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Negative Regulation of Lymphocyte Activation by the Adaptor Protein LAX

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The membrane-associated adaptor protein LAX is a linker for activation of T cells (LAT)-like molecule that is expressed in lymphoid tissues. Upon stimulation of T or B cells, it is phosphorylated and interacts with Grb2 and the p85 subunit of PI3K. LAX, however, is not capable of replacing LAT in the TCR signaling pathway. In this study we report that upon T or B cell activation, the LAX protein was up-regulated dramatically. Although disruption of the LAX gene by homologous recombination had no major impact on lymphocyte development, it caused a significant reduction in CD23 expression on mature B cells. Interestingly, naïve LAX−/− mice had spontaneous germinal center formation. Compared with normal T and B cells, LAX−/− T and B cells were hyperresponsive and had enhanced calcium flux, protein tyrosine phosphorylation, MAPK and Akt activation, and cell survival upon engagement of the T or B AgRs. Our data demonstrate that LAX functions as a negative regulator in lymphocyte signaling. The Journal of Immunology, 2005, 174: 5612–5619.

Adaptor proteins play important roles in lymphocyte signaling (1–3). One such protein is the transmembrane adaptor protein, linker for activation of T cells (LAT)4 (4, 5). LAT is palmitoylated and localized to lipid rafts (6). Upon T cell activation, it is phosphorylated by the ZAP-70 tyrosine kinase and interacts with Grb2, Gads, and phospholipase Cγ1 (4, 7, 8). Published studies have clearly demonstrated that LAT is essential for T cell activation, thymocyte development, and homeostasis (9–12).

Despite the importance of LAT in T cells, LAT is only present in T cells, NK cells, mast cells, and platelets (4, 13). Recently, LAT was also found in pre-B cells (14). We along with others attempted to identify whether LAT-like molecules exist in B cells and other cell types; two LAT-like molecules, non-T cell activation linker (NTAL)/linker for activation of B cells (LAB) and LAX, were identified (15–17). Non-T cell activation linker/LAB, which is expressed in B cells, NK cells, monocytes, and mast cells, is localized to lipid rafts like LAT and is tyrosine phosphorylated upon BCR and FeR stimulation (15, 16). LAX is expressed in T cells, B cells, and monocytes; it has multiple Grb2 motifs very similar to those in LAT. In contrast to LAT and LAB, LAX is not localized to lipid rafts (17). Upon stimulation of Jurkat T cells via the TCR, LAX is phosphorylated and interacts with Grb2, Gads, and the p85 subunit of PI3K. It is also phosphorylated upon BCR engagement. Although LAX contains multiple Grb2-binding motifs, it is not capable of correcting defective signaling pathways in LAT-deficient cells, indicating that LAX functions differently from LAT. Overexpression of LAX in Jurkat cells inhibits TCR-mediated p38 MAPK and NFAT/AP-1 activation. Therefore, in contrast to LAT, LAX probably functions to negatively regulate TCR signaling pathway.

To study LAX function in vivo, we generated LAX-deficient mice by homologous recombination. LAX deficiency had no major impact on lymphocyte development. T and B cells were hyperresponsive upon AgR stimulation. Signaling from the AgRs was enhanced in LAX−/− cells. Thus, our results indicate that LAX functions as a negative regulator.

Materials and Methods

Generation of LAX−/− mice by gene targeting

The targeting construct used for disruption of the LAX gene is shown in Fig. 1A. After linearization with NotI, this construct was used to transfet embryonic stem (ES) cells. ES transfectants were selected in the presence of G418 and gancyclovir. Of the 230 G418-resistant clones, five clones underwent homologous recombination. Two ES clones were injected into blastocysts of C57BL/6 mice. LAX−/− mice were obtained by interbreeding LAX+/− mice. Southern blot analysis of genomic DNA from ES cells was performed using hybridization solution according to the manufacturer’s protocol (BD Clontech). PCR genotyping was performed using three primers (5′-GACATCGGACTCCAGAGCGATGG-3′, 5′-GGTGCGCTGAGTCACAGAGTATGG-3′, and 5′-TGCGACGCGCATCGCCTTCTA TCG-3′).

Antibodies

Anti-human LAX rabbit sera and mAbs were generated as described previously (17). Polyclonal Abs against mouse LAX were purified from anti-human LAX sera by affinity purification using a GST-LAX (mouse) column. Anti-mouse LAX hybridomas were made by fusion of splenocytes from mice immunized with GST-LAX (mouse) with NSO cells. Anti-phospho-Tyr antibodies include 4G10, PY20 (BD Pharmingen), and 70-kDa heat shock protein; MKK3, MAPK kinase 3; NP-CGG, nitrophenylacetyl chicken γ-globulin; LAB, linker for activation of B cells.

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2 Address correspondence and reprint requests to Dr. Weiguo Zhang, Department of Immunology, Jones Building, Room 112, Box 3010, Duke University Medical Center, Durham, NC 27710. E-mail address: zhang033@mc.duke.edu
3 Abbreviations used in this paper: LAT, linker for activation of T cells; 7AAD, 7-aminoactinomycin D; ES, embryonic stem; GC, germinal center; Hsp70, 70-kDa heat shock protein; MKK3, MAPK kinase 3; NP-CGG, nitrophenylacetyl chicken γ-globulin; LAB, linker for activation of B cells.
Up-regulation of LAX expression

Jurkat cells (5 x 10^5 cells) were stimulated for 0, 3, 6, and 15 h in six-well plates coated with anti-CD3ε Ab (OKT3 ascites, 1/500 dilution) before lysis. Mouse splenocytes or thymocytes (2 x 10^6 cells) were stimulated in plates coated with anti-CD3ε (2C11; 5 μg/ml), or stimulated with 20 μg/ml anti-mouse IgM F(ab’2) (ICN/Cappel Laboratories) for 0, 3, 6, and 15 h before lysis. Cells were lysed in lysis buffer (1% Triton, 25 mM Tris-Cl, 150 mM NaCl, and 5 mM EDTA), and postnuclear lysates were immunoprecipitated with anti-LAX Abs. Protein samples were resolved on SDS-PAGE and analyzed by Western blotting with anti-LAX Abs. To test whether up-regulation of the LAX protein requires de novo synthesis, Jurkat cells were cultured in the presence of actinomycin D or cycloheximide at 5 μg/ml starting from 30 min before stimulation. To examine whether the LAX transcript is up-regulated after activation, T and B cells from spleens were stimulated with anti-CD3 or anti-IgM, respectively, for 1 and 3 h. Total RNAs were then extracted from these cells and used in RT-PCR with LAX-specific primers (5’-TCTCATCGATGGTGGGTGCTA-3’ and 5’-CCATGATTATGTCAATATCC-3’) and actin-specific primers (5’-ACTCTTATGTGGGTGCTA-3’ and 5’-CAGG TCCAGAGCCAGGATGCC-3’).

Flow cytometry

Single cell suspensions were prepared from mouse thymus, spleen, lymph node, and bone marrow and were stained with the following Abs: FITC-conjugated anti-CD4, CD21, IgM, and IgD; PE-conjugated anti-CD3ε, CD23, Fas, and Fas ligand; and allophycocyanin-conjugated anti-CD8, B220, and IgM (BD Pharmingen). For detection of apoptosis, activated CD4+ T cells or B cells were stained with annexin V and propidium iodide. Single cell suspensions were stained with PE-conjugated anti-IgM, CD21, IgM, and IgD; FITC-conjugated anti-CD28 (1 μg/ml) and anti-mouse IgM F(ab’2) (20 μg/ml) for 0, 2, 5, 10, 30, and 60 min before lysis. Protein samples were separated by SDS-PAGE and transferred to nitrocellulose. After incubation with primary Abs, nitrocellulose membranes were washed three times and probed with either anti-mouse or rabbit IgG-conjugated to Alexa Fluor 680 (Molecular Probes) or IRDye800 (Rockland). Membranes were then visualized with the Li-Cor Bioscience Odyssey system.

For proliferation, purified CD4+ T cells (4 x 10^5 cells/well in a 96-well plate) were incubated in medium alone, anti-CD3 (0.5 or 5 μg/ml) and anti-CD28 (1 μg/ml), or PMA (40 ng/ml) plus ionomycin (500 ng/ml) for 48 h. Purified B cells were incubated in medium alone, anti-mouse IgM F(ab’2) (10, 20, and 40 μg/ml), or PMA (40 ng/ml) plus ionomycin (500 ng/ml) for 48 h. Cells were stimulated in triplicate, followed by a 6- to 8-h pulse with 1 μCi/well [3H]thymidine. Incorporation of [3H]thymidine was measured using a liquid scintillation luminescence counter (PerkinElmer). For IL-2 production, CD4+ T cells (1 x 10^5 cells/well in a 96-well plate) were incubated in medium alone, anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml), or PMA (40 ng/ml) plus ionomycin (500 ng/ml) for 24 h. IL-2 in the culture supernatant was assayed in triplicate by ELISA using an OptEIA mouse IL-2 kit (eBiosciences).

Ca^{2+} flux

Splenocytes from LAX−/− and LAX+/+ mice were first loaded with indo-1 in Ca^{2+} loading buffer (1X HBSS with 10 mM HEPES and 1% FBS) for 30 min, then stained with PE-Cy4 or PE-Cy5. Flow cytometry was used to measure Ca^{2+} flux by monitoring the fluorescence emission ratio at 405/495 nm. T cells were stimulated by addition of anti-CD3-biotin (5
Results
Up-regulation of LAX protein upon lymphocyte activation
Our previous results showed that LAX mRNA is expressed predominantly in spleen and thymus. To detect the LAX protein in thymocytes and splenocytes from C57BL/6 mice, we lysed these cells in 1% Brij lysis buffer and immunoprecipitated LAX with anti-LAX mAbs, followed by an anti-LAX Western blot. Surprisingly, LAX protein in these cells was difficult to detect. We speculated that LAX protein might be up-regulated upon T cell activation.

To probe this possibility, we first examined LAX protein expression using Jurkat cells. Jurkat cells were activated with anti-CD3ε (OKT3) for 0, 3, 6, and 15 h before immunoprecipitation of LAX. As shown in Fig. 1A, LAX was expressed in unstimulated Jurkat cells as we previously described (17); however, the amounts of LAX protein immunoprecipitated were increased dramatically 6 h after stimulation. Thus, LAX protein is up-regulated upon TCR engagement.

We next determined whether up-regulation of LAX protein requires de novo transcription or protein synthesis. We immunoprecipitated LAX from Jurkat cells activated with OKT3 in the presence of actinomycin D, an inhibitor of transcription, or cycloheximide, an inhibitor of protein synthesis. As shown in Fig. 1B, up-regulation of LAX protein was suppressed by addition of either of these two inhibitors, suggesting that activation of Jurkat cells probably induces transcriptional activation of the LAX gene and synthesis of LAX protein.

We also examined whether LAX is up-regulated upon activation of murine splenocytes and thymocytes. Similar to the results from Jurkat cells, LAX protein in splenocytes or thymocytes was increased after stimulation with anti-CD3ε (2C11) for 3 h, whereas LAT remained unchanged (Fig. 1C). We also examined whether LAX protein is up-regulated upon stimulation of splenocytes with anti-IgM F(ab)2. Indeed, cross-linking via the BCR up-regulated LAX protein (Fig. 1D). We determined whether LAX RNA is up-regulated after activation of T and B cells. RNAs were extracted from cells activated for 0, 1, and 3 h; reverse transcribed; and then amplified by PCR using LAX- and actin-specific primers. Amplification of the actin transcript indicated that similar amounts of cDNAs were used. As suggested in Fig. 1E, the LAX RNA level was increased after stimulation. These data indicate that LAX is up-regulated upon AgR stimulation through de novo transcription and protein synthesis.

Generation of LAX-deficient mice
To study the in vivo function of LAX in lymphocyte development and signaling, we disrupted the LAX gene in mouse ES cells by homologous recombination using the standard gene-targeting approach. Bacterial artificial chromosome clones were screened using LAX cDNA. Sequencing of the LAX gene revealed that it contains five exons (Fig. 2A). Exon 2 contains the transmembrane domain. Exon 5 is the largest exon, which contains most of the tyrosine motifs in the cytoplasmic domain of LAX. The targeting vector was constructed by substituting three exons (exons 2, 3, and 4) with a Neoβ-containing cassette. After transfection with the targeting construct, ES cells were selected with G418 and screened by PCR. Positive ES clones were analyzed by Southern blot analysis using a probe, as indicated in Fig. 2A. The sizes of the DNA fragments of the targeted allele and WT allele were as predicted (Fig. 2B).

Targeted ES cells were injected into blastocysts to produce chimeric mice. Crossing these chimeras with C57BL/6 mice produced heterozygous mice (LAX+/−). Heterozygous mice (LAX+/−) were inbred to obtain homozygous mice for the disrupted LAX gene (LAX−/−). The targeted LAX allele was successfully transmitted from the chimeric mice to their offspring, as detected by PCR (Fig. 2C). LAX−/− mice were born at the expected Mendelian frequency and remained viable and apparently healthy. LAX−/− and LAX+/− mice were indistinguishable in appearance and size.

To confirm that LAX protein is absent in LAX−/− mice, we immunoprecipitated LAX from resting or anti-CD3ε stimulated splenocytes. As shown in Fig. 2D, LAX protein was detected in stimulated LAX+/− splenocytes, but it was absent in LAX−/− splenocytes, indicating that the LAX gene is successfully disrupted in LAX−/− mice.

Lymphocyte development in LAX−/− mice
Examination of LAX−/− mice showed that their thymuses, spleens, and lymph nodes were of normal size. We assessed T cell development by examining CD4 and CD8 expression in the thymus and peripheral lymphoid organs. As shown in Fig. 3A, there were no major alterations in T cell populations from the thymus, spleen, or lymph nodes (not shown). The percentages of CD4+CD8− or CD4− and CD8+ cells were similar in LAX−/− and LAX+/− mice. Analysis of bone marrow cells and splenocytes showed that LAX−/− mice had a normal development of B cells based on the expression of B220, IgM, and CD43 (Fig. 3A and data not shown).

Additional analysis showed that LAX−/− B cells expressed normal levels of IgM and IgD (Fig. 3B). The percentages of...
IgM<sup>high</sup>IgD<sup>low</sup> (T1 B cells), IgM<sup>low</sup>IgD<sup>high</sup> (T2 B cells), and IgM<sup>low</sup>IgD<sup>high</sup> (mature B cells) subsets were similar in LAX<sup>−/−</sup> mice, indicating that development of these B cell subsets is normal in LAX<sup>−/−</sup> mice. In addition, there was a decrease in the percentage of CD23<sup>−/−</sup> B cells compared with that in LAX<sup>+/+</sup> B cells. Staining splenocytes with PE-labeled anti-CD23 showed that CD23<sup>−</sup> B cells in LAX<sup>−/−</sup> mice had a mean fluorescence intensity of 203.5. In addition, there was an increase in the percentage of CD23<sup>+</sup> IgM<sup>−/−</sup> cells in spleens from LAX<sup>−/−</sup> mice, a likely consequence of CD23 down-regulation. Because splenic B cells in LAX<sup>−/−</sup> and LAX<sup>+/+</sup> mice had no significant differences in IgM and IgD expression, two markers that are used to define different B cell subsets, it is likely that LAX deficiency only affects CD23 expression in B cells, rather than the development of different B cell subsets.

We next examined Ab production in naive and immunized LAX<sup>−/−</sup> and LAX<sup>+/+</sup> mice. Levels of different Ig isotypes in the sera of naive mice were determined by ELISA. Although there were apparent differences in serum IgG3 and IgA concentrations, they were not statistically significant by t-test. There were no significant differences in levels of IgM and IgG2b; however, IgG1 and IgG2a levels were higher in LAX<sup>−/−</sup> mice (~2- to 3-fold). Most noticeably, LAX<sup>−/−</sup> mice had approximately eight times more IgE than LAX<sup>+/+</sup> mice (Fig. 4A).

We also determined whether LAX deficiency affects T-dependent and -independent Ab responses. LAX<sup>−/−</sup> and LAX<sup>+/+</sup> mice were immunized i.p. with NP-CGG with alum. We did not detect any significant differences in NP-specific IgM and IgG production in these mice during the primary and secondary responses (Fig. 4B). T-independent responses were similar in LAX<sup>−/−</sup> and LAX<sup>+/+</sup> mice (data not shown). LAX<sup>−/−</sup> mice were capable of forming germinal centers (GC) like LAX<sup>+/+</sup> mice after immunization (Fig. 4C). Surprisingly, small GC were already present in naive LAX<sup>−/−</sup> mice, whereas no or very few GC were detected in naive LAX<sup>+/+</sup> mice (Fig. 4C). Consistent with this observation,

FIGURE 3. Lymphocyte development in LAX<sup>−/−</sup> mice. A, Single cell suspensions from thymus, spleen, and bone marrow of LAX<sup>−/−</sup> and LAX<sup>+/+</sup> mice were stained with FITC-CD4 and allophycocyanin-CD8 or FITC-IgM and allophycocyanin-B220 and analyzed by FACS. B, Expression of IgM, IgD, CD23, and CD21 on splenic B cells. Splenocytes from LAX<sup>−/−</sup> and LAX<sup>+/+</sup> mice were stained with FITC-IgD and PE-CD21, PE-CD23, and allophycocyanin-IgM and analyzed by FACS. The FACS plot shown is from one representative mouse. Analysis of five LAX<sup>−/−</sup> and five LAX<sup>+/+</sup> mice show no significant differences in the percentage of different B cell subsets.

FIGURE 4. Ab production and GC formation in LAX<sup>−/−</sup> mice. A, Basal levels of Ig isotypes in sera from LAX<sup>−/−</sup> mice (●) and LAX<sup>+/+</sup> (○) were analyzed by ELISA. The mean value of each isotype is shown at the top of the data group. *, p < 0.05; **, p < 0.001. B, T-dependent Ab response. LAX<sup>−/−</sup> and LAX<sup>+/+</sup> mice were immunized with NP-CGG and challenged on day 21 with the same Ag. Sera were collected on days 0, 7, 14, 21, 26, and 31. NP-specific IgM and IgG titers were determined by ELISA. C, Histological analysis of the spleens from naive and immunized LAX<sup>−/−</sup> and LAX<sup>+/+</sup> mice. GL7<sup>+</sup> and B220<sup>+</sup> cells are shown as blue and red, respectively.
FACS analysis showed that naive LAX−/− mice had more GL-7+ cells in spleens than naive LAX+/+ mice (data not shown). Even though naive LAX−/− mice have spontaneous germinal formation, they showed no signs of autoimmune diseases. Histological examination of kidneys from LAX−/− mice revealed no evidence of glomerulonephritis, and anti-dsDNA Ab titers in 6-mo-old LAX−/− mice were normal (data not shown).

**Hypersensitive T and B cells in LAX-deficient mice**

Our previous data suggest that LAX functions as a negative regulator when it is overexpressed in the Jurkat T cell line. If this is true in vivo, we would expect to see enhanced T cell proliferation upon stimulation via the TCR in the absence of LAX. We first examined CD3-mediated T cell proliferation by [3H]thymidine incorporation. Purified CD4+ T cells from spleens of LAX−/− and LAX+/+ mice were stimulated with anti-CD3 and -CD28 or PMA plus ionomycin. Although thymidine incorporation was similar in T cells from LAX−/− and LAX+/+ mice in response to PMA plus ionomycin (data not shown), it was increased in LAX−/− T cells that were stimulated via CD3 and CD28 (Fig. 5A). We also measured TCR-mediated IL-2 production. T cells from LAX−/− mice produced more IL-2 than those from LAX+/+ mice after activation with anti-CD3 and CD28 Abs (Fig. 5B).

Increased thymidine incorporation by LAX−/− T cells could be due to either reduced cell death or accelerated cell division after T cell activation. To distinguish these two possibilities, T cells were stimulated with anti-CD3 and CD28 or PMA plus ionomycin for 2 days, stained with 7-aminoactinomycin D (7AAD) and annexin V, and analyzed by FACS. As shown in Fig. 5C, more LAX−/− T cells were alive after stimulation with anti-CD3 and CD28 compared with LAX+/+ T cells. Reduced cell death was not due to altered Fas or Fas ligand expression in LAX−/− T cells, because FACS analysis showed similar levels of these two molecules in LAX−/− and LAX+/+ T cells (data not shown). We also labeled LAX−/− and LAX+/+ T cells with CFSE to monitor CD3 and CD28 or PMA- plus ionomycin-induced cell division. These T cells divided at a similar rate when they were stimulated (Fig. 6B). These data suggest that increased thymidine incorporation by LAX−/− T cells is due to reduced cell death.

Because LAX protein is also expressed in B cells, we measured B cell proliferation after activation with anti-IgM Abs. Thymidine incorporation was similar in B cells from LAX+/+ and LAX−/− mice when stimulated with PMA plus ionomycin (data not shown); however, it was augmented in LAX−/− B cells when different doses of anti-IgM were used (Fig. 6A). Like LAX−/− T cells, LAX−/− B cells also showed less cell death after anti-IgM stimulation compared with normal B cells (Fig. 6B). Division of LAX+/+ and LAX−/− B cells was similar when they were stimulated with anti-IgM (Fig. 6C). We also examined BCR-induced up-regulation of activation and costimulatory molecules in purified LAX+/+ and LAX−/− splenic B cells. Ligation of BCR by anti-IgM or anti-IgM plus IL-4 up-regulated comparable expression levels of both early activation marker CD69 and costimulatory molecule CD86 (B7.2) in LAX+/+ and LAX−/− splenic B cells (data not shown).
Enhanced AgR signaling pathways in LAX−/− mice

We examined whether disruption of the LAX gene affects TCR- and BCR-mediated signaling pathways. TCR- or BCR-mediated calcium flux was assayed. As shown in Fig. 7A, calcium flux in LAX−/− CD4+ T cells was increased upon engagement of CD3 and CD4. A similar increase was seen in LAX−/− B cells after cross-linking of IgM (Fig. 7B).

We next examined whether AgR-mediated protein tyrosine phosphorylation and MAPK activation are affected in LAX−/− cells. CD4+ T cells purified from LAX−/− and LAX+/+ mice were activated by anti-CD3 and CD28 Abs, whereas B cells were stimulated with anti-IgM for 0, 2, 5, 10, 20, 30, and 60 min before lysis. Total lysates were analyzed by Western blotting with anti-phosphotyrosine. Equal amounts of lysates were loaded on the SDS-PAGE as indicated by an anti-Hsp70 blot. As shown in Fig. 7C, TCR-mediated phosphorylation of cellular proteins was slightly enhanced in LAX−/− T cells. Proteins with Mᵦ of ~100, 70, and 38 kDa had an apparent increase in tyrosine phosphorylation. In LAX−/− B cells, proteins with Mᵦ of ~70 and 80 kDa had a subtle increase in tyrosine phosphorylation (Fig. 7D).

We also examined AgR-mediated MAPK activation by Western blotting with anti-phospho ERK, p38, and JNK Abs. Although there was no difference in JNK activation between LAX−/− and LAX+/+ T and B cells, ERK activation was slightly increased in LAX−/− cells. Moreover, p38 MAPK activation was much stronger and more sustained in LAX−/− T cells (Fig. 7C). BCR-mediated p38 activation was also enhanced in LAX−/− B cells (Fig. 7D). These data correlate with our previous finding that overexpression of LAX in Jurkat cells inhibits TCR-mediated p38 activation.

Because activation-induced cell death was reduced in LAX−/− T and B cells after stimulation via the AgRs, we also examined Akt activation in these cells. Akt is a key molecule that regulates cell survival. As shown in Fig. 7C, T cells from LAX−/− mice showed early and prolonged activation of Akt after anti-CD3 and CD28 stimulation. In LAX−/− B cells, there was increased Akt activation at 5 min after stimulation (Fig. 7D). Enhanced Akt and p38 activation correlated with increased cell survival (reduced cell death) of LAX−/− T and B cells.

Discussion

In this study we generated LAX-deficient mice by gene disruption. Mice with homozygous mutations at the LAX gene were viable, fertile, and healthy. Lymphocyte development was normal in these mice. However, T and B cells were hyperresponsive to stimulation via TCR or BCR. Furthermore, calcium flux, tyrosine phosphorylation, p38 MAPK and Akt activation, and cell survival were enhanced in LAX-deficient cells. These data are in agreement with our previous findings that overexpression of LAX in Jurkat cells inhibits p38 MAPK activation and TCR-mediated NFAT/AP-1 activation (17), indicating that LAX is a negative regulator of lymphocyte activation. Consistent with the idea of LAX as a negative regulator, the LAX protein was up-regulated dramatically upon stimulation of Jurkat cells or mouse thymocytes and splenocytes. This up-regulation was due to transcriptional activation of the LAX gene, because inhibitors for transcription and translation could block it.

The mechanism by which LAX inhibits T cell activation is not clear. Because TCR- or BCR-mediated signaling events, such as calcium flux, tyrosine phosphorylation, and MAPK activation, were enhanced in the absence of LAX, LAX most likely affects the early stage of T cell activation, for example, tyrosine kinase activation. It is possible that LAX binds other negative regulators, such as phosphatases, to turn off T cell activation; however, we have not been able to detect any interaction between LAX and Src homology region 2 domain-containing protein tyrosine phosphatase 1 or 2 (data not shown). Another possibility is that LAX competes for binding of Grb2, Gads, and p85 with proteins that they normally bind, such as LAT. In the cytoplasmic domain of LAX, there are one p85, two Gads, and five Grb2 binding sites (17). Mutation of these tyrosines abolishes binding of these signaling molecules and the inhibitory function of LAX, indicating that they are important in LAX function. However, the level of LAX protein was low in resting T cells (Fig 1A). Phosphorylation of LAX in activated Jurkat cells was much less than that of LAT (data not shown). Thus, it might be difficult for LAX to compete for LAT binding proteins at least in the initial stage of T cell activation. Perhaps after activation, increased LAX expression could further...
strengthen negative signaling pathways by binding to a negative regu-
lator or competing with LAT for binding of Grb2, Gads, or p85.

AgR-mediated signaling pathways were enhanced in LAX+/− T and B cells, as evidenced by enhanced Ca2+ flux, ERK, p38, and Akt activation. Interestingly, enhanced AgR signaling did not lead to increased apoptosis. Fas or Fas ligand expression was not altered in LAX−/− cells. Instead, LAX−/− cells survived better than normal cells. Because LAX associates with the p85 subunit of PI3K, we examined whether the PI3K pathway is affected in the absence of LAX. Activation of PI3K activates the serine/threonine kinase, protein kinase B/Akt, which is a key molecule in regulating cell survival (18, 19). Protein kinase B/Akt has been shown to antagonize the proapoptotic functions of BCL2-antagonist of cell death and caspase 9 and positively regulates activation of NFκB and expression of Bcl-xL (20–23). Interestingly, activation of Akt was enhanced in LAX−/− T and B cells, which is probably the reason for increased survival of these cells upon stimulation. When we examined whether the expression of Bcl-2 or Bcl-xL is increased in LAX−/− cells, we did not detect any significant difference in the expression of these proteins in T or B cells between LAX+/+ and LAX−/− mice. However, it is still possible that there is a difference in the expression of Bcl-2 or Bcl-xL, which might be difficult to detect by Western blotting. Increased cell survival in LAX−/− cells could also be due to enhanced p38 MAPK activation after activation. It has been shown in neutrophils that p38 MAPK can associate with caspase-8 and caspase-3, phosphorylate them, and inhibit their activities (24). This MAPK could play a role in lymphocyte survival after engagement of AgRs.

Our present study showed that p38 MAPK activation was prolonged after lymphocyte activation. This result agrees with our previous finding that overexpression of LAX in Jurkat cells inhibits p38 MAPK activation. The p38 MAPK, originally identified for its response to stress stimuli, is activated by ligation of TCR and costimulatory molecules (25, 26). Interestingly, activation of Akt was enhanced in LAX−/− T and B cells, which is probably the reason for increased survival of these cells upon stimulation. When we examined whether the expression of Bcl-2 or Bcl-xL is increased in LAX−/− cells, we did not detect any significant difference in the expression of these proteins in T or B cells between LAX+/+ and LAX−/− mice. However, it is still possible that there is a difference in the expression of Bcl-2 or Bcl-xL, which might be difficult to detect by Western blotting. Increased cell survival in LAX−/− cells could also be due to enhanced p38 MAPK activation after activation. It has been shown in neutrophils that p38 MAPK can associate with caspase-8 and caspase-3, phosphorylate them, and inhibit their activities (24). This MAPK could play a similar role in lymphocyte survival after engagement of AgRs.

Although LAX deficiency did not cause significant defects in lymphocyte development, we did observe some abnormalities in LAX−/− mice. Naive LAX−/− mice have spontaneous GC formation. The GC reaction is a characteristic feature of T cell-dependent, humoral immune responses. GC is necessary for the generation of memory B cells and is the site of Ig gene V region hypermutation (31, 32). Formation of GC in naive LAX−/− mice might be due to the presence of T and B cells that are hypersensitive to Ag stimulation in LAX−/− mice.

Unexpectedly, the expression of CD23 in mature LAX−/− B cells was decreased significantly compared with that in LAX+/+ cells. CD23 is the low affinity IgE receptor. This molecule might have several functions, including regulation of T-dependent IgE production and B cell proliferation (33, 34). The immunological roles of CD23 are not clear. In CD23-deficient mice, IgE production is increased (35). These mice have increased inflammation and airway hyperresponsiveness after sensitization and challenging with allergens (36). Interestingly, IgE level in LAX−/− mice was also increased as in CD23−/− mice. It is possible that LAX might also function as an adaptor protein in CD23-mediated signaling pathways and is involved in the regulation of IgE production. The CD23-mediated signaling pathway has not been extensively stud-

ied. It should be interesting to determine whether CD23-mediated signaling pathway is normal in LAX−/− mice once we know more about this pathway in the future.

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