Deletion of the LIME adaptor protein minimally affects T and B cell development and function

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LIME (Lck-interacting membrane protein) is a transmembrane adaptor that associates with the Lck and Fyn protein tyrosine kinases and with the C-terminal Src kinase (Csk). To delineate the role of LIME in vivo, LIME-deficient mice were generated. Although Lime transcripts were expressed in immature and mature B and T cells, the absence of LIME impeded neither the development nor the function of B and T cells. TCR transgenic mice deprived of LIME showed, however, a 1.8-fold enhancement in positive selection. Since B cells and activated T cells express LIME and the related adaptor NTAL, mice lacking both adaptors were generated. Double-deficient mice showed no defect in the development and function of B and T cells, and the lack of LIME had no effect on the autoimmune syndrome that develops in aged NTAL-deficient mice. In contrast to a previous report, we further showed that this autoimmune syndrome develops in the absence of T cells. Therefore, our in vivo results refute all the previous roles postulated for LIME on the basis of studies of transformed B and T cells and demonstrate that LIME has no seminal role in the signaling cassette operated by antigen receptors and coreceptors.

Introduction

Transmembrane adaptor proteins (TRAP) represent a group of molecules participating in immunoreceptor signaling. They possess a short extracellular domain, a transmembrane region, and a long cytoplasmic tail carrying up to ten potential tyrosine phosphorylation sites. In contrast to the signaling subunits that are associated with immunoreceptors and that contain immunoreceptor tyrosine activation motif (ITAM), the known TRAP carry no ITAM and do not associate directly with immunoreceptors. So far four TRAP have been identified as components of membrane lipid rafts and denoted as ‘linker of activation of T cells’ (LAT, also
called LAT1), phosphoprotein associated with glycolipid-enriched membranes (PAG, also known as Cbp), non-T cell activation linker (NTAL, also called LAB or LAT2), and Lck-interacting membrane protein (LIME) (reviewed in [1]). These TRAP possess two palmitoylated cysteine residues in the membrane proximal segment of their cytoplasmic tail that target them to lipid rafts. Upon tyrosine phosphorylation, which is mostly due to Src- and Syk-family protein tyrosine kinases, TRAP bind several SH2 domain-containing cytoplasmic signaling molecules. This results in the assembly of membrane proximal complexes that regulate immunoreceptor signaling in a positive or negative manner.

So far little is known about the biological functions of LIME [2–4]. In humans, LIME is expressed predominantly in T cells and becomes tyrosine phosphorylated by Src-family kinases. This phosphorylation occurs exclusively after cross-linking of the CD4 and CD8 coreceptors by antibodies, or by the HIV glycoprotein 120 in the case of CD4 [3]. Overexpression of LIME in Jurkat T cells amplifies TCR-mediated calcium flux, phosphorylation of Erk and transcriptional activation of the IL-2 gene promoter [3]. It is therefore possible that LIME plays a positive role in the sequential activation of Lck and Fyn that occurs in the lipid rafts of T cells [5]. Considering that inhibitory signals are induced when CD4 is cross-linked in the absence of TCR engagement [6], the ensuing tyrosine phosphorylation of LIME could also generate inhibitory signals. Finally, LIME may also account for the remarkable ability of certain anti-CD4 antibodies to induce a state of antigen-specific tolerance through the induction of regulatory T cells [7].

The published data on mouse LIME are somewhat different. High expression of LIME was observed in the lung. In T cells, LIME expression was rapidly up-regulated following TCR-cross-linking and LIME was localized at the contact site between T cells and antigen-presenting cells (APC) [2]. A recent report further indicated that LIME might be involved in BCR-mediated B cell activation [4]. Suppression of LIME expression by siRNA resulted in inhibition of BCR-mediated activation of MAPK, PI3K, calcium flux, NFAT, and NF-κB. To further elucidate the function of LIME in vivo, we have generated LIME-deficient mice and have analyzed them for the development and function of B and T lymphocytes.

Results

LIME expression in mouse B and T cells

Previous studies contain several inconsistencies with regards to the pattern of expression of LIME [2, 8]. To settle this issue, populations corresponding to several stages of mouse B and T cell differentiation were prepared by cell sorting and analyzed by quantitative RT-PCR for their relative levels of LIME transcripts (Fig. 1A). The same cell populations were also analyzed for the presence of Lat and Ntal transcripts. As shown in Fig. 1A, CD4+CD8+ thymocytes expressed LIME transcripts at lower levels than mature, resting CD4+ and CD8+ T cells. A recent study analyzing whole B cells isolated from the spleen showed that LIME is expressed in mouse B cells [4]. To more precisely determine the pattern of LIME expression within mature B cells, splenic B cells were sorted according to IgM and IgD expression. IgMhigh IgDlow or transitional 1 (T1) cells, are recent immigrant from the bone marrow that develop into IgMhigh IgDhigh transitional 2 (T2) cells, some of which differentiate further into mature IgMlow IgDhigh, or follicular recirculating B cells [9]. As shown in Fig. 1, LIME transcripts were expressed in T1, T2, and mature B cells, albeit at lower levels than in mature T cells. Plasma cells also expressed LIME transcripts at levels comparable to those found in their direct follicular B cell precursors (Fig. 1A). Moreover, LIME transcripts were expressed during early stages of B cell differentiation in the bone marrow (Fig. 1A).

T cell development in LIME-deficient mice

To explore the role of LIME in B and T cell development and function, we generated mice deprived of LIME. As depicted in Fig. 2, our knockout approach resulted in the replacement of the whole LIME coding sequence with a loxP sequence. Mice homozygous for this mutation, LIME−/−, were born at the expected Mendelian frequencies and were deprived of detectable LIME protein (Fig. 2F). Analysis of LIME−/− mice showed that their thymi, spleens, and lymph nodes were of normal size. Monitoring CD4, CD8, CD44, CD25, CD62L, CD5, TCRαβ, TCRγδ and CD49b (DX5) expression, showed no major alteration in the T cell, NK T cell, and NK cell populations from thymus and secondary lymphoid organs (Fig. 3 and data not shown). T cell proliferation and IL-2 production in response to graded concentrations of anti-CD3 antibody, or to a suboptimal dose of anti-CD3 antibody in combination with increasing concentrations of anti-CD28 antibody were similar in LIME-sufficient and -deficient T cells (Fig. 4A and B). Upon CD3 cross-linking, LIME−/− thymocytes and CD4+ T cells showed calcium responses of the same magnitude as those observed in wild-type counterparts (Fig. 4D and data not shown). In line with these data, the clinical course of experimental autoimmune encephalitis (EAE), a mouse model of human multiple sclerosis, was not influenced by the absence of LIME (data not shown). We also evaluated whether the loss of LIME affects the ability of the CD4 molecule to deliver inhibitory signals.
when cross-linked prior to the engagement of the TCR. When CD4 expressed on LIME-sufficient and -deficient T cells was cross-linked for 45 min before stimulation with anti-CD3 antibodies, the same inhibitory signals were delivered by the cross-linked molecules CD4 (Fig. 4C and D). Therefore, the absence of LIME does not detectably affect a large panel of events associated with T cell development and function.

LIME deficiency improves positive selection of Marilyn TCR transgenic thymocytes

To analyze with a higher sensitivity the effect of the LIME-deficiency on intrathymic positive and negative selection events, Lime–/– mice were backcrossed into transgenic mice expressing an MHC class II-restricted TCR and maintained on a RAG-deficient background. We used the Marilyn TCR transgenic line because it expresses a monoclonal population of Vβ6+ CD4+ T cells specific for Dbγ, a male H-Y antigen presented by I-Ab molecules, and permits positive and negative selection among the same littermates to be readily analyzed [10]. Moreover, since in vitro experiments showed that LIME is heavily tyrosine phosphorylated after CD4 cross-linking [3], using the Marilyn TCR transgenic line we could gauge the role of LIME in a CD4-dependent physiological set-up. Comparison of the thymi of LIME-sufficient and -deficient Marilyn female mice for their cellularity, CD4-CD8 staining profile, and levels of CD3 and of V[β6+] TCR showed that LIME deficiency improves the selection of mature CD4+ SP cells expressing transgenic TCR (Fig. 5A). Lime+/– Marilyn female mice showed a 1.8-fold increase in the number of clonotype-positive CD4+ SP cells [15.1 ± 4.5 × 106 in wild-type Marilyn female thymi (n=4) versus 27.5 ± 2.1 × 106 in Lime+/– Marilyn female thymi (n=6)]. Consistent with this observation, comparison of the spleen from LIME-deficient and -sufficient Marilyn female mice showed that the absence of LIME resulted in a threefold increase in the number of clonotype-positive CD4+ cells [1.6 ± 0.5 × 106 in wild-type Marilyn female spleen (n=4) versus 5.2 ± 1.6 × 106 in Lime+/– Marilyn female spleen (n=6)]. In LIME-sufficient, Marilyn male mice, negative selection results in the absence of TCR[high] DP and of CD4+ SP cells, and, as a consequence, the periphery is deprived of clonotype-positive CD4+ T cells. As shown in Fig. 5A, in LIME-deficient, Marilyn male mice, the lack of LIME had no measurable impact on negative selection. As documented in other TCR transgenic model, a minute population of clonotype-positive CD4+ T cells.

Figure 1. Comparative expression of Lime, Lat and Ntal transcripts throughout mouse B and T cell development. (A) RNA samples were prepared from wild-type mice and analyzed by quantitative RT-PCR for the presence of Lat, Ntal and Lime transcripts. Results are expressed as relative units of Lat, Ntal and Lime mRNA normalized using Hprt transcripts. Negative control samples are shown on lane 11 and corresponds to total spleen cells isolated from Lat–/– (Lat panel), Ntal–/– (Ntal panel) or Lime–/– (Lime panel) mice. (B) RNA samples were prepared from CD4+ Th2 effectors freshly isolated from LatY136F mice and from wild-type CD4+ T cells that were left unstimulated (resting) or stimulated (activated) with anti-CD3 plus anti-CD28 antibodies for 40 h prior to analysis by quantitative RT-PCR for the presence of Ntal transcripts. Positive (+) and negative (−) control samples correspond to mature B cells isolated from wild-type or Ntal–/– mice. Data shown are representative of two independent experiments.
positive, CD4+CD8– T cells is found in the periphery of Marilyn male mice and is not affected by the lack of LIME (Fig. 5A). Despite the presence of slightly lower levels of clonotypic TCR on their surface (Fig. 5A), the mature CD4+ cells found in the periphery of Lime+/– Marilyn female mice were capable of proliferating and producing IL-2 in response to their cognate antigen, and slightly higher responses were even observed in the absence of LIME (Fig. 5B and C).

Figure 2. Generation and identification of Lime deficient mice. (A–D) Strategy used to delete the Lime gene. (A) Partial restriction map of the wild-type Lime gene. Exons are shown as filled boxes. Exons containing the initiation (ATG) and stop codon are specified (E: EcoRI, H: HindIII). (B) Targeting vector used for the deletion of exons 1–5. Shaded and open boxes correspond to the thymidine kinase gene (TK) and to the loxP-flanked-Cre-Neor cassette, respectively. LoxP sites are shown as triangles. (C) Structure of the targeted allele following homologous recombination. (D) Final structure of the targeted allele after self-excision of the Cre-Neor cassette. Exons containing the initiation (ATG) and stop codon are specified (E: EcoRI, H: HindIII). (E) PCR genotyping of indicated. The position of the PCR primers used to genotype the allele after self-excision of the Cre-Neo r cassette. The 5' probe is specified. (F) Lack of LIME expression in T cells from Lime–/– resulting mice are indicated by arrows. (G) PCR genotyping of indicated. The position of the PCR primers used to genotype the allele after self-excision of the Cre-Neo r cassette. The 5' probe is specified.

B cell development and function in LIME-deficient mice

Although LIME is expressed in immature and mature B cells, analysis of splenic B cell populations showed that they are capable of properly reaching the T1, T2, and follicular stages in the absence of LIME (Fig. 6A). Consistent with these results, basal levels of IgG1, IgG2a, IgG2b, IgG3 and IgE, and antibody responses to a T-dependent antigen were normal in LIME-deficient mice (data not shown). Importantly, NTAL, another raft-associated TRAP is expressed in T1, T2, and follicular B cells [11]. Young mice deprived of NTAL showed no defect in B cell differentiation and function [11, 12]. Because NTAL and LIME are coincidently expressed in immature and mature B cells (Fig. 1), the product of the other may have backed up the function of the missing one. Therefore, to analyze whether some redundancy exists between NTAL and LIME in mouse B cells, Lime+/– and Ntal+/– mice were bred to generate double-deficient mice. Analysis of the B cell compartment in Lime+/– Ntal+/–, Lime+/– Ntal−/−, and Lime−/– Ntal+/– mutant mice revealed no major difference with wild-type littermates (Fig. 6A). Moreover, mature B cells isolated from wild-type, Lime+/– Ntal+/–, Lime+/– Ntal−/−, and Lime−/– Ntal−/− mutant mice showed similar proliferative responses upon stimulation with F(ab2)2 fragments of an anti-IgM antibody or lipopolysaccharide (Fig. 6B and data not shown), and similar Ca2+ influx following BCR cross-linking (Fig. 6C and data not shown). Thus, single or combined deletions of LIME and NTAL do not detectably affect B cell development and function.

Lack of autoimmunity in LIME-deficient mice

Even though NTAL is barely detectable in naive T cells, it could be detected in activated T cells [13]. As shown in Fig. 1B, Ntal transcripts are indeed expressed in activated T cells, including Th2 effector cells from Lat+/136F1 mice, albeit at lower levels than in mature B cells. Therefore, the NTAL and LIME TRAP are coincidently expressed in activated T cells. However, combined deletions of LIME and NTAL do not detectably affect early and late events of T cell activation (Fig. 6D and E).

Aged (>6-month-old) Ntal−/− mice showed hyper-activated T cells that were thought to activate B cells and thus be responsible for the presence of anti-DNA antibodies in the sera of these mice [13]. Using an independently derived line of Ntal−/− mice [14], we failed to document an enlargement of the spleen and the presence of hyperactivated T cells in 25–30-week-old Ntal−/− mice (Fig. 7A and data not shown). However, we confirmed the presence of anti-nuclear antibodies when the sera of 6-month-old Ntal−/− mice were used to stain fixed HEp-2000 cells. The presence of anti-nuclear antibodies in the sera of these mice [13]
antibodies in the absence of hyperactivated T cells questions the contribution of NTAL-deficient T cells to the development of anti-nuclear antibodies. Considering that Ntal−/− Lat−/− mice lack T cells but possess a normal B cell compartment [11], we further analyzed the sera of 6-month-old Ntal−/− Lat−/− mice for the presence of anti-nuclear antibodies. As shown in Fig. 7E, anti-nuclear antibodies were readily detected in the sera of 6-month-old Ntal−/− Lat−/− mice. Therefore, in contrast to the conclusions reached by Zhu and colleagues [13], NTAL has an intrinsic negative regulatory role within B cells that contributes to regulate their activity in aged mice. Importantly, aged Lime−/− mice showed no sign of T or B cell activation and their sera contained no detectable anti-DNA antibodies (Fig. 7E). Furthermore, the absence of LIME was without effect on the development of anti-nuclear antibodies that occurs in aged Ntal−/− mice (Fig. 7E).

**Discussion**

By assembling signalosomes, the four raft-associated TRAP (LAT, NTAL, PAG and LIME) coordinate intracellular programs that control the development and activation of immunocytes. Consistent with these views, LAT plays a seminal role during T cell development and function and is also critical for FcεRI signaling in mast cells [15–17]. In contrast, it has been difficult to define the role of the three other raft-associated TRAP and deletion of their genes resulted in either subtle or no detectable phenotype. NTAL functions primarily as a negative regulator of FcεRI-, BCR- and TREM-1-mediated signaling [11, 12, 14, 18]. Despite a wealth of biochemical data supporting an important role of PAG in regulation of Src-family protein tyrosine kinases, PAG appears dispensable for T cell development and function [19, 20]. We report here that LIME is also dispensable for the development and function of B and T cells. The only robust effect due to the lack of LIME was noted when a null mutation of the Lime gene was bred into mice in which αβ TCR variability was neutralized by expressing a TCR transgene previously calibrated in a LIME-sufficient thymus. Under those conditions, the loss of LIME enhanced both positive selection and the number of mature T cells found in the periphery. Therefore, under sensitized conditions LIME likely contributes to dampening TCR signals.

The loss of C-terminal Src kinase (Csk) at early stages of T cell development unleashed the activity of Lck and Fyn, and resulted in TCR-deficient T cells that accumulate in peripheral lymphoid organs [21]. Considering...
that the SH2 domain of Csk could bind both PAG and LIME [1], it remains possible that some functional complementation exists between LIME and PAG and thus accounts for the different phenotype observed in mice that lack Csk or have a deletion of the Pag or Lime gene. In that regard, generation of mice with a combined deletion of Lime and Pag should be particularly informative.

In this study, besides a 1.8-fold enhancement in T cell-positive selection, we have failed to document any blatant effect resulting from the loss of LIME as well as from the combined loss of LIME and NTAL. These results obtained using in vivo assays or freshly isolated T and B cells contrast with previous results obtained using transformed B and T cell lines and refute all the roles postulated for LIME on the basis of the analysis of transformed cell lines. In contrast, using a wealth of in vivo sensitized experimental conditions, LIME was found to have no seminal role in the signaling cassette operated by antigen receptors and coreceptors.

Materials and methods

Mice

Mice deficient in recombination activation gene 2 (Rag2−/− mice) [22], in CD3ε (Cd3ε5/5 mice) [23], in LAT (Lat−/− mice) [24] and in NTAL (Ntal−/− mice) [11] have been described. Mice homozygous for a mutation that replaced tyrosine 136 of LAT with phenylalanine (LatY136F mice) have been described [25]. The Marilyn TCR transgenic line was obtained from O. Lantz [10]. Mice have been crossed onto a C57BL/6 back-

Figure 4. Proliferative responses, IL-2 production, and Ca2+ responses in lymph node T cells from wild-type and Lime−/− mice. (A) T cells purified from 7-week-old wild-type and Lime−/− mice were stimulated with graded concentrations of anti-CD3 antibody. After 72 h, the extent of T cell proliferation was measured by determining levels of [3H]thymidine uptake. (B) Purified T cells were stimulated with a fixed concentration (1 µg/mL or 0.1 µg/mL) of anti-CD3 antibody with or without graded concentrations of anti-CD28 antibody. Also shown is the level of T cell proliferation obtained following stimulation with phorbol-myristate-acetate and ionomycin (PMA/Iono). (C) CD4+ T cells purified from wild-type and Lime−/− mice were stimulated with anti-CD3 antibodies. When specified [a-CD4 (prelig)], CD4 molecules were cross-linked for 45 min before stimulation with anti-CD3 antibodies. The extent of proliferation was measured after 72 h of culture. Values have been normalized as % of the proliferation value observed following stimulation with PMA and Iono. In A–C, the data shown are representative of five independent experiments and the values correspond to the mean ± SD from quadruplicate samples. (D) Calcium flux analysis in response to TCR stimulation in wild-type and Lime−/− lymph node CD4+ T cells. The diagram depicts calcium flux (y axis) as a function of time (x axis). Arrows indicate the time point of addition of streptavidin (SAV) to cross-link the biotinylated anti-CD3 antibody, or of ionomycin (Iono). In the right panels, CD4 molecules were cross-linked for 45 min prior to stimulation with anti-CD3 antibodies. Data shown are representative of four independent experiments.

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Figure 5. T cell development in wild-type and Lime<sup>−/−</sup> mice expressing the Marilyn transgenic TCR. (A) Thymocytes and splenocytes from LIME-sufficient (WT) and -deficient (Lime<sup>−/−</sup>) female or male mice expressing the Marilyn transgenic TCR were analyzed by flow cytometry for expression of CD4, CD8, and CD3<sup>e</sup>. The genotypes and the total number of cells in the thymus or spleen (averaged from 4 WT and 6 Lime<sup>−/−</sup> age-matched mice) are indicated above dot plots. The percentages of cells within each quadrants or gates are indicated. Single-color histograms represent CD3<sup>e</sup> staining on total thymocytes and splenocytes. The percentage of CD3<sup>int</sup> and CD3<sup>high</sup> thymocytes are indicated. In the case of the spleen, the percentage and mean fluorescence (italicized values) of CD3<sup>+</sup> cells are indicated. All the mice were maintained on a Rag-2<sup>−/−</sup>-deficient background, and were between 6 and 8 weeks of age. (B, C) CD4<sup>+</sup> T cells found in the periphery of WT and Lime<sup>−/−</sup> Marilyn female mice were analyzed for their capacity to proliferate (B) and produce IL-2 (C) after stimulation with graded concentrations of agonistic peptide. CD4<sup>+</sup> T cells were purified from secondary lymphoid organs and incubated with A<sup>β</sup>-positive APC prepulsed with the Dby agonist peptide. The extent of cell proliferation (B) and IL-2 content (C) were determined after 40 h and 24 h, respectively. Data shown in (B) and (C) are representative of three independent experiments.
Figure 6. Analysis of B and T cell development and function in mice with single and combined deletion of LIME and NTAL. (A) Splenocytes from wild-type (WT), LIME-deficient (Lime–/–), NTAL-deficient (Ntal–/–) mice and mice lacking both LIME and NTAL (Lime–/– Ntal–/–) were stained with the indicated antibodies and analyzed by flow cytometry. Genotypes are specified over the dot plots. In the CD19 versus MHC class II staining, the percentages of B cells (CD19+MHCII+) are indicated. In the IgM versus IgD staining profiles, CD19+MHCII+-gated B cells were categorized into IgM\text{high} IgD\text{low}, IgM\text{high} IgD\text{high}, and IgM\text{low} IgD\text{high} cells. (B) Spleen B cells from mice of the specified genotypes were left unstimulated (0) or stimulated with lipopolysaccharide (LPS, 10 μg/mL), goat F(ab')\text{2} anti-mouse IgM antibody (IgM, 10 μg/mL), or with phorbol myristate acetate and ionomycin (P/I). After 40 h of culture, the extent of cell proliferation was determined. (D) Spleen T cells from mice of the specified genotype were left unstimulated (0) or stimulated with anti-CD3\varepsilon antibody at two different doses (1 μg/mL and 5 μg/mL), and with a mix of anti-CD3\varepsilon and anti-CD28 antibodies. After 40 h of culture, the extent of cell proliferation was determined. (C, E) Calcium responses analysis following to antigen receptor stimulation of B (C) and T (E) cells isolated from mice of the specified genotypes.
ground for at least seven generations, and housed in a specific pathogen-free animal facility. All procedures were in accordance with protocols approved by French Laws and the European Directives.

**Vector construction**

The targeting construct used for disruption of the *Lime* gene is shown in Fig. 2. The 5′ homologous sequences correspond to a gene segment encompassing nucleotide positions 8079–9024 (sequence available from GenBank/EMBL/DDBJ under accession number F131462). The 3′ homologous sequences correspond to a gene segment encompassing nucleotide positions 13 800–18 812 (sequence available from GenBank/EMBL/DDBJ under accession number F131462). In the targeting vector, exons 1–5 of the *Lime* gene were replaced by a *loxP*-flanked, Cre-Neo r auto-deleter cassette [26].

**Isolation of recombinant embryonic stem cell clones and production of mutant mice**

After electroporation of CK35 129/Sv embryonic stem cells [27] and selection in G418 and gancyclovir, colonies were screened for homologous recombination by Southern blot analysis. The 5′ single-copy probe corresponds to a 392-bp fragment that encompasses nucleotide position 6857–7249. It was amplified using the following oligonucleotides: 5′-CGG AAT TCG TGG CAC CAC ACT GTA GAG AG-3′ and 5′-GCT CTA GAC TGT GCA CAC TCC TGA CAA CTC-3′. When tested on HindIII-digested DNA, it hybridized either to a 7.4-kb wild-type fragment or to a 2.7-kb recombinant fragment. The presence of an appropriate homologous recombination event at the 3′ side was assessed using a 357-bp fragment encompassing nucleotide positions 19201–19558. It was amplified using the following oligonucleotides: 5′-CGG AAT TCT GGC TGC AGA CAT GAA CAC TCC TGA CAA CTC-3′ and 5′-GCT CTA GAT TGT AGG AAG GCT ATT TCA GG-3′. When tested on EcoRI-digested DNA, this fragment hybridized either to a 16.6-kb wild-type fragment or to a 9.6-kb recombinant fragment. A Neo probe was also used to ensure that adventitious non-homologous recombination
events had not occurred in the selected clones. Screening of mice for the presence of the Lime null mutation was performed by PCR using the following oligonucleotides: (a): 5'-ACC TGC TTT GTT CAC CTC ATT AAT GGG TAT-3', (b): 5'-ACA GCC AGG AAG ATC TTT GTG GCC TGC TGG-3', and (c): 5'-GTG ACC ACC CTG TCC TGC CCA ACC TAG AGG-3'. The wild-type Lime allele is visualized as a 229-bp fragment using the a-b pair of oligonucleotides, whereas the mutant allele is visualized as a 530-bp fragment using the a-c pair of oligonucleotides.

Immunoblotting

Splenocytes from wild-type or Lime−/− mice were solubilized in the presence of 1% lauryl maltoside. Lysates were subjected to SDS/PAGE followed by Western blotting. The blot was immunostained with a rabbit antiserum directed to the cytoplasmic domain of mouse LIME (amino acids 86–202), followed by goat anti-rabbit Ig conjugated to horseradish peroxidase.

Flow cytometry and intracellular staining

Lymphocyte suspensions were prepared from thymus or lymphoid peripheral organs and stained with the specified antibodies (BD Pharmingen and Caltag laboratories). Cells were analyzed on FACSCalibur and BDLSRI flow cytometers (Becton Dickinson). Data acquisition and analysis were performed using CellQuest (Becton Dickinson) or FlowJO (Treestar) software.

RNA preparation and quantitative RT-PCR

Purification of immature and mature B and T cell fractions, RNA isolation and real-time RT-PCR were performed as described [11]. The pairs of primers used to amplify the Hprt, Lat, and Ntal transcripts have been reported [11]. Lime transcripts were amplified with the following oligonucleotides: Lime sense, 5'-GAA GCA CAA AGG GAC AGA GC- 3' and Lime antisense, 5’-CAG GTT CAG CTG GTG CTG TGT GA- 3’. Relative expression values were expressed as 2−ACT where ACT is the difference between the mean Ct value of duplicates of the test sample and of the endogenous HPRT control.

Cell purification

Splenic T cells were purified by negative selection using magnetic cell sorting (Miltenyi Biotec). Purity was analyzed by flow cytometry and was >90%.

Ca2+ flux measurement

For B cells, Ca2+ flux measurements were as described [11], and data acquisition and analysis were performed with CellQuest (Becton Dickinson) or FlowJO (Treestar), respectively. T cells were incubated for 30 min with 3.75 µg/mL indo-1-AM (Molecular Probes) in phenol red-free RPMI 1640 medium (GIBCO BRL) containing 10% fetal calf serum. Cells were subsequently stained with CD4 and CD8 and incubated with 10 µg/mL biotinylated anti-CD3 antibody (clone 145–2C11) at 4°C. Calcium fluxes were induced by cross-linking the TCR/CD3 complex with 75 µg/mL streptavidin and were measured using an LSR flow cytometer (BD Biosciences). In some instances, prior to stimulation with anti-CD3 antibodies, CD4 molecules were cross-linked for 45 min using a biotinylated rat anti-mouse CD4 antibody (clone RM 4–5) and streptavidin.

Proliferative responses and IL-2 production of T cells

Purified T cells were cultured in 96-well round-bottom plates (2.5 × 103 cells per well) and stimulated with graded concentrations of rat anti-mouse CD3 antibodies. After 72 h, plates were pulsed for 8 h with 0.5 µCi [3H]thymidine per well. Incorporation of [3H]thymidine into DNA was measured by liquid scintillation counting (1450 MicroBeta Trilux, Perkin Elmer Wallac GmbH). To assess the effect of CD28 costimulation, graded concentrations of anti-CD28 antibodies were added to T cells stimulated with a fixed concentration (0.1 or 1 µg/mL) of rat anti-mouse CD3 antibodies. When specified, T cells were stimulated with PMA (0.05 µg/mL) and ionomycin (2 nM). In the case of Marilyn TCR transgenic T cells, antigen-induced proliferation was measured using I-Aβ-positive spleen cells from CD45/AS mice as APC. Spleen cells were irradiated and pulsed with various concentration of the Dby peptide [28]. Purified CD4+ cells (5 × 105) were stimulated with 5 × 105 antigen-pulsed APC. After 40 h, cultures were assayed for their content of ATP. Briefly, 100 µL CellTiterGlo reagent (Promega) was added to the culture and the resulting luminescence, which is proportional to the ATP content of the culture, was measured with a Victor2 luminometer (Wallac, Perkin Elmer Life Science). The level of IL-2 contained in the supernatant after 20 h was determined using the CTL-L line. Values were converted into IL-2 units using a recombinant mouse IL-2 standard (Pharmingen).

B cell responses and ELISA

They were performed as described [11].

Detection of anti-nuclear antibodies

The presence of anti-nuclear antibodies was determined using human HEp-2000 cells (Immunocorens). HEp-2000 cells were incubated with the indicated mouse serum dilution for 30 min. Slides were then washed and incubated for 30 min with a FITC-labeled goat F(ab')2 anti-mouse IgG antibody (Beckman-Coulter). Slides were analyzed by fluorescence microscopy.

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