

SUPPLEMENTARY MATERIALS AND METHODS

Generation of LIME^{-/-} Mice

For the 5' arm, an 8.0-kb Sall-EcoRI fragment upstream of the first exon was obtained from a LIME phage clone obtained by screening a lambda library made from the 129 Sv mouse strain (Stratagene). For the 3' arm, a 2.8-kb NotI-NotI fragment containing exon 4 and exon 5 was cloned by PCR using the genomic DNA of murine ES cells originating from 129/OlaHsd mice as the template. The 5' arm was subcloned into the Sall-EcoRI site of a pBluescriptIIKS(+) (Stratagene) backbone vector containing a 1.6-kb pgk-neo gene. Subsequently, the 9.6-kb fragment containing the 5' arm and PGK-neo was substituted for the pgk-neo of the pBluescriptSK(+) vector containing Diphtheria Toxin A (DTA) as a negative selection marker. The 3' arm was inserted into the NotI site between pgk-neo and DTA. This targeting vector was linearized by Sall and introduced into E14K ES cells by electroporation. G418 (250 µg/ml)-resistant clones carrying the mutated allele were selected, and homologous recombination was confirmed by Southern blot analysis using a flanking probe. A targeted ES clone was injected into C57BL/6 blastocysts, which were then transferred into pseudo-pregnant foster mothers. The resulting male chimeric mice were bred to C57BL/6 females to obtain heterozygous mice. Germline transmission was verified by Southern blot analysis of DNA obtained from tail genomic DNA of F₁ offspring. Following heterozygous matings, homozygous LIME-deficient mice were identified by Southern blot analysis and PCR. Care of animals was in accordance with institutional guidelines.

Intracellular calcium measurement

For calcium flux measurement, T cells were purified by Thy1.2 magnetic beads (Miltenyl Biotec). Cells were preloaded with 0.5 µM of cell-permeable Fura-2/AM (Molecular Probes) in serum-free RPMI medium at 37°C for 40 min. For stimulation, cells were incubated with hamster anti-CD3_ε mAb (145-2C11, 5 µg/ml; BD Pharmingen) on ice for 30 min, washed, and resuspended with HBSS/FBS (HBSS with 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 1% FBS) containing anti-hamster IgG (G94-56, 10 µg/ml; BD Pharmingen). Intracellular calcium concentration was monitored with an Rf-5301PC spectrofluorophotometer (Shimazu, Kyoto, Japan).

Preparation of various T cell subsets

For preparation of naïve T cell subsets, CD4⁺CD62L⁺ or CD8⁺CD62L⁺ naïve T cells were isolated from lymph nodes using a CD4⁺ or CD8⁺ T cell isolation kit (Miltenyl Biotec). Non-polarized T cell blasts (B) were prepared by stimulating the naïve T cells with plate-coated anti-CD3 mAb (2C11, 5 µg/ml; BD Bioscience) and soluble anti-CD28 mAb (37.51, 2 µg/ml; BD Bioscience) for 24 h. For preparation of Th1, Th2 and Th17 cells, CD4⁺ lymph node T cells were stimulated *in vitro* with plate-coated anti-CD3 mAb (5 µg/ml) plus soluble anti-CD28 mAb (2 µg/ml) for 3 days in the presence of mIL-

12 (10 ng/ml; R&D Systems) and anti-mIL-4 mAb (10 µg/ml, clone 11B11; BD Bioscience) for Th1 differentiation, mIL-4 (10 ng/ml; R&D Systems), anti-mIL-12 mAb (10 µg/ml, clone C17.8; BD Bioscience) and anti-mIFN-γ mAb (10 µg/ml, clone XMG1.2; BD Bioscience) for Th2 differentiation, and TGF-β (3 ng/ml), rIL-6 (20 ng/ml), anti-IL-4 mAb (10 µg/ml), and anti-IFN-γ mAb (10 µg/ml) for Th17 differentiation. Then, cells were split and cultured in each differentiation condition with the cytokines and anti-cytokine mAb for further 2-3 days. Tc1 cells were generated by culturing naïve CD8⁺ T cells under Th1 differentiation conditions.

Measurement of [³H]-thymidine uptake and cytokine production by ELISA

For the measurement of T cell proliferation, [³H]-thymidine uptake measured using a scintillation counter (Perkin Elmer). Briefly, purified CD4⁺ T cells (2 × 10⁵) were cultured in each differentiation condition toward effector subsets as described in Materials and Methods section. Cells were pulsed with [³H]-thymidine (0.5 µCi/well) for 9 h of final culture period. For cytokine quantification by ELISA, culture supernatants were harvested 24 h after re-stimulation with Anti-CD3 mAb plus anti-CD28 mAb or PMA (100 ng/ml) plus ionomycin (0.5 µg/ml) and examined by ELISA kit (R&D systems).

Histological analysis of ear pinnae in CHS response

In vivo migration of leukocytes was analyzed in CHS response experiment. Briefly, following the rechallenge of the dorsal mouse ear with 20 µl of 0.25% DNFB, biotin-labeled lectin was injected into the tail vein and allowed to circulate through the whole body for 2 min, which allows lectin attached to highly glycosylated proteins on leukocytes and inner endothelia of blood vessels. Following this, mice were euthanized and all blood was perfused with PBS and 1% formaldehyde. The ears were excised and split into dorsal and ventral halves. Dorsal ear halves were then processed with avidin-biotin complex/diaminobenzidine (ABC/DAB) histochemistry (Vector Lab) to stain the leukocytes.

Analysis of lymphocyte homing to secondary lymphoid organ

Freshly isolated CD4⁺CD62L⁺ naïve lymph node T cells from WT and LIME^{-/-} mice were labeled with 10 µM of CMTMR or 0.5 µM of CFSE, respectively. Equal numbers of WT and LIME^{-/-} T cells were mixed and injected intravenously into recipient wild-type mice. After 4 h, the mice were killed and peripheral (axillary and inguinal) lymph nodes, mesenteric lymph nodes, spleen, and Peyer's patches were collected. The percentage of CD3⁺CFSE⁺ or CD3⁺CMTMR⁺ cells was assessed by Fluorescent Activated Cell Sorting (FACS) analysis.

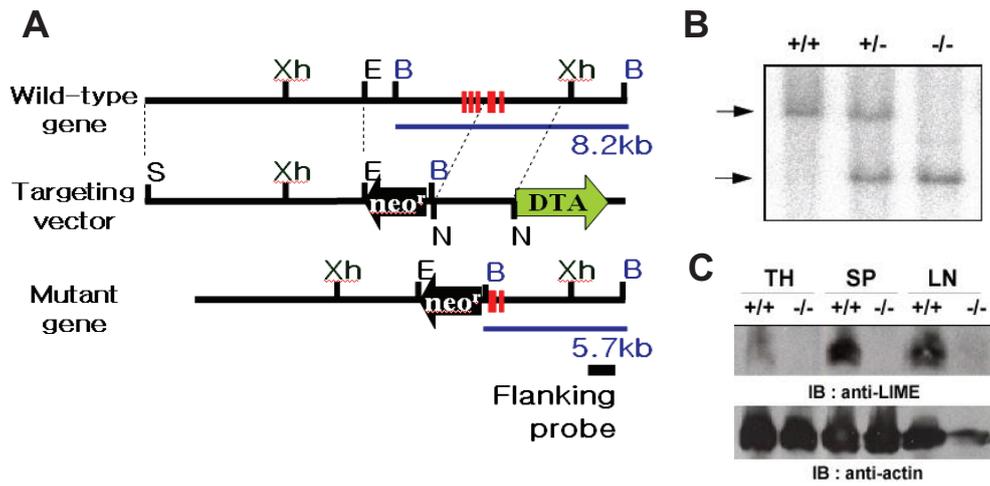
Cell adhesion assay

Flat-bottomed MaxiSorp surface microplates (Nunc) were coated with fibronectin (10 µg/ml; Sigma), laminin (10 µg/ml; Sigma), or recombinant ICAM-1 (2 µg/ml; BD PharMin-

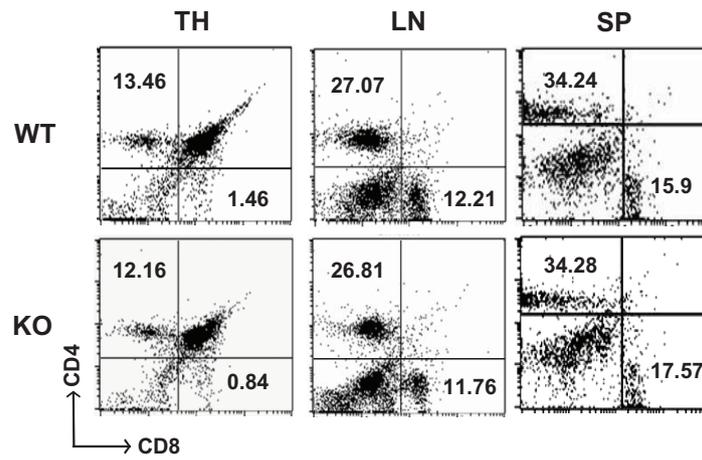
gen) and blocked with 3% BSA (Sigma) in PBS. Activated 4×10^5 lymph node T cells in 0.5% BSA/RPMI were plated in triplicate and allowed to settle for 1 h at 4°C. Subsequently, samples were incubated at 37°C for 30 min and washed with PBS containing 0.5% BSA. Adhered cells were stained with hematoxylin and counted. The experiments were performed three times in duplicate.

Intracellular cytokine staining in T cells

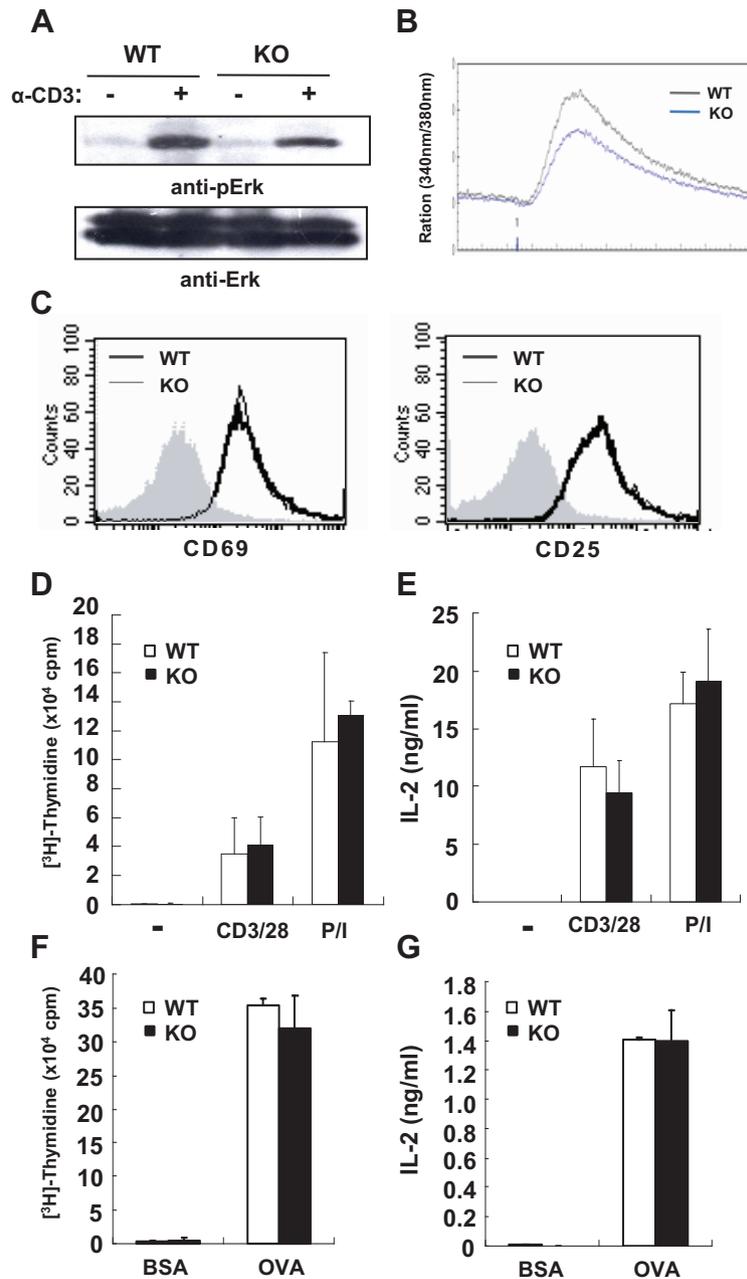
For intracellular cytokine staining, draining lymph node cells isolated from EAE-elicited mice were restimulated with PMA (100 ng/ml) plus ionomycin (0.5 µg/ml) for 6 h in the presence of Golgistop™ (BD Bioscience). Cells were harvested, fixed with 4% para-formaldehyde for 15 min at room temperature, permeablized and stained with indicated antibody in 0.5% saponin/PBS for 30 min at 4°C. After washing twice with 0.5% saponin/PBS and once with PBS, cells were analyzed by flow cytometer.



Supplementary Fig. S1. Generation of LIME^{-/-} mice. (A) Schematic representation of the LIME genomic locus, targeting vector and targeted locus. Hatched boxes represent exons. neo^r, neomycin resistant gene; DTA, Diphtheria toxin. (B) Southern blot analysis of genomic DNA from wild-type (+/+), heterozygous (+/-) and homozygous LIME mutant (-/-) mice. (C) Western analysis of tissue lysates from homozygous LIME mutant (-/-) mice. Alpha-actin was probed as a quantitative control. TH, thymus; SP, spleen; LN, lymph node.

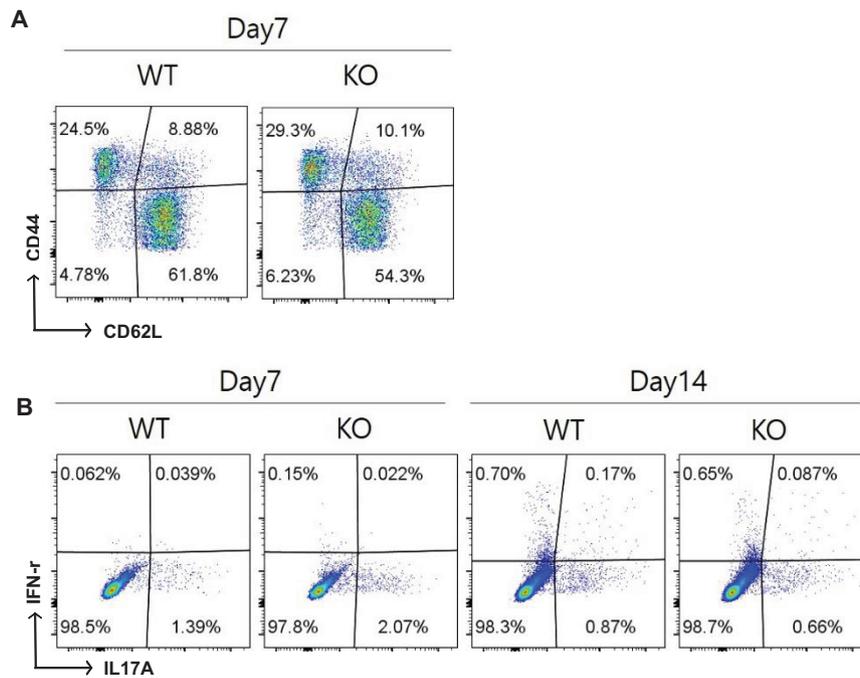


Supplementary Fig. S2. Flow cytometric analysis of T cell development and peripheral T cell subsets in 8-week-old WT (upper panels) and LIME^{-/-} mice (lower panels). Thymus (TH), lymph node (LN), and spleen (SP) cells were stained with anti-CD4 and anti-CD8 mAb. Numbers indicate the percentage of cells in each quadrant.



Supplementary Fig. S3. Analysis of TCR-mediated early T cell activation, proliferation and IL-2 production in WT and LIME^{-/-} T cells.

(A) Lymph node T cells were stimulated with anti-CD3 mAb. Cell lysates were immunoblotted with anti-phosphoERK antibody. (B) Naïve CD4⁺ T cells were pre-loaded with Fura-2/AM at a final concentration of 5 μM and stimulated with anti-CD3 Ab. Intracellular calcium concentration was monitored with an Rf-5301PC spectrofluorophotometer. (C) Naïve T cells from WT or LIME^{-/-} mice were stimulated with plate-coated anti-CD3 mAb (2C11) and anti-hamster IgG (G94-56) for 24 h and were analyzed for the activation markers CD69 and CD25 by flow cytometry. (D-G) For the proliferation assay and measurement of IL-2 cytokine production, lymph node T cells from WT and LIME^{-/-} mice were stimulated with plate-coated anti-CD3 mAb (5 μg/ml) plus soluble anti-CD28 mAb (2 μg/ml) (D and E), and lymph node T cells from LIME^{+/+}/OTII or LIME^{-/-}/OTII mice were stimulated with pOVA-loaded splenic APCs (F and G) for three days. Subsequently, T cell proliferation (D and F) and IL-2 production (E and G) were measured by [³H]-thymidine incorporation and ELISA, respectively.



Supplementary Fig. S4. Effector T cell differentiation in EAE response. (A) At day 7 after MOG35-55 peptide immunization, surface expression of CD44 and CD62L on Thy1.2⁺CD4⁺-gated cells was analyzed by flow cytometer. CD44⁻CD62L⁺ cells, CD44⁺CD62L⁻ cells, and CD44⁺CD62L⁺ cells represent naïve, effector, and memory phenotype T cell population, respectively. (B) At day 7 or 14 after MOG35-55 peptide immunization, draining lymph nodes were isolated and stimulated with PMA (100 ng/ml) plus ionomycin (0.5 μ g/ml) for 6 h in the presence of GolgistopTM, and intracellular cytokine was assessed by flow cytometry analysis. Data are representative of two independent experiments with 4 mice per group.