcription factor involved in development of Treg cells (Fontenot et al., 2003). We examined expression of Foxp3 in thymocytes and splenocytes from *Lat2*$^{+/+}$ and *Lat2*$^{-/-}$ mice by intracellular staining with a Foxp3 antibody followed by flow cytometry analysis. Similar percentages of Foxp3$^+$ cells were seen in these mice (Figure 1D). In addition, *Lat2*$^{+/+}$ and *Lat2*$^{-/-}$ Treg cells showed a similar suppression of proliferation of CD4$^+$CD25$^-$ cells in vitro (data not shown). These data suggested that the autoimmunity in aged *Lat2*$^{-/-}$ mice was not due to abnormal Treg cell development or function.

Hyperactivated T Cells in Aged *Lat2*$^{-/-}$ Mice

We then examined the activation status of B and T cells in these mice by analyzing expression of activation markers, CD86, CD80, and MHC class II (I-A^b) for B cells, and CD25, CD69, CD44, CD62L, and CD45RB for T cells. In 6-week-old mice, we did not observe substantial differences in expression of these surface markers on B or T cells from *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice (data not shown). In contrast, in 6-month-old mice, CD4$^+$ T cells from *Lat2*$^{-/-}$ mice showed increased expression of CD25, CD69, and CD44 and decreased expression of CD62L and CD45RB in comparison with those from *Lat2*$^{+/+}$ mice (Figure 2A). Similar differences in expression of these surface markers were also seen in CD8$^+$ T cells (data not shown). However, B cells from *Lat2*$^{-/-}$ mice had similar amounts of CD86, CD80, and MHC class II as those from *Lat2*$^{+/+}$ mice (data not shown). These data indicated that there were more activated or memory T cells in aged LAB-deficient mice.

Next, we performed intracellular staining to examine whether *Lat2*$^{-/-}$ T cells produced more cytokines than *Lat2*$^{+/+}$ T cells. Splenocytes from aged *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice were stimulated by PMA (phorbol myristate acetate) and ionomycin for 1 hr. After addition of Golgistop, these cells were further stimulated for 3 hr. Cells were then stained with anti-CD4 and CD8, fixed, permeablized, and stained again with antibodies against different cytokines. T cells from 5- to 6-week-old *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice produced very little IL (interleukin)-2, IL-4, IL-10, and IFN (interferon)-γ (data not shown). However, T cells from 6-month-old *Lat2*$^{-/-}$ mice produced more IL-2, IL-10, and IFN-γ compared with T cells from age-matched *Lat2*$^{+/+}$ mice (Figure 2B), whereas similar percentages of IL-4-producing T cells were detected (data not shown).

We further examined proliferation of B and T cells in response to stimulation via the antigen receptors. B and T cells were purified from splenocytes from aged *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice via autoMACS (automatic magnetic cell sorting) and were then stimulated with different concentrations of anti-IgM or anti-CD3ε, respectively. Anti-IgM-induced [^{3}H]thymidine incorporation was similar between *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ B cells (data not shown); however, thymidine incorporation into T cells from aged *Lat2*$^{-/-}$ mice was enhanced when low concentrations of anti-CD3 (0.05 and 0.5 μg/ml) were used (Figure 2C). The enhanced thymidine uptake could be due to accelerated proliferation or decreased cell death. To distinguish between these two possibilities, T cells were labeled with CFSE (carboxy fluorescein diacetate succinimide ester) before being stimulated with different concentrations of anti-CD3. CFSE intensity should be diluted after each cell division. As shown in Figure 2D, *Lat2*$^{-/-}$ T cells divided faster than *Lat2*$^{+/+}$ cells when 0.05 μg/ml anti-CD3 was used. We also assayed anti-CD3-induced cell death by staining T cells with Annexin V. No apparent differences were seen between *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ T cells. These data indicated that T cells in aged *Lat2*$^{-/-}$ mice were hyperresponsive to stimulation via the TCR.

Expression of LAB in Activated T Cells

Results from Figure 2 indicated that T cells from aged *Lat2*$^{-/-}$ mice were hyperactivated and produced more cytokines. This result was not expected because previously published data showed that LAB is not expressed in naive T cells (Brdicka et al., 2002; Janssen et al., 2003). Thus, we hypothesized that while naive T cells do not express or express a trace amount of LAB, activated T cells might upregulate LAB. To examine LAB expression in T cells, naive CD4$^+$ and CD8$^+$ cells from *Lat2*$^{+/+}$ mice were purified by MACS. For activated T cells, splenocytes from *Lat2*$^{+/+}$ mice were stimulated in anti-CD3-coated plates for 2 days and cultured in IL-2 media for an additional 2–3 days before purification by MACS. Real-time PCR was performed with cDNAs prepared from these cells. The amount of LAB RNA was normalized relative to β-actin. As shown in Figure 3A, LAB expression was indeed low in naive CD4$^+$ and CD8$^+$ T cells; however, the amount of LAB RNA was increased by $\sim$17-fold in CD4$^+$ and $\sim$35-fold in CD8$^+$ T cells activated in vitro via CD3 in the presence of IL-2.

Because T cells from aged *Lat2*$^{-/-}$ mice were hyperactivated, it is possible that in aged T cells, LAB is upregulated to suppress T cell activation. To examine this possibility, CD4$^+$ and CD8$^+$ T cells from 1-month-old and 8-month-old mice were purified by FACS sorting. B cells were also sorted from these mice as a control. cDNAs were prepared from these cells and were then used in real-time PCR. As seen in Figure 3B, the LAB RNA was increased by approximately 5-fold in T cells from 8-month-old mice while it was not changed substantially in B cells. We further purified memory (CD4$^+$CD62L$^-$CD25$^-$), activated (CD4$^+$CD62L$^-$CD25$^+$), and naive (CD4$^+$CD62L$^+$) T cells from 8-month-old mice and quantitated the amount of LAB RNA by real-time PCR. The amount of LAB transcript in memory and activated T cells was $\sim$6 and 8.5 times higher, respectively, than in naive T cells (Figure 3C).

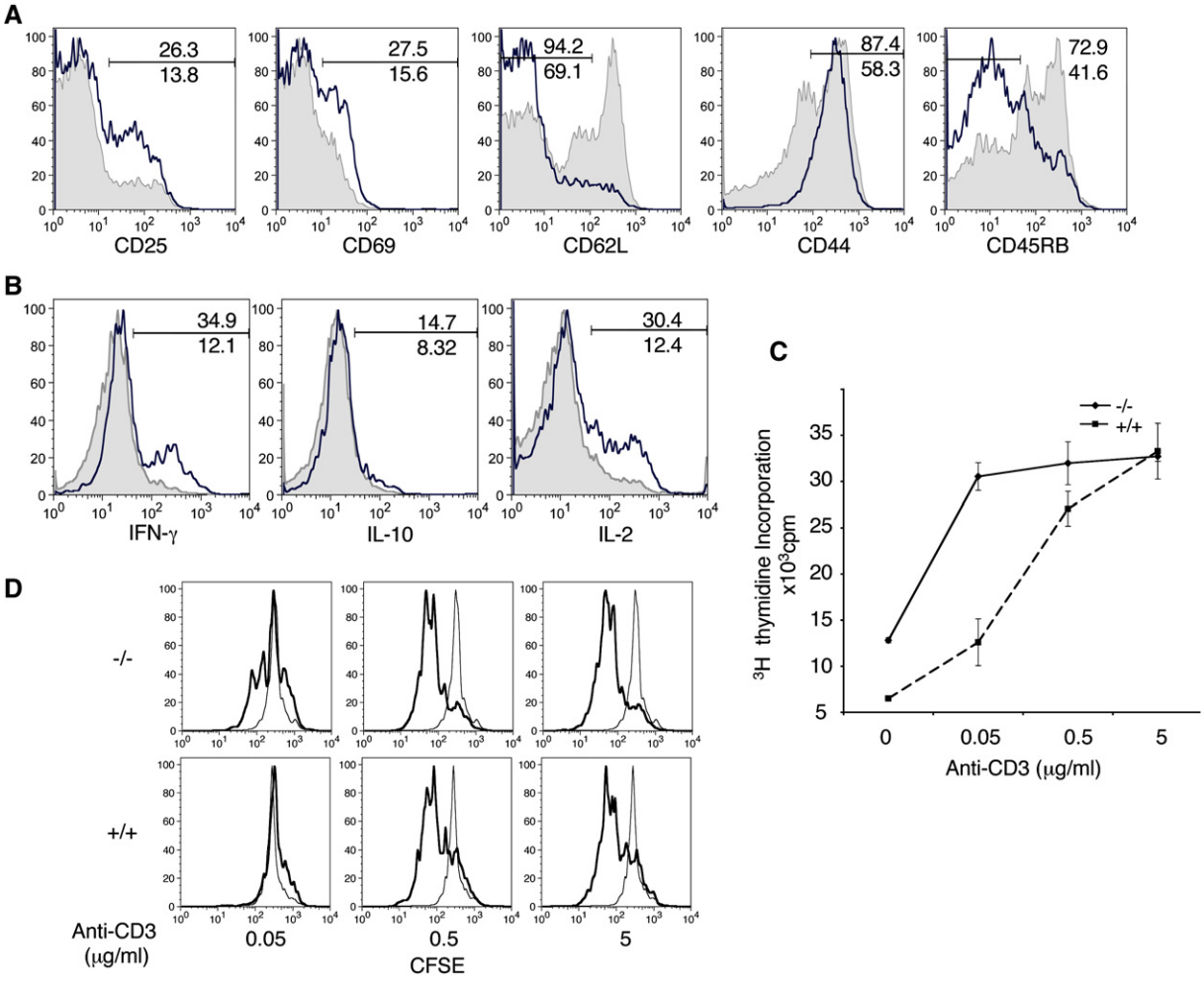

Figure 2. Hyperactivated T Cells in Aged *Lat2*$^{-/-}$ Mice

(A) Splenocytes from 6-month-old *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice were stained with anti-CD4, CD8, and different T cell activation markers as indicated in the figure. The shadowed histogram represents CD4$^+$ T cells from *Lat2*$^{+/+}$ mice, and the dark-lined histogram represents CD4$^+$ T cells from *Lat2*$^{-/-}$ mice. The figure shown is a representative of five mice for each genotype. Numbers indicate the percentages of gated *Lat2*$^{-/-}$ (top) and *Lat2*$^{+/+}$ (bottom) cells.

(B) Cytokine production in CD4$^+$ T cells from 6-month-old *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice. Splenocytes from 6-month-old *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice were analyzed by staining with anti-CD4 and CD8 followed by intracellular staining with antibodies against IFN-γ, IL-10, and IL-2. The shadowed histogram represents *Lat2*$^{+/+}$ T cells. The dark-lined histogram represents *Lat2*$^{-/-}$ T cells. Numbers indicate the percentages of cytokine-producing *Lat2*$^{-/-}$ (top) and *Lat2*$^{+/+}$ (bottom) cells. The figure shown represents three independent experiments.

(C) Thymidine incorporation. Purified T cells from *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice were stimulated with 0, 0.05, 0.5, and 5 μg/ml anti-CD3 in the presence of anti-CD28 (0.5 μg/ml) for 24 hr before assayed for [^{3}H]thymidine incorporation. The figure shown is representative of three independent experiments performed in triplicate. Bars indicate mean ± SD.

(D) Increased proliferation of T cells from aged *Lat2*$^{-/-}$ mice. CFSE-labeled T cells from *Lat2*$^{-/-}$ and *Lat2*$^{+/+}$ mice were stimulated with 0, 0.05, 0.5, and 5 μg/ml anti-CD3 in the presence of anti-CD28 (0.5 μg/ml) for 48 hr before being analyzed by FACS. The thin-lined histogram represents cells without anti-CD3 stimulation. The figure shown is representative of three independent experiments.

We next determined whether the amount of LAB protein in those cells correlated with the abundance of LAB RNA. Naive T cells and T cells activated in vitro were lysed, and postnuclear lysates were subjected to immunoprecipitation with anti-LAB. We also immunoprecipitated LAB from B cells as a positive control. Lysates from corresponding cell populations from *Lat2*$^{-/-}$ mice were used as negative controls. Immunoprecipitates were analyzed by an anti-LAB immunoblot. As shown in Figure 3D, LAB protein could be clearly detected in activated CD4$^+$ and CD8$^+$ cells.

We further examined whether LAB could be phosphorylated upon stimulation via the TCR. CD4$^+$ and CD8$^+$ T cells were activated in vitro and were restimulated with anti-CD3 and anti-CD4 or anti-CD3 and anti-CD8, respectively, before lysis. LAB was immunoprecipitated from cell lysates and analyzed by immunoblotting with anti-pTyr and anti-LAB. As shown in Figure 3E, LAB was clearly phosphorylated after TCR engagement. Taken together, these data indicated that LAB protein was indeed expressed in activated T cells.

Enhanced TCR-Mediated Signaling in *Lat2*$^{-/-}$ T Cells

Since LAB was expressed in activated T cells and could be phosphorylated upon stimulation via the TCR, we next examined whether LAB deficiency affected

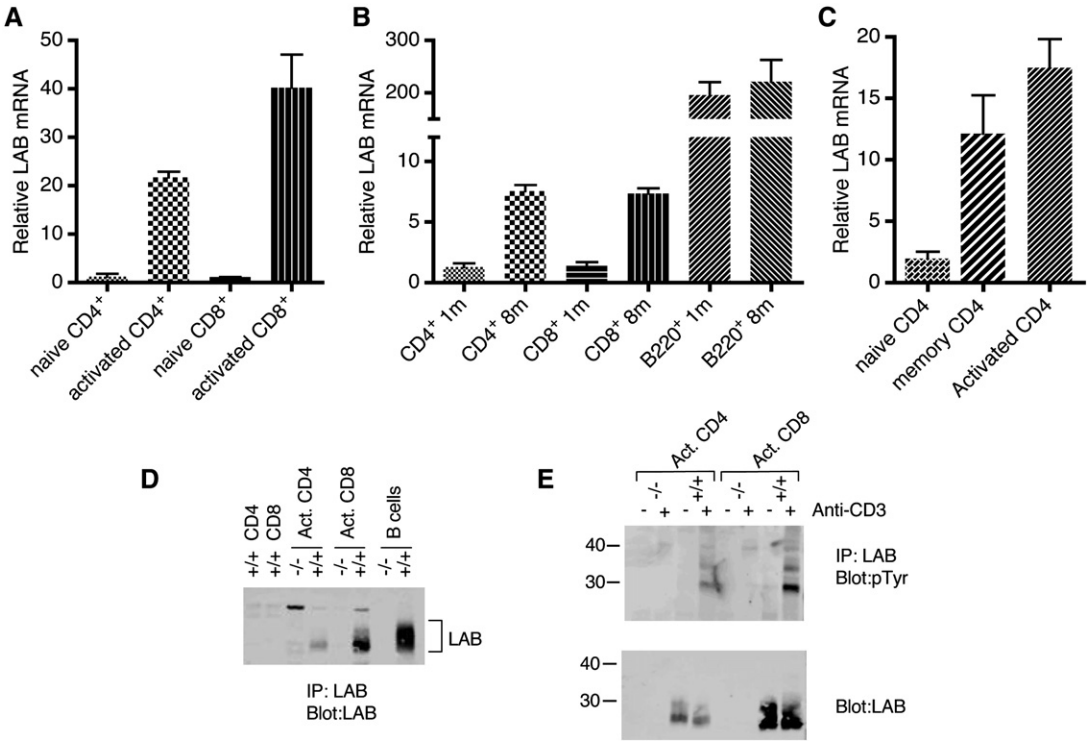

Figure 3. Expression of LAB in Activated T Cells

(A) Real-time PCR to detect LAB expression in T cells. Cells were sorted from spleens directly or after activation in vitro with a CD3ε antibody. The amount of LAB RNA in each population was normalized by the β-actin RNA. The figure shown in (A)–(C) is representative of three independent experiments performed in triplicate. Bars indicate mean ± SD.

(B) $CD4^+$, $CD8^+$, and $B220^+$ cells were sorted by FACS from 1-month-old and 8-month-old mice and used in real-time PCR.

(C) Naive ($CD4^+CD62L^+$), memory ($CD4^+CD62L^-CD25^-$), and activated ($CD4^+CD62L^-CD25^+$) T cells were sorted from 11-month-old mice and used in real-time PCR.

(D) LAB protein in activated T cells. Activated T cells were purified as in (A). B cells were isolated directly from spleens by MACS. Cells were lysed in RIPA buffer and lysates were immunoprecipitated with anti-LAB serum followed by immunoblotting with a LAB monoclonal antibody. Cells from $Lat2^{-/-}$ mice were used as negative controls.

(E) Phosphorylation of LAB in activated T cells. Activated $CD4^+$ or $CD8^+$ T cells from $Lat2^{-/-}$ and $Lat2^{+/+}$ mice were rested and restimulated by crosslinking CD3 and CD4 or CD3 and CD8 for 1.5 min before lysis in RIPA buffer. Lysates were immunoprecipitated with anti-LAB sera and analyzed by immunoblotting with either a pTyr or LAB antibody. The figure shown is representative of three independent experiments.

TCR-mediated signaling in activated T cells. Splenocytes from $Lat2^{-/-}$, $Lat2^{+/+}$, and $Lat2^{Tg}$ mice were activated with anti-CD3 and were expanded in IL-2 medium for 3 days. In T cells from $Lat2^{Tg}$ mice, LAB was overexpressed under control of the human CD2 promoter in $Lat^{+/+}$ T cells (Janssen et al., 2004). Since most of the T cells under this culture condition were $CD8^+$ T cells, only $CD8^+$ T cells were purified and used in analysis of TCR-mediated signaling. First we examined overall tyrosine phosphorylation of proteins after stimulation via the TCR for 0, 1.5, 5, and 10 min. Total lysates were analyzed by anti-pTyr immunoblotting. As seen in Figure 4A, the most obvious difference in tyrosine phosphorylation of proteins in T cells from different mice was LAT phosphorylation. Compared with $Lat2^{+/+}$ T cells, $Lat2^{-/-}$ T cells had increased LAT phosphorylation. In contrast, $Lat2^{Tg}$ T cells had dramatically reduced LAT phosphorylation. The effect of LAB expression on LAT phosphorylation was further confirmed by immunoblotting with an antibody against LAT phosphorylated at pY191 (Figures 4B and 4C).

Two signaling events downstream of LAT phosphorylation are phosphorylation of PLC-γ1 and activation of Erk. Correlated with LAT phosphorylation, PLC-γ1 phosphorylation and Erk activation was slightly increased in $Lat2^{-/-}$ T cells, but reduced in $Lat2^{Tg}$ T cells. In addition, phosphorylation of Akt (protein kinase B), an important protein in the PI3K pathway, was also enhanced in $Lat2^{-/-}$ cells, while it was suppressed in $Lat2^{Tg}$ cells (Figures 4B and 4C). We examined TCR-mediated phosphorylation of ZAP-70 tyrosine kinase and c-Cbl, a protein that negatively regulates T cell activation. Cbl phosphorylation was similar in these cells. Phosphorylation of ZAP-70 was also similar in $Lat2^{-/-}$ T cells; however, it was slightly reduced in $Lat2^{Tg}$ T cells (Figures 4B and 4C). We also examined TCR-mediated Ca^{2+} flux, a consequence of PLC-γ1 activation, by crosslinking CD3 and CD4 or CD3 and CD8. Calcium flux in activated $Lat2^{-/-}$ $CD4^+$ and $CD8^+$ T cells was enhanced. In contrast, it was reduced in $Lat2^{Tg}$ T cells (Figure 4D). These data suggested that LAB likely functions to inhibit LAT phosphorylation and LAT-mediated signaling events in T cells.

LAT Raft Localization, Cytokine Production, and Proliferation

Our previous data indicate that LAT and LAB may compete for localization to lipid rafts in mast cells. To

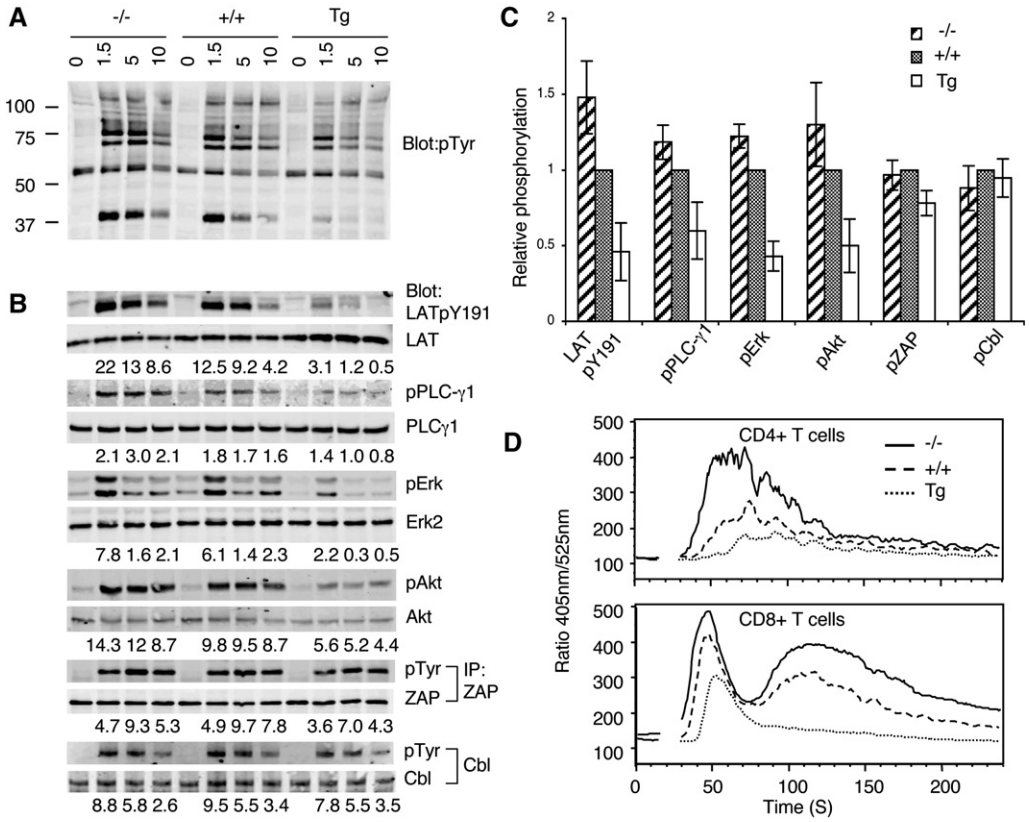

Figure 4. LAB in TCR-Mediated Signaling

(A and B) Tyrosine phosphorylation of proteins. Activated CD8$^+$ T cells were restimulated with anti-CD3 (5 μg/ml) and anti-CD8 (1 μg/ml) for indicated time points (minutes) before lysis in RIPA buffer. Cell lysates were analyzed by immunoblotting with anti-pTyr, phospho-specific, or pan antibodies against LAT, PLC-γ1, Erk, and Akt. Phosphorylation of ZAP-70 and Cbl was assayed by immunoprecipitation followed by immunoblotting with anti-pTyr. Each band was quantified, and the relative intensities of the phosphorylated form after normalization to the total amount of each protein were shown. Data shown are from one representative of three experiments.

(C) Quantitative analysis of changes in protein phosphorylation. Relative intensities of the phosphorylated form of each protein at 1.5 min after activation of *Lat2*$^{-/-}$ or *Lat2*^{Tg} T cells were normalized to those in *Lat2*$^{+/+}$ T cells. Data from three independent experiments were used in calculation. The figure shows mean relative intensity of each protein from three independent experiments. Bars indicate mean ± SD.

(D) Ca^{2+} mobilization. Activated CD4$^+$ or CD8$^+$ T cells were loaded with Indo-1. Ca^{2+} flux was induced by anti-CD3 (5 μg/ml) and CD4 (1 μg/ml) or CD8 (1 μg/ml) crosslinking. Data shown are from one representative of three experiments.

examine whether LAB also affects LAT localization to rafts in T cells, we isolated proteins in lipid rafts and nonraft fractions from activated *Lat2*$^{-/-}$, *Lat2*$^{+/+}$, and *Lat2*^{Tg} T cells by sucrose gradient ultracentrifugation. After centrifugation, each fraction was analyzed by immunoblotting with a LAT antibody. As shown in Figure 5A, in activated T cells from *Lat2*$^{-/-}$, *Lat2*$^{+/+}$, and *Lat2*^{Tg} mice, LAT was present in both raft (fractions 3–5) and nonraft (fractions 7–12) fractions; however, the relative amount of LAT in raft and nonraft fractions varied between cells tested. Compared with *Lat2*$^{+/+}$ T cells, *Lat2*$^{-/-}$ T cells had more LAT protein in raft fractions and less in nonraft fractions. In contrast, *Lat2*^{Tg} T cells had less LAT in raft fractions and more in nonraft fractions. These results suggested that in T cells, LAT and LAB might compete for raft localization or palmitoylation. In the absence of LAB, more LAT protein moved to lipid rafts, leading to increased tyrosine phosphorylation of LAT and enhanced LAT-mediated signaling.

We also assayed cytokine production in activated *Lat2*$^{+/+}$, *Lat2*$^{-/-}$, and *Lat2*^{Tg} T cells. Since most of the activated T cells under our culture condition were CD8$^+$ T cells, we purified these activated CD8$^+$ *Lat2*$^{-/-}$, *Lat2*$^{+/+}$, and *Lat2*^{Tg} T cells by MACS and quantitated cytokines secreted by these T cells after restimulation with anti-CD3 and anti-CD28 or PMA and ionomycin for 4 hr via a multiplex cytokine assay. As shown in Figure 5B, compared with *Lat2*$^{+/+}$ T cells, *Lat2*$^{-/-}$ T cells produced more IL-2, IL-10, TNFα, IL-12, and IFN-γ upon stimulation with anti-CD3 and anti-CD28, while they produced a similar or lower amount of cytokines when stimulated with PMA and ionomycin. In contrast, *Lat2*^{Tg} T cells produced very little of these cytokines, especially when they were stimulated with anti-CD3 and anti-CD28.

We further examined whether TCR-mediated proliferation of activated *Lat2*$^{-/-}$ T cells activated in vitro was also enhanced like *Lat2*$^{-/-}$ T cells from aged mice. Activated T cells from *Lat2*$^{+/+}$, *Lat2*$^{-/-}$, and *Lat2*^{Tg} mice were stimulated with different concentrations of anti-CD3 and 0.5 μg/ml anti-CD28. Proliferation of these T cells was assayed by [^{3}H]thymidine incorporation. As shown in Figure 5C, compared with *Lat2*$^{+/+}$ T cells, *Lat2*$^{-/-}$ T cells were hyperresponsive to anti-CD3 stimulation while *Lat2*^{Tg} T cells were hyporesponsive. Together, these

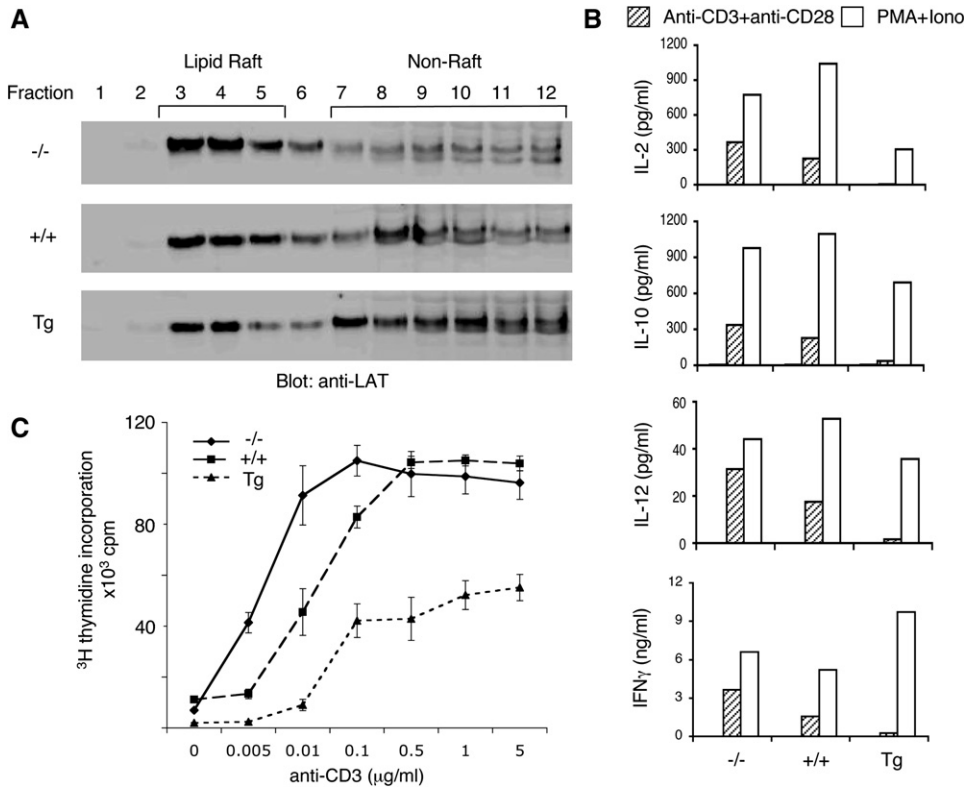

Figure 5. LAT Localization in Lipid Rafts, Cytokine Production, and T Cell Proliferation

(A) Localization of LAT in lipid rafts in activated CD8 T cells from *Lat2*$^{-/-}$, *Lat2*$^{+/+}$, and *Lat2*^{Tg} mice. Activated CD8$^+$ T cells were lysed in 1% Triton before ultracentrifugation on a sucrose gradient. 12 fractions were collected starting from the top of the sucrose gradient. Each fraction was resolved on SDS-PAGE and blotted with a LAT antibody.

(B) Cytokine production. T cells from *Lat2*$^{-/-}$, *Lat2*$^{+/+}$, and *Lat2*^{Tg} mice were activated as in Figure 4. CD8$^+$ T cells were purified, rested, and restimulated with anti-CD3 (5 μg/ml) and CD28 (0.5 μg/ml), PMA+ionomycin, or left untreated. Cytokines secreted into the tissue culture supernatants were quantitated with a multiplex cytokine kit.

(C) T cell proliferation. Activated CD8$^+$ T cells were stimulated with different concentrations of anti-CD3 antibodies in the presence of anti-CD28 (0.5 μg/ml) for 24 hr followed by a 6 hr pulse with [^{3}H]thymidine. Incorporation of [^{3}H]thymidine was measured with a liquid scintillation counter. The figure shown is representative of three independent experiments performed in triplicate. Bars indicate mean ± SD.

data indicated that LAB in T cells inhibited TCR-mediated signaling, cytokine production, and T cell proliferation.

Aggravation of the Autoimmune Disease in *Lat*^{Y136F} Mice by LAB Deficiency

Since our results suggested that LAB plays a negative role in regulating T cell activation, we further investigated the role of LAB in vivo via an autoimmune mouse model, the *Lat*^{Y136F} knockin mouse (Aguado et al., 2002; Sommers et al., 2002), to see whether deletion of the *Lat2* gene leads to aggravation or lessening of the disease. In the *Lat*^{Y136F} mice, a critical tyrosine of LAT (Y136) responsible for binding PLC-γ1 is mutated to phenylalanine. This mutation leads to development of a severe autoimmune disease with enormous expansion of CD4$^+$ T cells, splenomegaly, and production of autoantibodies. We crossed *Lat2*$^{-/-}$ mice with the *Lat*^{Y136F} mice to generate *Lat2*$^{-/-}$*Lat*$^{m/m}$ mice (m = Y136F). 11-week-old *Lat2*$^{-/-}$*Lat*$^{m/m}$ mice, together with age-matched *Lat2*$^{+/+}$, *Lat2*$^{-/-}$, and *Lat*$^{m/m}$ mice, were examined. *Lat2*$^{+/+}$ and *Lat2*$^{-/-}$ mice had similar sizes of spleen and lymph nodes at this age. *Lat*$^{m/m}$ mice had enlarged spleen and lymph nodes, as previously reported. Compared with these mice, *Lat2*$^{-/-}$*Lat*$^{m/m}$ mice had much bigger spleens and lymph nodes (data not shown). Total numbers of splenocytes from these mice are: *Lat2*$^{-/-}$*Lat*$^{m/m}$, (3.6 ± 0.2) × 10^8; *Lat*$^{m/m}$, (2.0 ± 0.2) × 10^8; *Lat2*$^{-/-}$, (2.9 ± 0.1) × 10^7; *Lat2*$^{+/+}$, (2.7 ± 0.2) × 10^7. FACS analysis of splenocytes showed that ~44% splenocytes were CD4$^+$ in *Lat*$^{m/m}$ mice resulting from uncontrolled expansion of CD4$^+$ T cells. However, in *Lat2*$^{-/-}$*Lat*$^{m/m}$ mice, there was even more expansion of CD4$^+$ T cells and ~68% of splenocytes were CD4$^+$ (Figure 6A). In addition, *Lat2*$^{-/-}$*Lat*$^{m/m}$ mice had high titers of dsDNA antibodies in their sera starting at as early as 6 weeks old, while *Lat*$^{m/m}$ mice started to have dsDNA antibodies at 10 weeks old. The autoantibody titers in *Lat2*$^{-/-}$*Lat*$^{m/m}$ mice were also higher than in *Lat*$^{m/m}$ mice (Figure 6B). Although there were differences in autoantibody production, IgE and IgG1 concentrations were elevated similarly in *Lat2*$^{-/-}$*Lat*$^{m/m}$ and *Lat*$^{m/m}$ mice (data not shown) as previously reported (Wang et al., 2005), suggesting that there is a dissociation between hypergammaglobulinemia and the presence of autoantibodies. Our data indicated that the autoimmune phenotype in *Lat*$^{m/m}$ mice was aggravated by LAB deficiency and suggested a negative role of LAB in T cells.

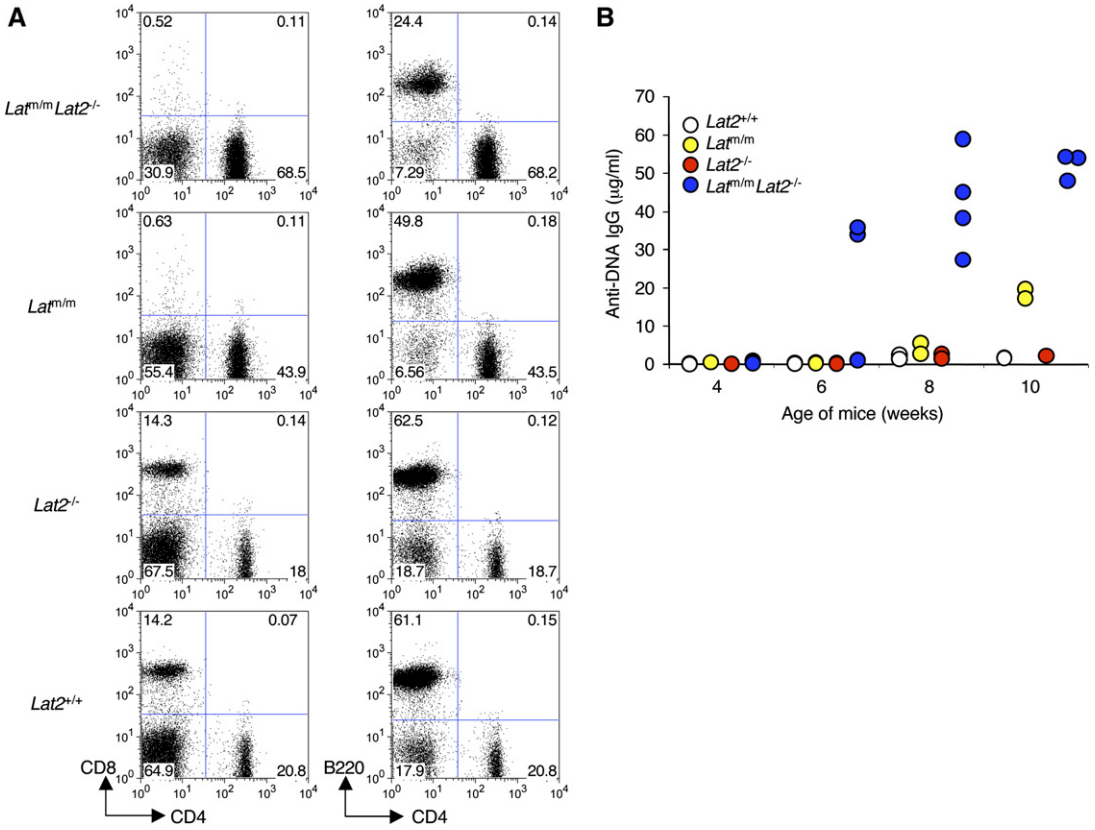

Figure 6. Aggravation of the Autoimmune Disease in the *Lat*^{Y136F} Mice by LAB Deficiency

(A) FACS analysis of CD4, CD8, and B220 on splenocytes from 11-week-old *Lat2*$^{-/-}$*Lat*$^{m/m}$ (m = Y136F), *Lat*$^{m/m}$, *Lat2*$^{-/-}$, and *Lat2*$^{+/+}$ mice. Numbers indicate the percentages of cells in quadrants. Data shown are from one of three independent experiments.

(B) The concentrations of anti-dsDNA IgG in *Lat2*$^{-/-}$*Lat*$^{m/m}$, *Lat*$^{m/m}$, *Lat2*$^{-/-}$, and *Lat2*$^{+/+}$ mice at 4, 6, 8, and 10 weeks old. Each circle represents the concentration of anti-dsDNA IgG from each mouse measured by ELISA.

Dysfunctional T Cells Caused the Autoimmunity in *Lat2*$^{-/-}$ Mice

LAB is expressed in B cells, mast cells, NK cells, and activated T cells as demonstrated here. To determine a specific role of LAB in each cell type, we generated conditional knockout mice in which the *Lat2* gene could be deleted upon expression of the Cre recombinase. We used two different recombinase systems to remove the neomycin-resistant gene and two critical exons. These two exons to be deleted encode the transmembrane domain and the palmitoylation sites. In the targeting construct, two FLP recombinase recognition sequences (FRT) flanked the PGK-Neo DNA fragment. One LoxP site was placed at the 5′ end of FRT and the other one was placed at the 3′ end of the exons to be deleted. This construct was used to transfect ES (embryonic stem) cells. Chimeric mice were produced with positive ES cells and were crossed with transgenic mice expressing FLP to remove the *Neo* gene. After deletion of the PGK-Neo fragment, these mice were then crossed with LckCre transgenic mice to specifically delete the *Lat2* gene in T cells. As shown in Figure 7A, cells from LckCre^{+}*Lat2*$^{f/f}$ mice expressed a similar amount of LAB protein compared with B cells from *Lat2*$^{+/+}$ mice. To determine whether the *Lat2* gene was deleted in T cells from LckCre^{+}*Lat2*$^{f/f}$ mice, we activated T cells from *Lat2*$^{+/+}$, LckCre^{+}*Lat2*$^{f/f}$, and *Lat2*$^{-/-}$ mice with anti-CD3 and expanded these cells in IL-2 medium. Activated T cells were purified and lysed. Lysates were subjected to anti-LAB immunoprecipitation. As shown in Figure 7A, LAB protein was absent in activated T cells from LckCre^{+}*Lat2*$^{f/f}$ and *Lat2*$^{-/-}$ mice while it was expressed in T cells from *Lat2*$^{+/+}$ mice. These data indicated that the *Lat2* gene was successfully deleted in T cells from LckCre^{+}*Lat2*$^{f/f}$ mice.

We next examined whether LckCre^{+}*Lat2*$^{f/f}$ mice also developed an autoimmune syndrome like *Lat2*$^{-/-}$ mice. Indeed, 6-month-old LckCre^{+}*Lat2*$^{f/f}$ mice had splenomegaly compared with the age-matched *Lat2*$^{+/+}$ mice (data not shown). Analysis of activation markers of T cells, CD25, CD69, CD44, CD62L, and CD45RB showed that there were more activated CD4^{+} T cells in LckCre^{+}*Lat2*$^{f/f}$ mice like in *Lat2*$^{-/-}$ mice (Figure 7B). However, different from *Lat2*$^{-/-}$ mice, CD8^{+} T cells in LckCre^{+}*Lat2*$^{f/f}$ mice had a similar expression of these markers (data not shown). Similar to T cells in aged *Lat2*$^{-/-}$ mice, T cells from aged LckCre^{+}*Lat2*$^{f/f}$ mice produced more IL-2, IL-10, and IFN-γ compared with those from *Lat2*$^{+/+}$ mice (Figure 7C). In addition, LckCre^{+}*Lat2*$^{f/f}$ mice also had high concentrations of dsDNA antibodies (Figure 7D). These data suggested that abnormal T cells cause autoimmunity in *Lat2*$^{-/-}$ mice.

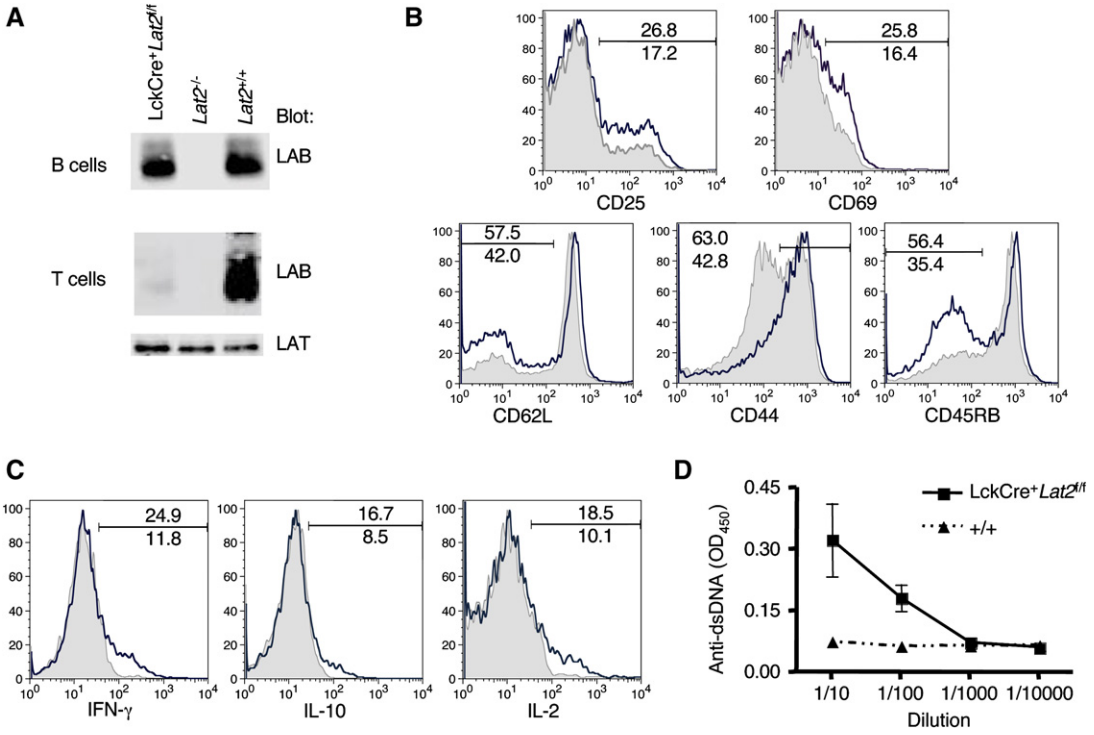

Figure 7. Specific Deletion of LAB in T Cells

(A) Deletion of *Lat2* in T cells. Splenocytes from LckCre^{+}*Lat2*$^{f/f}$, *Lat2*$^{-/-}$, and *Lat2*$^{+/+}$ mice were activated via CD3 and expanded in the medium with IL-2. Cells were lysed and subjected to anti-LAB immunoprecipitation followed by an anti-LAB immunoblot. T cell lysates were also blotted with anti-LAT. Data shown are from one of three independent experiments.

(B) Splenocytes from 6-month-old LckCre^{+}*Lat2*$^{f/f}$ and *Lat2*$^{+/+}$ mice were stained with anti-CD4, CD8, and different T cell activation markers as indicated in the figure. The shadowed histogram represents CD4^{+} T cells from *Lat2*$^{+/+}$ mice. The dark-lined histogram represents CD4^{+} T cells from LckCre^{+}*Lat2*$^{f/f}$ mice. The numbers indicate the percentages of activated cells from LckCre^{+}*Lat2*$^{f/f}$ (top) and *Lat2*$^{+/+}$ (bottom) mice. The figure shown is representative of five mice for each genotype.

(C) Cytokine production in CD4^{+} T cells from 6-month-old LckCre^{+}*Lat2*$^{f/f}$ and *Lat2*$^{+/+}$ mice. The shadowed histogram represents *Lat2*$^{+/+}$ T cells, and the dark-lined histogram represents LckCre^{+}*Lat2*$^{f/f}$ T cells. The numbers indicate the percentages of cytokine-producing cells for LckCre^{+}*Lat2*$^{f/f}$ (top) and *Lat2*$^{+/+}$ (bottom) mice. The figure shown is representative of three experiments.

(D) The presence of dsDNA antibodies in the sera from LckCre^{+}*Lat2*$^{f/f}$ mice. Sera from 6-month-old LckCre^{+}*Lat2*$^{f/f}$ and *Lat2*$^{+/+}$ mice were diluted and used in ELISA for detection of dsDNA antibody. Data show mean of OD_{450} in each dilution of sera from five different mice in each group. Bars indicate mean ± SD.

Discussion

In this report, we showed that a transmembrane adaptor protein, LAB, could negatively regulate T cell activation, likely by antagonizing LAT function. Previous studies show that LAB is not expressed in naive T cells. LAB deficiency was therefore not expected to have an effect on T cell function. However, in aged *Lat2*$^{-/-}$ mice, T cells were hyperactivated and produced more cytokines, leading to development of a spontaneous autoimmune syndrome. The autoimmunity in these mice is most likely a consequence of abnormal T cell function, as indicated by the fact that the conditional knockout mice with the *Lat2* gene deleted in T cells showed similar phenotypes to those of *Lat2*$^{-/-}$ mice. Although our data suggested that deficiency of LAB in T cells caused the autoimmune syndrome in *Lat2*$^{-/-}$ mice, we could not totally exclude the possibility that deficiency of LAB in B cells or other cell types also contribute to the autoimmunity in *Lat2*$^{-/-}$ mice. LAB was more abundant in B cells (Figures 3B and 3D) or macrophages (data not shown) than in activated T cells. In the absence of LAB, B cells or macrophages could also be functionally abnormal. This possibility will be tested in the future via LAB conditional knockouts in each of these cell types.

Analysis of splenocytes from aged LAB-deficient mice showed that splenomegaly was not due to just expansion of T cells. The percentages of T and B cells were similar to those in age-matched wild-type mice, indicating that both T and B cell populations were expanded proportionally in *Lat2*$^{-/-}$ mice. It is possible that cytokines produced by hyperactivated T cells could promote expansion of B cells. *Lat2*$^{-/-}$ B cells could also undergo faster expansion than *Lat2*$^{+/+}$ B cells without any influence from *Lat2*$^{-/-}$ T cells.

LAB is not expressed in naive T cells. Why did LAB deficiency cause abnormal T cell function? Our data indicated that T cell activation induced upregulation of LAB, which might function to turn off T cell activation. Our in vitro data indicated that in activated T cells without LAB protein, TCR-mediated signaling events, such as LAT phosphorylation, Akt activation, and calcium mobilization, were enhanced. In contrast, overexpression of LAB in T cells suppressed TCR-mediated signaling. A negative role for LAB in T cells was supported by our in vivo experiments. LAB deficiency aggravated the

development of the autoimmune disease and enhanced expansion of $CD4^+$ T cells in the Lat^{Y136F} mice. In addition, we have also examined NP-specific antibody response in LAB-deficient mice. While there were no differences in T-independent antibody response or T-dependent primary antibody response between $Lat2^{-/-}$ and $Lat2^{+/+}$ mice as previously reported (Wang et al., 2005), T-dependent secondary response was substantially enhanced in $Lat2^{-/-}$ mice (data not shown). Furthermore, T-dependent, NP-specific secondary antibody response was also enhanced in $LckCre^+Lat2^{f/f}$ mice (data not shown). These data further supported our conclusion that LAB negatively regulates T cell function.

TCR-mediated signaling was enhanced in $Lat2^{-/-}$ T cells. In the absence of LAB, more LAT moved to lipid rafts, leading to enhanced LAT phosphorylation and calcium mobilization. How LAB deficiency affects LAT localization to lipid rafts is not clear. While the importance of lipid rafts in TCR-mediated signaling is still a topic of debate, we believe that more LAT localized to lipid rafts might also be a reflection of changes in LAT palmitoylation status, because only palmitoylated LAT could localize to rafts. It is likely that LAT and LAB might be palmitoylated by the same enzyme(s). When LAB is missing, more LAT gets palmitoylated. We have attempted to examine the palmitoylation status of LAT in activated WT and $Lat2^{-/-}$ T cells by metabolic labeling with $[^3H]$palmitate, and we did not observe substantial differences in LAT palmitoylation. It is possible that in vitro labeling with $[^3H]$palmitate for 3 hr is not sensitive enough to detect the differences in palmitoylation. Because LAB tyrosine phosphorylation was much weaker than LAT phosphorylation upon engagement of the TCR in activated T cells, it is less likely that LAB competes for binding proteins that LAT normally binds, such as Grb2 and Gads. While competing for localization to lipid rafts or palmitoylation could be the mechanism by which LAB negatively regulates T cell activation, it is also possible that LAB might be actively involved in regulation of LAT function, for example by recruiting proteins to lipid rafts to target LAT for degradation or phosphatases to dephosphorylate LAT.

The immune system uses peripheral tolerance to prevent self-reactive lymphocytes from attacking host tissues. Several mechanisms that contribute to peripheral tolerance include activation-induced cell death, suppression by regulatory T cells, and T cell anergy (Heissmeyer and Rao, 2004). Anergy induction leads to upregulation of several E3 ubiquitin ligases (Heissmeyer et al., 2004) or reduced LAT palmitoylation (Hundt et al., 2006). Our data indicated that the autoimmune syndrome in $Lat2^{-/-}$ mice was not due to reduced activation-induced cell death or abnormal Treg cell development and function. In addition, negative selection was also normal in male $Lat2^{-/-}$ HY transgenic mice (data not shown). In $Lat2^{-/-}$ T cells, more LAT was present in lipid rafts, and TCR-mediated signaling was enhanced. Because LAT-mediated signaling is decreased in anergic T cells, it is possible that LAB deficiency enhances LAT function, leading to a reduction in T cell anergy.

Both $Lat2^{-/-}$ and the transgenic mice overexpressing LAB in $Lat^{-/-}$ T cells develop a spontaneous autoimmune syndrome (Janssen et al., 2004). Although this seems puzzling, the mechanisms for causing the syndrome are likely different in these mice. The phenotype of transgenic mice overexpressing LAB were very similar to that of the Lat^{Y136F} mice (Aguado et al., 2002; Sommers et al., 2002). The autoimmune conditions in Lat^{Y136F} mice and the LAB transgenic mice are more severe than in $Lat2^{-/-}$ mice. Studies with the Lat^{Y136F} mice show that negative selection and development of Treg cells are affected in the absence of the LAT-PLCγ1 interaction (Koonpaew et al., 2006; Sommers et al., 2005). However, in $Lat2^{-/-}$ mice, negative selection and development of Treg cells were normal likely due to the presence of WT LAT. This is expected because LAB expression is very low or absent in thymocytes and naive T cells. Since TCR signaling was enhanced in activated $Lat2^{-/-}$ T cells, LAB deficiency might cause prolonged T cell activation and gradual accumulation of autoreactive T cells in mice, leading to development of autoimmunity. In conclusion, we provide evidence that LAB deficiency leads to enhanced TCR signaling and T cell activation and development of autoimmunity. Our findings here, together with previously published studies, demonstrate a critical role for transmembrane adaptor proteins in integrating and fine-tuning signaling downstream of the TCR.

Experimental Procedures

Mice

$Lat2^{-/-}$ and $Lat2^{Tg}$ mice have been described previously (Janssen et al., 2004; Zhu et al., 2004). $Lat2^{-/-}$ mice have been crossed onto C57Bl/6 background for at least 10 generations. The Lat^{Y136F} mice ($Lat^{m/m}$) were kindly provided by L. Samelson. The *Lat2* conditional knockout construct was made with a 2835 bp XbaI and BamHI fragment from the *Lat2* BAC clone as the short arm, a 978 bp BamHI and EcoRV fragment as the fragment to be deleted upon expression of the Cre recombinase, and a 6943 bp BamHI and XbaI fragment as the long arm. After transfection of ES cells with the targeting construct, positive clones were identified by PCR. Positive ES cell clones were injected into blastocysts to generate chimeric mice. All mice were used in accordance with the National Institutes of Health guidelines. The experiments described in this study were reviewed and approved by the Duke University Institutional Animal Care Committee. Mice were housed in specific pathogen-free conditions.

LAB Expression by Real-Time PCR and Immunoblot

Total RNAs from cells sorted by FACS or purified by MACS were extracted with the Trizol reagent. cDNAs were synthesized with SuperScript reverse transcriptase (Invitrogen) with oligo-dT as a primer. Quantitation of the LAB RNA was performed by real-time PCR with SYBR Green Supermix (Bio-Rad). The following primers were used to amplify LAB: 5′-CCCTCACCCTCAGCCTTA-3′ and 5′-AGCAGCA ATAATCCCGACA-3′. Real-time PCR was performed in triplicates. The amount of LAB RNA was normalized relative to the β-actin RNA. For detection of the LAB protein, cells were lysed in RIPA lysis buffer. Postnuclear lysates were immunoprecipitated with rabbit anti-LAB sera. For immunoblotting, samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. After incubation with primary antibodies, membranes were probed with either goat anti-mouse or anti-rabbit Ig conjugated with Alexa Fluor 680 (Molecular Probes) or IRDye 800 (Rockland). Membranes were then visualized and quantified with an infrared fluorescence imaging system (LI-COR Bioscience).

FACS Analysis and Detection of Autoantibodies

Single-cell suspensions were prepared from mouse spleens and were stained with the following antibodies: FITC-conjugated anti-CD4, PE-Cy5-conjugated anti-CD8 and CD44, and phycoerythrin (PE)-conjugated anti-CD25, CD69, CD62L, CD45RB, and B220

(eBioscience). For intracellular staining of cytokines, splenocytes were stimulated with 40 ng/ml of PMA and 500 ng/ml of ionomycin for 1 hr. After addition of Golgi-Stop, cells were allowed to be stimulated for additional 3–4 hr. The antibodies used for staining of intracellular cytokines were the following: FITC-anti-IFN-γ, IL-10, and PE-anti-IL-2 (eBioscience). Intracellular staining of Foxp3 and detection of autoantibodies were done as we did previously (Koonpaew et al., 2006). For CFSE labeling, purified T cells were incubated with 5 μM CFSE in 5% FBS in PBS for 5 min at 37°C and were then stimulated with different concentrations of anti-CD3 in the presence of 0.5 μg/ml of anti-CD28 for 2 days before FACS analysis.

Proliferation and Cytokine Production of Activated T Cells

To prepare activated T cells, splenocytes were cultured in CD3 antibody (2C11)-coated plates in the presence of murine IL-2 (10 ng/ml) for 2 days and then were moved into new plates to expand for 3 more days in the presence of IL-2. Activated $CD4^+$ or $CD8^+$ cells were purified by positive selection with MACS beads (Miltenyi Biotec).

For assay of T cell proliferation, purified $CD4^+$ and $CD8^+$ T cells (10^5 cells/well in 96-well plates) or purified activated $CD8^+$ T cells (2×10^4 cells/well in 96-well plates) were incubated either with medium alone, with anti-CD3 (0.05–5 μg/ml) and anti-CD28 (0.5 μg/ml), or with PMA (40 ng/ml) plus ionomycin (500 ng/ml) for 24 hr. Cells were stimulated in triplicates followed by a 6 hr pulse with 1 μCi [^{3}H]thymidine per well. Incorporation of [^{3}H]thymidine was measured with a liquid scintillation luminescence counter (Perkin Elmer). For cytokine production, purified activated $CD8^+$ T cells were rested for 6 hr before stimulation with anti-CD3 (5 μg/ml) and anti-CD28 (0.5 μg/ml), or PMA and ionomycin for 4 hr. The concentrations of cytokines secreted into the culture supernatants were determined with the Bio-Plex mouse cytokine Th1/Th2 kit according to the manufacturer's protocol (Bio-Rad).

MAPK Activation and Calcium Mobilization

Purified activated T cells were rested in medium without IL-2 for 6 hr before being stimulated with anti-CD3 (5 μg/ml) and anti-CD4 (1 μg/ml) or with anti-CD3 (5 μg/ml) and anti-CD8 (1 μg/ml) for 0, 1.5, and 5 min. Cells were lysed with RIPA buffer. Protein samples were resolved on the SDS-PAGE and blotted with anti-phospho-PLC-γ1, PLC-γ1, phospho-p44/42 Erk, Erk2, phospho-Akt (Ser473), pan-Akt (Cell Signaling), and other antibodies as indicated in each figure.

For calcium mobilization in activated T cells, activated splenocytes were first loaded with indo-1 in Ca^{2+} loading buffer (1× HBSS with 10 mM HEPES and 1% FBS) for 30 min and then stained with PE-CD4 or PE-CD8. Ca^{2+} flux was assayed by monitoring the fluorescence emission ratio at 405/495 nm with FACS. Calcium flux was initiated by addition of anti-CD3-biotin (5 μg/ml) and anti-CD4-biotin (1 μg/ml) or anti-CD8 biotin (1 μg/ml) followed by cross-linking with streptavidin (25 μg/ml).

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