Molecules and Cells

The Transmembrane Adaptor Protein LIME Is Essential for Chemokine-Mediated Migration of Effector T Cells to Inflammatiory Sites

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Lck-interacting transmembrane adaptor 1 (LIME) has been previously identified as a raft-associated transmembrane protein expressed predominantly in T and B lymphocytes. Although LIME is shown to transduce the immunoreceptor signaling and immunological synapse formation via its tyrosine phosphorylation by Lck, a Src-family kinase, the *in vivo* function of LIME has remained elusive in the previous studies. Here we report that LIME is preferentially expressed in effector T cells and mediates chemokine-mediated T cell migration. Interestingly, in LIME$^{-/-}$ mice, while T cell receptor stimulation-dependent proliferation, differentiation to effector T cells, cytotoxic T lymphocyte (CTL) function and regulatory T lymphocyte (Treg) function were normal, only T cell-mediated inflammatory response was significantly defective. The reduced inflammation was accompanied by the impaired infiltration of leukocytes and T cells to the inflammatory sites of LIME$^{-/-}$ mice. More specifically, the absence of LIME in effector T cells resulted in the reduced migration and defective morphological polarization in response to inflammatory chemokines such as CCL5 and CXCL10. Consistently, LIME$^{-/-}$ effector T cells were found to be defective in chemokine-mediated activation of Rac1 and Rap1, and dysregulated phosphorylation of Pyk2 and Cas. Taken together, the present findings show that LIME is a critical regulator of inflammatory chemokine-mediated signaling and the subsequent migration of effector T cells to inflammatory sites.

Keywords: chemokine, effector T cells, Lck-interacting transmembrane adaptor 1, migration

INTRODUCTION

Transmembrane adaptor proteins (TRAPs) play essential roles in the formation of membrane proximal-signaling complexes to connect cell surface receptors with intracellular signaling pathways (Simeoni et al., 2005). TRAPs form a unique group of adaptor proteins with common structural features: a short extracellular domain, single membrane-spanning alpha-helices, and multiple immunoreceptor tyrosine-based activation motifs (ITAMs) within a long cytoplasmic tail. After ligation of immunoreceptors and other signal-transducing receptors, TRAPs become phosphorylated on tyrosine residues in the cytoplasmic domain, bind SH2 domain-containing cytoplasmic signaling proteins, and thereby activate downstream signaling (Kliche et al., 2004; Simeoni et al., 2005).

Despite common structural features, the localization, expression patterns and functions of TRAPs are specialized. Several TRAPs are expressed in T lymphocytes and, depending

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on their regulatory functions with respect to T cell activation, TRAPs can be grouped as positive or negative regulators. Linker for activation of T cells (LAT) and LIME are regarded as positive regulators of T cell activation (Brdickova et al., 2003; Hur et al., 2003; Weber et al., 1998), while SHP2-interacting transmembrane adaptor protein (SIT), phosphoprotein associated with GEMs (PAG), and linker for activation of X cells (LAX) are proposed to be negative regulators (Kawabuchi et al., 2000; Marie-Cardine et al., 1999; Pfrepper et al., 2001; Zhu et al., 2002).

Originally, LIME was identified as a binding partner of Lck and acts as a positive regulator of immunoreceptor signaling (Brdickova et al., 2003; Hur et al., 2003). Upon T cell antigen receptor (TCR) stimulation, unlike LAT, which is phosphorylated mainly by ZAP-70 or Syk (Sarkar, 1998; Zhang et al., 1998), LIME interacts with and is phosphorylated by Lck, and subsequently recruits cytoplasmic proteins such as Lck, Fyn, p85PI3K, Grb2, Gads, Shp-2, Vav and Csk to membrane-proximal sites (Kliche et al., 2004; Simeoni et al., 2005; Son et al., 2012). When ectopically overexpressed in Jurkat T cells, LIME promoted TCR-mediated signaling pathways that drove IL-2 production (Kliche et al., 2004). LIME also becomes phosphorylated by Lck after cross-linking of the CD4 and CD8 coreceptors and associates with Lck and its negative regulator, Csk, suggesting that LIME also mediates T cell activation though coreceptors (Simeoni et al., 2005). In contrast to these cell line studies, LIME-deficient mice show no significant alteration in T cell development and TCR stimulation-dependent T cell proliferation, calcium flux or IL-2 secretion, which suggests that LIME is dispensable for the activation of naïve T cells (Gregoire et al., 2007). In addition, LIME is also expressed in B cells and promotes B cell antigen receptor (BCR)-mediated activation of NFAT and NF-κB pathways in transformed B cell line (Ahn et al., 2006), while B cell development and BCR-mediated proliferation and Ca^{2+} influx are not altered in $LIME^{-/-}$ mice (Gregoire et al., 2007). Therefore, the in vivo function of LIME has been elusive.

Here, we show a novel function of LIME which is preferentially required for effector T cell-migration in response to chemokine signals. While TCR stimulation-dependent proliferation, differentiation to effector T cells, cytokine production and CTL function were not affected, T cell-mediated inflammatory responses were specifically compromised in the absence of LIME. We also observed the loss of cellular polarization and directional migration in response to chemokines, such as CCR5 and CXCL10, and dysregulated phosphorylation of Pyk2 and Cas signaling protein as well as reduced activation of small-GTPase proteins, such as Rac1 and Rap1 in $LIME^{-/-}$ effector T cells. These results implicate that LIME has critical function for transducing chemokine signaling events in effector T cells. Taken together, we suggest that LIME is an effector T cell-specific adaptor protein required for inflammation response by fine tuning the phosphorylation of signaling proteins and subsequent transduction of chemokine signaling pathways for GTPase activation.

MATERIALS AND METHODS

Mice

$LIME^{-/-}$ mice were generated as described in Supplementary Materials and Methods. Six- to eight-week-old mice were used in the experiments. All $LIME^{-/-}$ mice used in this study were backcrossed more than seven times to C57BL/7 mice. Care of the animals was in accordance with Institutional Animal Care and Use Committee (IACUC) (No. #IACUC 18-078) of Ewha Woman's University.

Flow cytometry analyses

Ab-stained cells were analyzed using FACScaliber (BD Biosciences, USA) CellQuest Software. Anti-CD4-PE, anti-CD4-APC, anti-CD8-FITC, anti-CD25-FITC, anti-CD44-PE, anti-CD62L-APC, anti-CD69-FITC, anti-CCR5-PE, anti-CXCR3-PE, and anti-CXCR4-PE, anti-IFNγ-BV421, anti-IL17A-PE, anti-Thy1.2-BV605 were purchased from BD Biosciences.

Preparation of effector 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, USA). Preparation of T cell blasts and Th1, Th17 and Tc1 cells was performed as described previously (Fang et al., 2010). Briefly, $CD4^+$ T cells or $CD8^+$ T cells were enriched by using $CD4^+$ or $CD8^+$ T cell isolation kit (Miltenyl Biotec) from lymph nodes of 6- to 8-week age $LIME^{+/+}$ or $LIME^{-/-}$ mice according to the manufacturers' instructions. As determined by flow cytometry, > 95% of the enriched cells were $CD4^+$ or $CD8^+$ cell. Enriched $CD4^+$ T cells were cultured at a concentration of 1×10^6 cells/ml in complete RPMI medium supplemented with various cytokines and anti-cytokine mAb as described in Supplementary Materials and Methods. The non-polarized T cell blast (B) and the differentiated effector T cells are harvested after 24 h and 5 days, respectively.

Delayed-type hypersensitivity (DTH) response

DTH response to ovalbumin was analyzed as previously described (Ohta et al., 1997). Briefly, mice were sensitized to pOVA by an intradermal injection of 100 μg of pOVA emulsified with complete Freund's adjuvant (Sigma, USA) at two sites on the back trunk. Seven days after the immunization, the mice were challenged by an injection of 20 μg of pOVA in phosphate-buffered saline (PBS) into one rear footpad, while the other rear footpad received a comparable volume of PBS as a control. Twenty-four hours after the challenge, footpad swelling was measured using a dial caliper (Mitutoyo, Japan). The magnitude of the DTH response was determined as the difference in footpad thickness between pOVA- and PBS-injected footpads.

Contact hypersensitivity (CHS) response

DNFB (2,4-dinitro-1-fluorobenzene)-induced CHS was assayed as described previously (Nakae et al., 2002). In brief, mice were sensitized epicutaneously on the shaved abdomen with 0.2% DNFB (Sigma) dissolved in acetone:olive oil (4:1) on day 0 and 1 and then challenged on the ear on day 5 with 0.25% DNFB. Twenty-four hours after rechallenge, ear thick-

ness was measured. Data shown are from the time point of maximal swelling (24 h). To assay CHS after adoptive transfer, wild-type (WT), LIME$^{+/-}$ and LIME$^{-/-}$ mice were sensitized with 0.2% DNFB and, on day 5, drained lymph nodes from three mice were harvested and pooled, and Thy1.2^{+} T cells were isolated using MACS magnetic beads. After washing in PBS, 1 × 10^{7} T cells were adoptively transferred into WT recipient mice. Adoptively-transferred mice were immediately challenged on the ear with DNFB and ear thickness was measured after 24 h.

Induction of experimental autoimmune encephalomyelitis (EAE)

Myelin oligodendrocyte glycoprotein (MOG) peptide (35-MEVGWYRSPFSRVVHLYRNGK-55) was synthesized by PeproTech (Korea). Ten-week-old female mice were immunized by subcutaneous injection at the base of the tail with 150 μg of MOG peptide emulsified with complete Freund's adjuvant (Sigma-Aldrich, USA) containing 600 μg of heat-inactivated *Mycobacterium tuberculosis* (Difco Laboratories, USA). The immunized mice were injected intraperitoneally with 400 ng of pertussis toxin (List Biological Laboratories, USA) on day 0 and day 2 after immunization and were scored daily for clinical signs of EAE, as follows: 0, normal; 0.5, distal limp tail; 1, complete limp tail; 1.5, limp tail and hind limb weakness; 2, unilateral partial hind paralysis; 2.5, bilateral hind limb paralysis; 3, complete bilateral hind limb paralysis; 3.5, complete bilateral hind limp paralysis and unilateral forelimb; 4, total paralysis of fore and hind limbs; 5, death. The mean onset day of EAE was calculated by averaging the first day of clinical signs for individual mice. The mean maximum score was calculated by adding the highest score for each mouse.

Cytotoxicity assays

The conventional standard [^{51}Cr]-release assay was performed as follows. In brief, WT and LIME$^{-/-}$ mice were injected intraperitoneally with 10^{6} plaque forming unit (PFU) of lymphocytic choriomeningitis virus (LCMV, Amstrong strain). Five days after immunization, CD8^{+} T cells were purified and mixed with gp33-41 peptide-pulsed, [^{51}Cr]-loaded target cells (2 × 10^{4} cells/well) in triplicate at the indicated effector/target cell ratio in 200 μl complete RPMI medium. [^{51}Cr]-labeling of target cells were performed by incubating EL4 lymphoma cells with 200 μCi (3.7 MBq) of Na_2 ^{51}CrO4 (Perkin Elmer, USA) for 1 h at 37°C. After 4 h of co-culture in U-bottomed 96 well plates (Corning, USA), 100 μl of supernatant was collected and the released radioactivity was measured by γ-counter (Perkin Elmer). The percentage of released radioactivity was calculated from the total radioactivity of loaded target cells per well.

Analysis of T cell migration to inflammatory sites

WT and LIME$^{-/-}$ mice were sensitized with DNFB on day 0 and 1, and CD4^{+} T cells were isolated from draining lymph nodes on day 5. Subsequently, WT and LIME$^{-/-}$ CD4^{+} T cells were loaded with carboxyfluorescein diacetate-succinimidyl ester (CFSE; Molecular Probes, USA) or 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR; Molecular Probes), respectively, mixed at a 1:1 ratio, and intravenously transferred to the WT recipient mice (n = 3). Prior to transfer, recipient mice were sensitized with DNFB on their shaved abdomens at day -6 and -5 and rechallenged on their ears at day -1. Twenty four hours after adaptive transfer, distinctly labeled WT and LIME$^{-/-}$ T cells from frozen ear tissue sections were counted in at least 10 randomly selected fields and mean values were calculated from these counts.

Analysis of chemokine-dependent migration

In vitro migration assay was performed as described previously (Park et al., 2007). Briefly, a total of 1 × 10^{6} T cells in 100 μl of serum-free RPMI enriched with 0.3% BSA were loaded in the upper wells of 5-μm pore-size, 24-well Transwell plates (Corning). The Transwell plates were then inserted into wells filled with medium containing CXCL12 (500 ng/ml), CXCL10 (50 ng/ml), or CCL5 (50 ng/ml) and incubated for 3 h at 37°C. Migration was quantified by counting the number of cells that migrated to the lower well. The experiments were performed at least three times in triplicate.

Analysis of chemokine-mediated T cell polarization

WT and LIME$^{-/-}$ Th1 cells were loaded with CFSE (0.5 μM) or CMAC (2 μM), alternatively, and mixed at a 1:1 ratio. Cells were treated with 20 ng/ml CXCL-10 and incubated at 37°C for the indicated time period. After fixation with paraformaldehyde, cells were plated on poly-L-lysine–coated glass slides and stained with allexa-568–labeled phalloidin to visualize actin filaments. Slides were examined with a confocal microscope and up to 10 randomly selected pictures were evaluated in each group.

Analysis of Rac1 and Rap1 activation

Th1 cells were prepared as described above, but after 4 h of serum starvation, 1 × 10^{7} Th1 cells were either left untreated or treated with 50 ng/ml of CXCL10 or CCL5 for 10 min, immediately harvested by centrifugation, and lysed in 500 μl of lysis buffer. To pull down GTP-bound Rac1, cell lysates were incubated with 20 μg of GST-PAK-RBD bound to glutathione-Sepharose beads for 1 h at 4°C. Beads were washed with the same lysis buffer 5 times and bound proteins were analyzed by immunoblotting with ant-Rac1 antibody (Upstate). To isolate GTP-bound Rap1, a GST-RalGDS-RBD pull-down assay was performed using the Active Rac1 Pull-Down and Detection Kit (Thermo Scientific, USA) following the manufacturer's instructions. Proteins isolated using this kit were then analyzed by immunoblotting with included anti-Rap1 antibody.

Statistical analysis

Statistical analysis was performed using the unpaired Student's *t*-test using GraphPad Prism 5 (GraphPad Software, USA). *P* value of < 0.05 was considered significant. All results are expressed as means, with error bars representing SD.

Additional materials and methods

Further details regarding experimental procedures are described in Supplementary Materials and Methods.

RESULTS

T cell development and early activation are unaltered in LIME$^{-/-}$ mice

LIME$^{-/-}$ mice were generated as described in Supplementary Materials and Methods and illustrated in Supplementary Fig. S1A. Correct targeting of the LIME locus was confirmed by genomic Southern and Western blot analysis (Supplementary Figs. S1B and S1C). In LIME$^{-/-}$ mice, no significant abnormalities in the T cell populations from the thymus and secondary lymphoid organs were observed (Supplementary Fig. S2), suggesting that LIME is dispensable in the developmental stage of T cells. Next, we analyzed TCR-mediated activation of LIME$^{-/-}$ T cells. Upon CD3 cross-linking, Erk phosphorylation (Supplementary Fig. S3A) and intracellular calcium flux (Supplementary Fig. S3B) were only marginally affected by the absence of LIME. In addition, upon CD3/CD28 engagement, the expression levels of the activation markers CD69 and CD25 were comparable in WT and LIME$^{-/-}$ T cells (Supplementary Fig. S3C). Upon CD3/CD28 cross-linking, LIME$^{-/-}$ T lymphocytes proliferated normally (Supplementary Fig. S3D), and IL-2 production (Supplementary Fig. 3E), one of the major downstream events of the Ras/MAPK and calcium pathway, was also unaltered in LIME$^{-/-}$ T lymphocytes. Moreover, upon stimulation of CD4$^+$ T cells from LIME$^{-/-}$/OT-II TCR transgenic mice with pOVA-loaded splenocytes, neither defects in proliferation (Supplementary Fig. S3F) nor in IL-2 production (Supplementary Fig. S3G) were observed. Thus, LIME seems not to be required for TCR-mediated activation of naïve T cells. These findings are consistent with a previous report that T cell development and activation are not affected by LIME deficiency (Gregoire et al., 2007).

LIME expression is upregulated in effector T cells

As the first step in our investigation of LIME function, we determined the protein expression levels of LIME at various stages of T cell differentiation. In the CD4$^+$ lineage, LIME expression was barely detected in CD4$^+$CD62L$^+$ naïve T cells but was induced in blast cells prepared by CD3/CD28 cross-linking of naïve T cells for 12 h (Fig. 1A). Furthermore, LIME expression was further upregulated in Th1, Th2, or Th17 effector T cells (Figs. 1A and 1B). Also, in the CD8$^+$ lineage, LIME expression was not detected in naïve CD8$^+$ T cells, but was induced in blast cells and significantly upregulated in Tc1 cells. The expression levels of LIME in Th1, Th2, Th17, and Tc1 cells were comparable and, at least two separate bands of approximately 29 kDa and 31 kDa were detected, possibly representing forms generated by alternative splicing or differential phosphorylation. In contrast to the upregulation of LIME expression in effector T cells, LAT expression was constitutive throughout the T cell differentiation stages (Fig. 1C). The finding that LIME expression was significantly upregulated as T cells differentiated to effector cells implies that LIME is involved in signaling events in effector T cell subsets rather

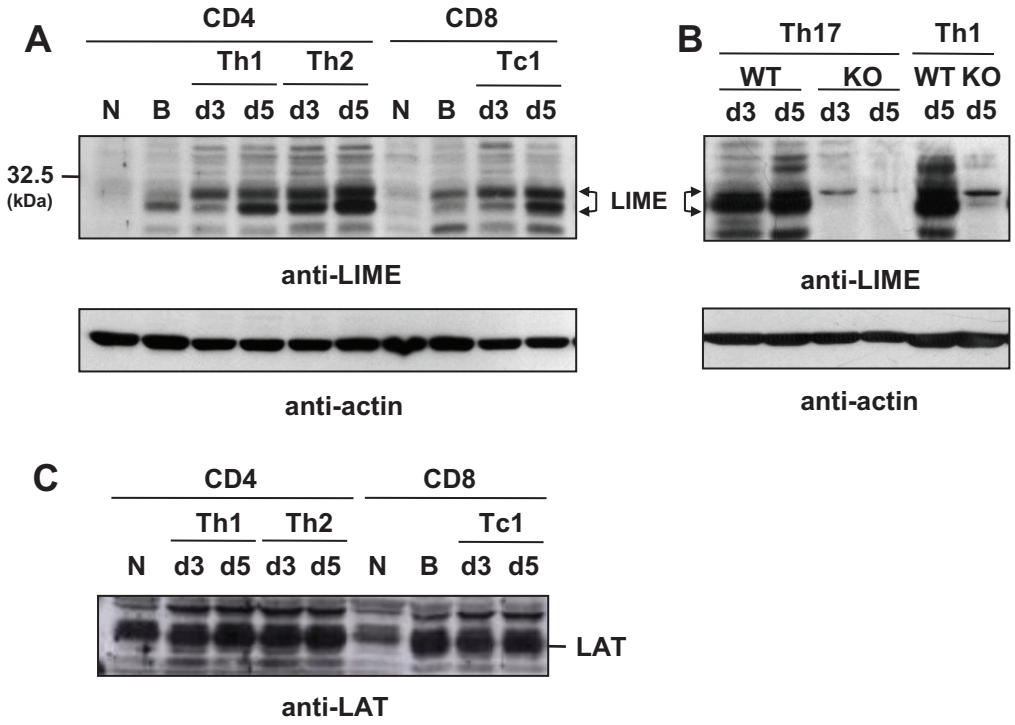


Fig. 1. LIME expression is upregulated following T cell activation. (A and B) Analysis of the expression levels of LIME in different T cell lineages. Various T cell subsets were prepared as described in the Materials and Methods section and include CD4$^+$CD62$^+$ or CD8$^+$CD62$^+$ naïve T cells (N), T cell blasts (B), and Th1, Th2, Th17, and Tc1 cells. In each lane, 25 μg of lysate was loaded and immunoblotted using rabbit polyclonal anti-LIME Ab. As a loading control, the expression level of actin was examined. (C) Analysis of the expression levels of LAT in various T cell lineages.

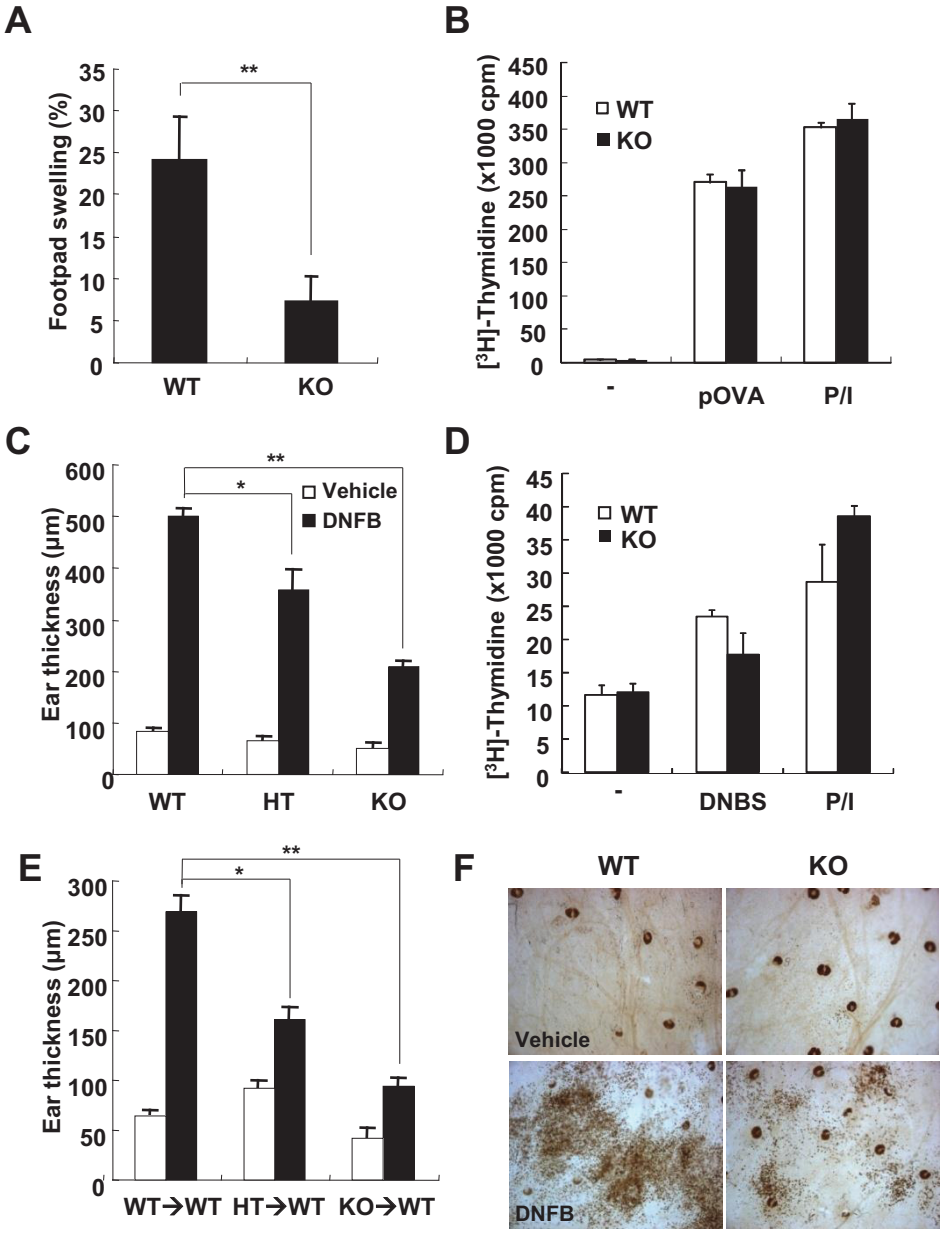


Fig. 2. Impairment of inflammatory responses in LIME$^{-/-}$ mice. (A) DTH responses to pOVA/CFA in WT and LIME$^{-/-}$ (KO) mice were analyzed as described in the Materials and Methods section. Data shown represent the mean percentage of footpad swelling ± SD of each group (n = 5 for WT and n = 6 for LIME$^{-/-}$ mice). The result shown is a representative of three individual experiments. (B) Five days after the initial immunization with pOVA/CFA, CD4$^+$ T cells were isolated from the draining lymph nodes and were then incubated with pOVA-pulsed splenic APCs or PMA/ionomycin (P/I). After three days, proliferation was analyzed by measuring [^{3}H]-thymidine incorporation. (C) CHS responses to DNFB were analyzed in WT, LIME$^{+/-}$, or LIME$^{-/-}$ mice (n = 10 for WT, n = 6 for LIME$^{+/-}$, and n = 9 for LIME$^{-/-}$ mice). Data shows combined data from 2 different experiments. (D) On day 5 after the sensitization with DNFB, lymph node T cells were isolated and incubated with DNBS (50 μg/ml) or P/I (50 ng/ml, 0.5 μg/ml, respectively). After 36 h, cells were pulsed with [^{3}H]-thymidine for 12 h and proliferation was analyzed by measuring the amount of incorporated [^{3}H]-thymidine (n = 5 for WT, n = 3 for LIME$^{+/-}$, and n = 5 for LIME$^{-/-}$ mice). (E) CHS responses were analyzed after adoptive transfer of sensitized T cells. T cells were isolated from the draining lymph nodes of DNFB-pretreated WT, LIME$^{+/-}$ (HT), or LIME$^{-/-}$ (KO) mice and adoptively transferred to naïve WT recipient mice. Recipient mice were rechallenged on the ear with DNFB and ear thickness was measured after 24 h. The result shown is a representative of three individual experiments (n = 3 of each). (F) Histological analysis of CHS-induced ear tissue of WT and LIME$^{-/-}$ mice. Whole mounts of ears were processed as described in the Materials and Methods section and infiltrated leukocytes were stained by DAB staining (50× total magnification). $*P < 0.05$, $**P < 0.01$. Statistical analysis was performed using an unpaired Student's *t*-test.

than in naïve T cells.

DTH and CHS responses are suppressed in LIME$^{-/-}$ mice

As LIME expression is upregulated in effector T cells, we were interested in investigating the involvement of LIME in effector functions of T cells. First, we analyzed the DTH response, which is a typical CD4$^+$ T cell-mediated inflammatory response (Cher and Mosmann, 1987). WT and LIME$^{-/-}$ mice were sensitized by subcutaneous injection of OVA peptide (pOVA) emulsified in Complete Freund's Adjuvant (CFA). Seven days later, pOVA was injected into the right footpad and, 24 h later, the thickness of the footpad was measured. In comparison with WT mice, LIME$^{-/-}$ mice showed significantly reduced footpad swelling responses (< 35%) (Fig. 2A). Notably, pOVA-stimulated [^{3}H]-thymidine incorporation (Fig. 2B) and IFN-γ production (unpublished data) by CD4$^+$ T cells isolated from the draining lymph nodes of WT and LIME$^{-/-}$ mice were comparable seven days after pOVA/CFA immunization, suggesting that the reduced DTH response in LIME$^{-/-}$ mice is not due to an alteration in T cell expansion or differentiation in the sensitizing phase.

To confirm the importance of LIME in the inflammatory response, we also examined the CHS response to reactive hapten, DNFB, in WT and LIME$^{-/-}$ mice. Compared with LIME$^{+/+}$ (WT), LIME$^{+/-}$ (HT), and LIME$^{-/-}$ (KO) mice showed significantly reduced ear swelling responses (up to 69% and 41% of WT, respectively) (Fig. 2C). At five days after the sensitization with DNFB, the activated T cell population (CD62^{low} CD44^{high}) and total T cell number in the draining lymph nodes of LIME$^{-/-}$ mice were similar to those of WT mice, indicating that early T cell activation and expansion occurred normally (unpublished data). In addition, the hapten-specific proliferation of lymph-node T cells of WT and LIME$^{-/-}$ mice mediated by 2,4-Dinitrobenzenesulfonic acid (DNBS), the soluble form of DNFB, was comparable (Fig. 2D).

Subsequently, we assessed the specific function of LIME in the T cell-mediated inflammatory response by excluding the influence of other leukocytes that are involved in acute inflammatory responses. For this, we transferred T cells isolated from the DNFB-sensitized LIME$^{+/+}$ or LIME$^{-/-}$ mice to the recipient WT mice and analyzed CHS response in these recipient mice. Twenty-four hours after rechallenging the rear ear with DNFB, the mice that received LIME$^{+/-}$ (HT) or LIME$^{-/-}$ (KO) T cells showed significantly reduced ear swelling compared with those that received WT T cells (up to 38% and 27% of WT mice, respectively) (Fig. 2E). Taken together, these results show that LIME is critical to T cell-mediated inflammation, and the observed inhibition of the CHS response in LIME$^{-/-}$ mice is due to intrinsic defects in LIME$^{-/-}$ T cells.

In addition, we analyzed the migration of leukocytes to inflammatory sites by histological examination of the ear undergoing CHS (Fig. 2F). Leukocytes were stained brown with DAB substrate through the action of endogenous peroxidase, and blood vessels in the ear pinna were visualized by staining the endothelial glycoproteins as described in the Materials and Methods section. Significant leukocyte infiltration was observed around the blood vessels in DNFB-sensitized WT mice (Fig. 2F, lower left panel). On the other hand, leukocyte infiltration was significantly reduced in LIME$^{-/-}$ mice and leukocytes were restricted near the blood vessels (Fig. 2F, lower right panels). In the vehicle-treated ear (Fig. 2F, upper panels), used as a control, few leukocyte infiltrations were observed, indicating that CHS responses were elicited in an antigen-specific manner. These data show that the defect in inflammatory response observed in LIME$^{-/-}$ mice is largely due to the impaired migration of leukocytes.

Cytokine production by effector CD4$^+$ T cells, cytolytic activity of effector CD8$^+$ T cells and the suppressor function of regulatory T cells (Tregs) are not affected by LIME deficiency

Having found that LIME plays an essential role in inflammatory responses, we sought the mechanism underlying this role. We first investigated whether the proliferation capacity and cytokine production capacity of differentiated Th1 or Th2 cells were intact upon restimulating the polarized T helper cells with anti-CD3ε and anti-CD28 monoclonal antibodies for 24 h. As with naïve T cells, [^{3}H]-thymidine incorporation in both WT and LIME$^{-/-}$ Th1- and Th2-polarized effector T cells was comparable, indicating that the proliferation capacity of effector T cells is not affected by the absence of LIME (Figs. 3A and 3B). Likewise, IL-2 production was also comparable for both WT and LIME$^{-/-}$ Th1 and Th2 T cells (Fig. 3C). In addition, IFN-γ production by Th1 cells, IL-4 production by Th2 cells, and IL-17 production by Th17 cells were not grossly altered in restimulated LIME$^{-/-}$ effector T cells (Figs. 3D-3F). Moreover, *in vitro* suppression assay revealed that the regulatory function of CD4$^+$CD25$^+$ Treg cells was comparable between WT and LIME$^{-/-}$ T cells (Fig. 3G).

In addition, the effector function of CD8$^+$ T cells, as analyzed by *in vitro* cytolytic assays, was similar to or rather slightly increased for LIME$^{-/-}$ cytotoxic T lymphocytes (CTLs) compared with WT CTLs (Fig. 3H), indicating that LIME is not required for the cytolytic activity of CTLs. Taken together, LIME appears to be dispensable for effector functions such as proliferation and cytokine production by polarized Th1, Th2, and Th17 cells, suppressive effects of CD4$^+$ Tregs, and antigen-specific cytolytic activity of CD8$^+$ T cells.

LIME-deficient T cells are defective in their ability to migrate to inflammatory sites

As the proliferation and cytokine production of effector T cells turned out to be normal despite the observed inhibition of DTH and CHS response in LIME$^{-/-}$ mice, we next investigated whether the migration of T cells is affected by LIME deficiency. T cell migration was investigated by tracing the migration of adoptively transferred T cells in inflamed skin undergoing a CHS response. WT and LIME$^{-/-}$ T cells from the DNFB-sensitized mice were loaded with two distinguishable fluorescent dyes, CFSE (green) and CMTMR (orange), mixed at a 1:1 ratio and adoptively transferred to WT recipient mice that had been pre-sensitized with DNFB 24 hours before adoptive transfer. Twenty-four hours after the adoptive transfer, fluorescence microscopic analysis of the skin sections revealed that LIME$^{-/-}$ T cells (CMTMR positive) were observed in much lower frequencies (< 58% of WT T cells) compared with WT T cells (CFSE positive) (Fig. 4A). These results show that LIME is critical for the *in vivo* migration of effector T cells

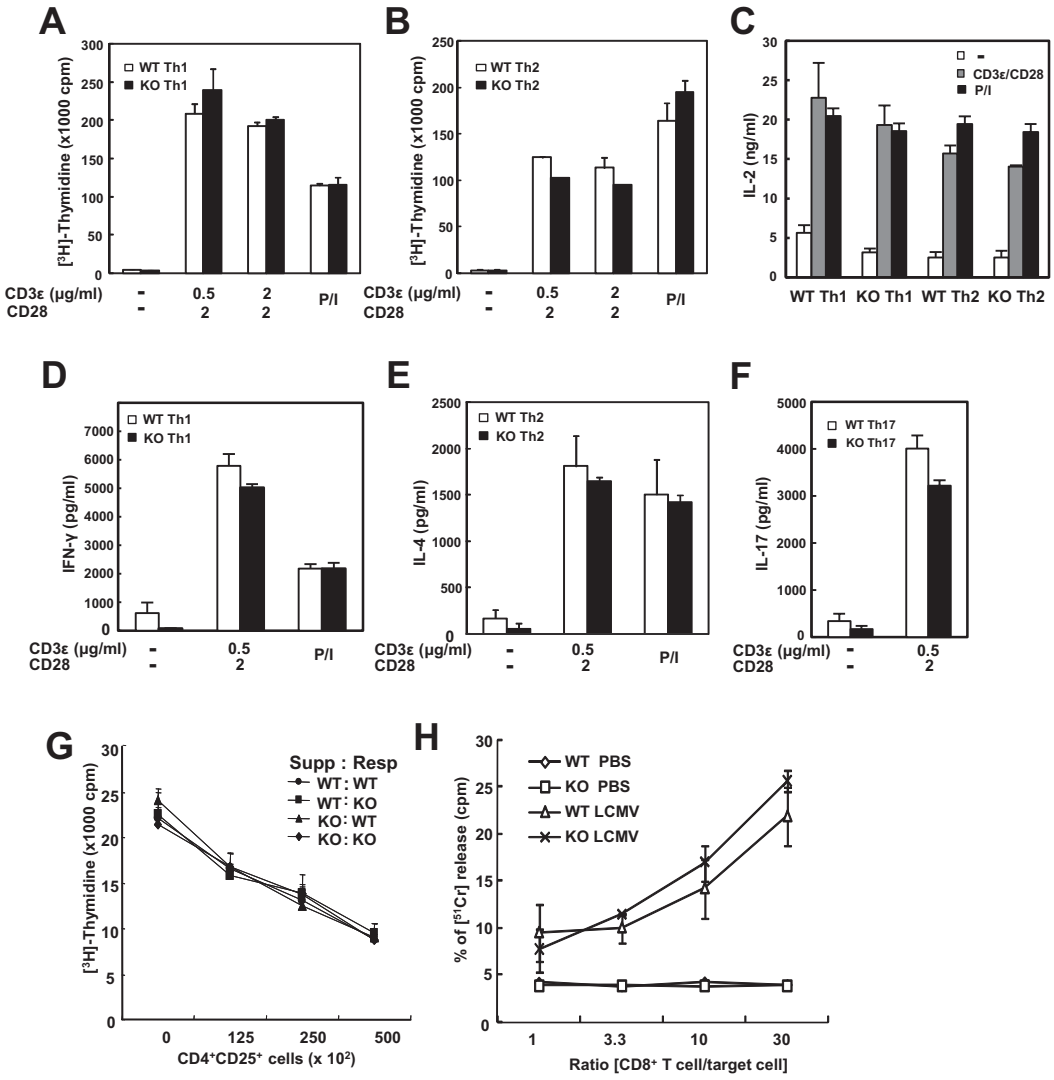


Fig. 3. Cytokine production by effector CD4$^+$ T cells, CTL activity of effector CD8$^+$ T cells and the suppressor function of regulatory T cells are not affected by LIME deficiency. (A and B) Th1 and Th2 cells were generated as described in the Materials and Methods section. Subsequently, Th1 and Th2 cells were restimulated with the indicated concentrations of plate-coated anti-CD3ε and soluble anti-CD28 mAb for 24 h, and proliferation was analyzed by measuring [^{3}H]-thymidine (0.5 μCi/well) incorporation. As a control, cells were stimulated with PMA (50 ng/ml) and ionomycin (0.5 μg/ml) (P/I). (C-E) Th1 and Th2 cells were restimulated with anti-CD3ε (2 μg/ml) and anti-CD28 (2 μg/ml) for 24 h, and the culture supernatant was analyzed for the levels of IL-2 (C), IFN-γ (D), and IL-4 (E) by ELISA. (F) Lymph node CD4$^+$ T cells were cultured under Th17 polarizing conditions for three days, and the amount of IL-17 in the supernatant was measured by ELISA. (G) Analysis of the suppressive function of regulatory T cells. For *in vitro* suppression assays, 1 × 10^5 CD4$^+$CD25$^-$ responder T cells (Resp) together with 1 × 10^5 irradiated WT splenocytes as antigen-presenting cells were incubated with the indicated numbers of CD4$^+$CD25$^+$ T cells as suppressor (Supp) T cells from WT mice or LIME$^{-/-}$ mice. Cells were stimulated with 1 μg/ml anti-CD3ε mAb. Data are presented as mean [^{3}H]-thymidine incorporation. (H) Antigen-specific cytolytic activity of effector CD8$^+$ T cells. WT and LIME$^{-/-}$ mice were intraperitoneally injected with PBS or LCMV and, five days later, splenic CD8$^+$ T cells were isolated and incubated with gp33-41 peptide-pulsed, [^{51}Cr]-loaded EL4 cells at the indicated ratio. Cytolytic activity was measured by the chromium release assay with 4 h-culture supernatant. All of the results shown in this figure are representatives of at least three independent experiments.

into inflammatory sites.

As LIME expression was not detected in naïve T cells, we next investigated whether the homing of naïve T cells to lymphoid organs is affected by LIME deficiency. As shown in Fig. 4B, short-term (4 h) homing of adoptively transferred naïve LIME$^{-/-}$ lymphocytes was similar to that of WT T cells for all examined peripheral lymphoid organs, including Peyer's patches (PP), peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN), and spleens (SP) (Fig. 4B). These results show that LIME is required for the migration of effector T cells to inflam-

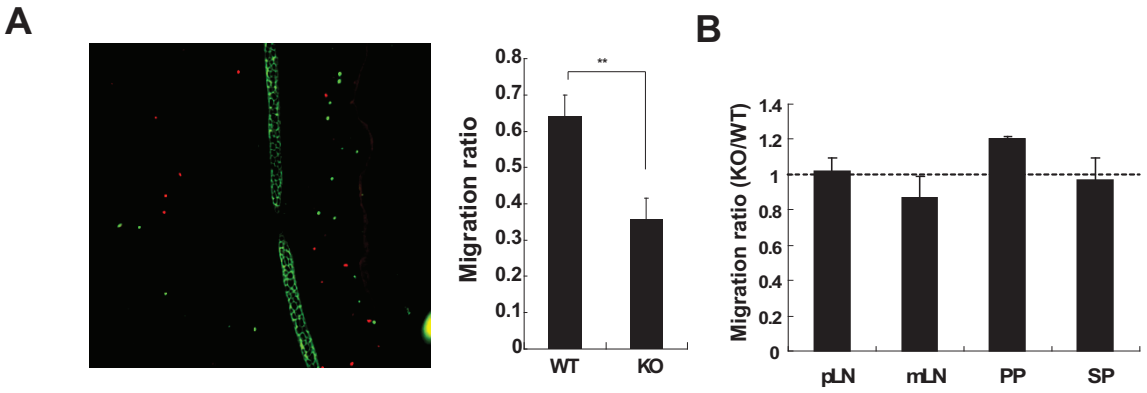


Fig. 4. LIME$^{-/-}$ leukocytes and T cells show defects in *in vivo* migration to inflammatory sites. (A) LIME$^{-/-}$ CD4^{+} T cells were defective in migration to inflamed skin during the CHS response. DNFB-sensitized CD4^{+} T cells from WT and LIME$^{-/-}$ mice were loaded with CFSE and CMTMR, alternatively. Subsequently, a 1:1 mixture of each loaded CD4^{+} T cell sample was injected into the tail vein of a DNFB-pretreated recipient mouse (n = 3). Twenty-four hours after the adoptive transfer, frozen sections of ear skin were analyzed for donor T cell migration with fluorescence microscopy (100× total magnification). Five frozen section of ear skin was analyzed in each experiment, and a representative experiment data is plotted as the ratio of WT or LIME$^{-/-}$ donor cells in each microscopic field (left panel) vs total counted cells (right panel). (B) *In vivo* homing assay of naïve T lymphocytes. Lymph node CD4^{+}CD62L^{+} T cells from WT and LIME$^{-/-}$ mice were loaded with CFSE or CMTMR, alternatively. The same number of loaded cells was mixed and intravenously transferred to WT mice (n = 2). After 4 h, mice were sacrificed and donor cells that had migrated to mesenteric lymph nodes (mLN), peripheral lymph nodes (pLN), Peyer's patches (PP), and spleen (SP) were examined by FACS analysis. The homing ratio represents the ratio of WT vs LIME$^{-/-}$ cells that migrated. Results are representative of two independent experiments. $**P < 0.01$. Statistical analysis was performed using an unpaired Student's *t*-test.

matory sites but not for the homing of naïve T cells.

LIME$^{-/-}$ effector T cells are defective in chemotaxis towards inflammatory chemokines

The migration of effector T cells into inflammatory sites is controlled by specific chemokines (Bonecchi et al., 1998; Mackay, 2001). Especially, the increasing expression of chemokines such as CXCL10 (IP-10) and CCL5 (RANTES) in the subepidermal region drives infiltration of Th1-type CD4^{+} T cells to inflammatory sites (Goebeler et al., 2001). Therefore, the observed defect in migration of LIME$^{-/-}$ effector T cells to inflammatory sites led us to test whether LIME is involved in CXCL10- or CCL5-dependent migration of Th1 cells. The numbers of migrated cells in the lower chamber of transwells were 27.5% ± 1.25% and 15% ± 0.05% of input in response to CXCL10, and 16% ± 0.03% and 7.7% ± 1.7% of input in response to CCL5, for WT and LIME$^{-/-}$ Th1 cells, respectively. When the basal non-specific migration without chemokine (control) is excluded, the number of migrated LIME$^{-/-}$ Th1 cells was significantly decreased to 48.6% and 33% of wild-type Th1 cells in response to CXCL10 and CCL5, respectively (Fig. 5A). On the other hand, migration of naïve T cells toward one of the homing chemokines, CXCL12, was found to be comparable between WT and LIME$^{-/-}$ T cells (Fig. 5B). In these experiments, the expression levels of CXCR4, the receptor for CXCL12, were similar between WT and LIME$^{-/-}$ naïve T cells (Fig. 5C). In addition, the levels of CXCR3 and CCR5, major chemokine receptors for CXCL10 and CCL5 on the Th1 cells, respectively, were upregulated to equal levels in WT and LIME$^{-/-}$ Th1 cells at the time point when the migration assay was performed (Fig. 5C). These results show that the inducible expression of LIME is crucial for Th1 cell migration towards the inflammation-specific chemokines CXCL10 and CCL5. However, the responsiveness of naïve T cells to chemokines is not affected by LIME-deficiency, which is consistent with the finding that *in vivo* homing is not affected by LIME deficiency (Fig. 4B).

In addition to chemokine-related responses, integrin-dependent adhesion of T cells to vascular endothelial cells and to extracellular matrix (ECM) is known to be required for migration to inflammatory sites. Therefore, we also compared integrin-mediated adhesion ability between WT and LIME$^{-/-}$-activated T cells. Peripheral CD4^{+} T cells were isolated and activated by CD3/CD28 crosslinking for 24 h and were assayed for adhesion to fibronectin, laminin and ICAM-1. As shown in Fig. 5D, LIME$^{-/-}$-activated T cells showed reduced adhesion ability to ECM substrates, suggesting that LIME is also involved in integrin-mediated adhesion by activated T cells.

LIME augments chemokine-mediated polarization of effector T cells

After stimulation with chemokines, lymphocytes become polarized, with the leading edge characterized by the accumulation of polymerized F-actin, and these morphological changes are closely related to their migration capacities (Coates et al., 1992). Therefore, we next examined the effect of LIME deficiency on the morphological changes of Th1 cells in response to inflammatory chemokines. WT and LIME$^{-/-}$ Th1 cells were labeled with the differential fluorescent dyes, CFSE (green) and CMAC (blue), respectively, mixed at a 1:1 ratio and incubated with or without CXCL10 for 10 min. After fixing

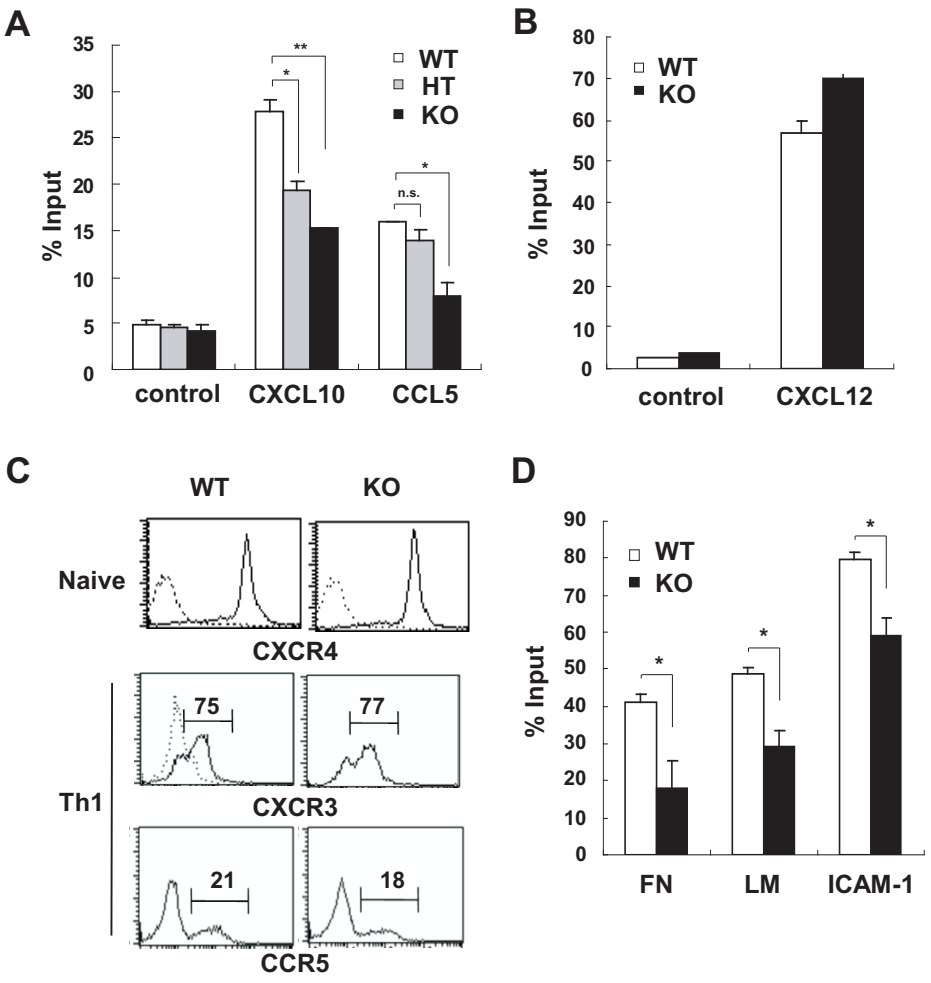


Fig. 5. LIME$^{-/-}$ Th1 cells are defective in migration towards inflammatory chemokines and in integrin-dependent adhesion. (A) Analysis of Th1 effector cell migration towards the inflammatory chemokines CXCL10 and CCL5. The migration assay was performed using Transwell plates (Corning) in the presence of the indicated chemokines, and the percentages of cells that had migrated were plotted. (B) Analysis of the migration of naïve lymph node CD4$^+$CD62L$^+$ T cells towards homeostatic chemokine CXCL12. The percentages of migrated cells (out of total cells tested) are plotted. (C) The expression levels of chemokine receptors in Th1 or naïve T cells from WT and LIME$^{-/-}$ mice. Naïve CD4$^+$CD62L$^+$ T cells isolated from lymph nodes were stained with anti-CXCR4-PE. Th1 cells generated *in vitro* were stained with anti-CXCR3-PE or anti-CCR5-PE. Subsequently, flow cytometric analysis was performed. (D) Analysis of the adhesion of activated T cells to fibronectin (FN), laminin (LM), or ICAM-1. CD4$^+$ T cells from WT and LIME$^{-/-}$ mice were stimulated on anti-CD3 mAb-coated plates with soluble anti-CD28 mAb for 12 h. The harvested CD4$^+$ T cells were allowed to adhere to the FN-, LM-, and ICAM-1-coated 96-well plates. After washing to remove the unattached cells, adhered cells were counted using hematoxylin staining and subsequent microscopic analysis. The experiments described in this figure were performed at least three times in duplicate. $*P < 0.05$, $**P < 0.01$, n.s, not significant. Statistical analysis was performed using an unpaired Student's *t*-test.

and plating on slides, polymerized actin was stained with alexa-568-labeled phalloidin (red). In the absence of chemokine treatment, both WT and LIME$^{-/-}$ Th1 cells showed unpolarized round shapes with evenly distributed filamentous actin in the cytoplasm (Fig. 6A). After 10 min of CXCL10 treatment, WT Th1 cells showed highly polarized shapes and intense F-actin staining was detected in the lamellipodia (> 73% of the cells in the field). However, the majority of LIME$^{-/-}$ Th1 cells remained unpolarized and F-actin accumulation was observed in only a few cells (< 23% of cells in the field), and to a much weaker extent. Similar results were obtained for CCL5 treatment (unpublished data). These observations indicate that LIME is required for the leading edge formation in effector T cells that accompanies chemokine-mediated migration.

In effector T cells, chemokine-mediated activation of the Rac1 and Rap1 small GTPases, Pyk2 and Cas are perturbed in the absence of LIME

To elucidate the LIME-mediated signaling mechanism for promoting effector T cell migration, we examined several chemokine-mediated signaling pathways. Upon CXCL10 treatment, we did not observe differences in calcium flux, Erk

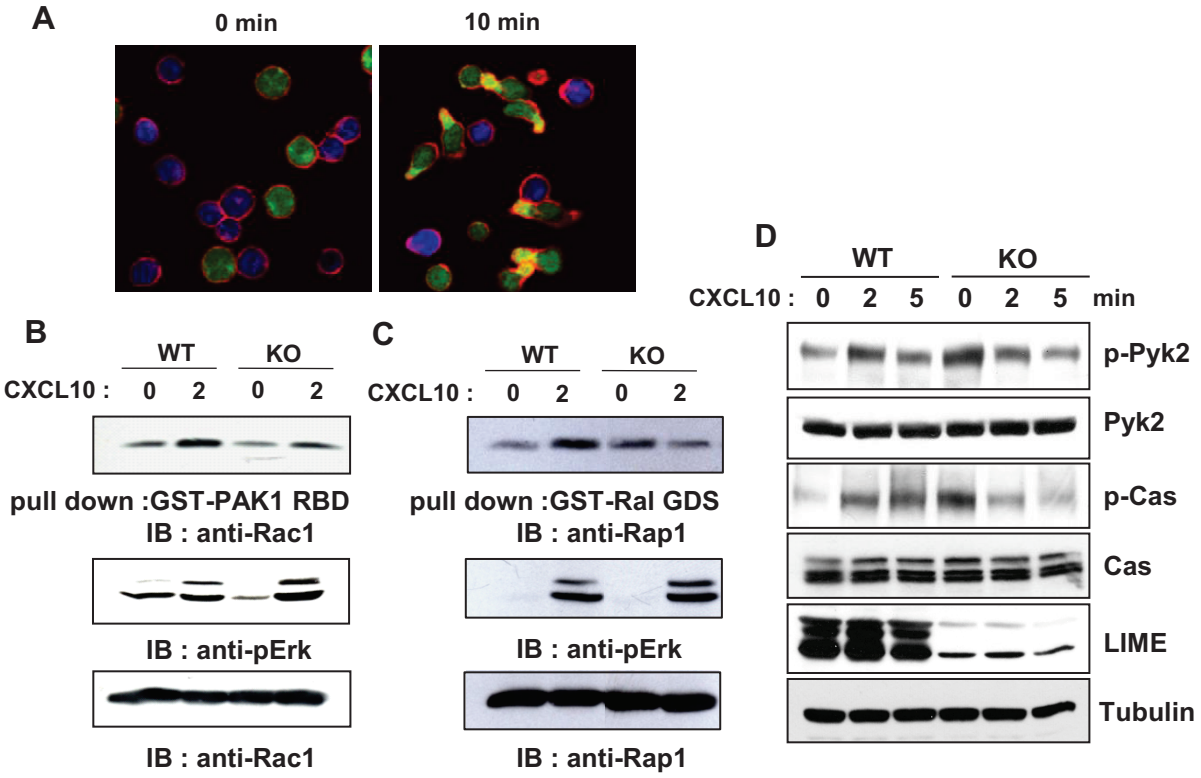


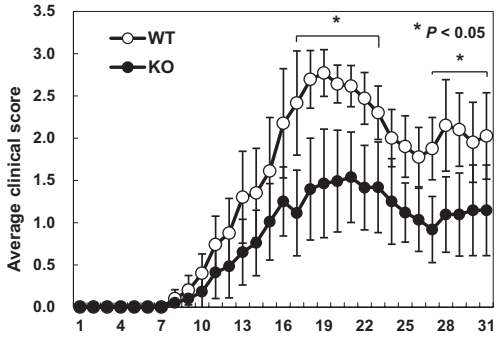
Fig. 6. LIME augments chemokine-mediated T cell polarization. (A) WT and LIME$^{-/-}$ Th1 cells were loaded with CFSE (0.5 μM, green) or CMAC (2 μM, blue), respectively, mixed at a 1:1 ratio, and incubated with or without 20 ng/ml of CXCL-10 at 37°C for 10 min. Cells were fixed and plated on poly-L-lysine-coated slides. Actin filaments were stained with Allexa568-labeled phalloidin (red). Magnification, ×1,000. (B and C) LIME$^{-/-}$ Th1 cells are defective in CXCL10-dependent activation of Rac1 and Rap1 GTPase. WT and LIME$^{-/-}$ Th1 cells were stimulated with or without CXCL10 (50 ng/ml) for 2 min at 37°C. Subsequently, cell lysates were incubated with 20 μg of purified GST-PAK-RBD or GST-Ral-GDS-RBD fusion proteins and glutathione-Sepharose beads for 1 h at 4°C. Pulled-down GTP-bound Rac1 and Rap1 were immunoblotted with anti-Rac1 and anti-Rap1 Ab, respectively (upper panel). As controls for Th1 cell activation, the same lysates were analyzed by immunoblotting with anti-phosphoErk1/2 mAbs (middle panels). Immunoblotting of whole-cell lysates with anti-Rac1 or anti-Rap1 Ab shows that equal amounts of lysates were assayed in each sample (lower panels). Representative results from three independent experiments are shown. (D) The chemokine-mediated phosphotylation of Pyk2 and Cas is altered in LIME$^{-/-}$ Th1 cells. WT and LIME$^{-/-}$ Th1 cells were stimulated with or without CXCL10 (50 ng/ml) for 2 min or 5 min at 37°C. whole cell lysates were examined for immunoblotting with the indicated antibodies. The equal amount of lysates were confirmed with anti-tubulin Ab.

phosphorylation, and Akt phosphorylation between WT and LIME$^{-/-}$ Th1 cells (unpublished data). Next, we examined the activation status of Rac1 and Rap1, small GTPases involved in actin polymerization and inside-out signaling pathways to increase integrin activation (Garcia-Bernal et al., 2005; Shimonaka et al., 2003). Although, considerable levels of GTP-bound activated Rac1 and Rap1 are detected even before chemokine treatment, stimulation of WT Th1 cells with CXCL10 led to the rapid enhancement of the levels of both activated Rac1 and Rap1 (Figs. 6B and 6C). On the other hand, following CXCL10 treatment, LIME$^{-/-}$ Th1 cells showed reduced levels of GTP-bound Rac1 and Rap1 compared with WT Th1 cells (Figs. 6B and 6C, upper panels). Equal loading of lysates was confirmed by immunoblotting with anti-Rac1 or anti-Rap1 antibodies (Figs. 6B and 6C, lower panels). We also analyzed the activation status of Pyk2 and Cas, signaling molecules involved in actin polymerization and inside-out signaling (Dikic et al., 1998; Regelmann et al., 2006). In WT Th1 cells, levels of phosphorylated Pyk2 and Cas increased upon CXCL10 treatment (Fig. 6D). However, in LIME$^{-/-}$ Th1 cells, even before chemokine treatment, basal levels of Pyk2 and Cas were found to be elevated to a level comparable to that observed after CXCL10 treatment in WT Th1 cells. And at 2 min and 5 min after CXCL10 treatment, the levels of phosphorylated Pyk2 and Cas decreased and were significantly lower compared with WT Th2 cells (Fig. 6D). Equal loading of lysates was confirmed by immunoblotting with anti-Pyk2, anti-Cas, and anti-tubulin Abs. The observed perturbation of normal activation of signaling molecules downstream of CXCL10 show that LIME is required for the proper activation of Rac1, Rap1, Pyk2, and Cas in response to chemokine.

Symptoms of EAE are attenuated in LIME$^{-/-}$ mice

We also assessed the severity of the clinical symptoms of EAE, a murine model of multiple sclerosis, as it is a well-established model of the T cell-mediated inflammatory autoimmune re-

A



B

EAE score in LIME knockout mice

Group	Incidence	Mean day of onset (mean ± SD)	Mean maximum score (mean ± SD)
WT	10 of 10 (100%)	10.3 ± 1.2	3.3 ± 0.4
KO	11 of 12 (91.7%)	12.2 ± 2.6	2.3 ± 1.1

Fig. 7. LIME$^{-/-}$ mice are partially resistant to EAE. (A) Using 10-week-old female mice, EAE was induced and mice were scored daily for clinical signs of EAE as described in the Materials and Methods section (n = 10 for WT, n = 12 for LIME$^{-/-}$). $*P < 0.05$. Statistical analysis was performed using an unpaired Student's *t*-test. (B) The mean day of onset of EAE was calculated by averaging the first day of clinical signs for individual mice.

sponse (Stromnes et al., 2008). Two groups of ten WT and twelve LIME$^{-/-}$ mice were immunized with myelin oligodendrocyte glycoprotein (MOG_{35-55}) in CFA and pertussis toxin, and mice were scored for clinical signs of EAE. As shown in Fig. 7, LIME$^{-/-}$ mice showed significantly attenuated symptoms of EAE. The mean day of onset (10.4 ± 0.8 for WT vs 12.7 ± 1.9 for LIME$^{-/-}$) was delayed and the mean maximal clinical score (3.17 ± 0.83 in WT vs 1.91 ± 1.14 in LIME$^{-/-}$) was significantly lower in LIME$^{-/-}$ mice compared with WT mice. In EAE experiment, the proportion of CD44^{+}/CD62L^{-} effector T cells in draining lymph nodes, assayed as a control, was comparable between WT and LIME$^{-/-}$ mice (Supplementary Fig. S4A). In addition, the frequency of effector Th1 and Th17 cells at day 7 and day 14 in the draining lymph nodes of LIME$^{-/-}$ mice was also similar or rather increased slightly compared to WT mice (Supplementary Fig. S4B), suggesting that the reduced disease progression was not due to the alteration in effector T cell differentiation in LIME$^{-/-}$ mice. These results support the above finding that LIME is an important component of the effector T cell-mediated inflammatory response.

DISCUSSION

Five of the eight currently identified TRAPs, LAT, LIME, TRIM, SIT, and Cbp/PAG, are expressed in T cells. Among these, only LAT and LIME positively integrate TCR signaling to downstream events (Brdickova et al., 2003; Hur et al., 2003; Zhang et al., 1998). LIME possesses several features in common with LAT. First, both LIME and LAT are constitutively localized in the lipid raft domains of the plasma membrane by the palmitoylated CXXC motif, which is conserved in raft-targeted TRAPs (Brdicka et al., 1998; Brdickova et al., 2003; Hur et al., 2003). Second, the majority of the binding partners of LIME also bind with LAT. These include Gads, p85 subunit of PI3K, and Grb2 (Hur et al., 2003; Zhang et al., 2000). Interestingly, however, in contrast to LAT, LIME expression is largely upregulated upon T cell activation (Hur et al., 2003). The biological meaning of the inducible expression of LIME has not been examined until now.

Here, to further study the function of LIME at animal level, we analyzed the phenotype of LIME-deficient mice and found that the immunological functions of LIME are different from those of LAT. Firstly, in contrast to LAT, LIME is dispensable in T cell development. LAT$^{-/-}$ mice lack mature single-positive cells in the periphery because LAT$^{-/-}$ thymocytes are blocked at the CD25^{+}CD44^{-} (DN3) stage, in which signaling through the pre-TCR drives the differentiation of CD4/CD8 double negative cells to double positive cells (Zhang et al., 1999). In contrast, LIME deficiency minimally affected to the development of thymocytes and differentiation into mature T cells, which is consistent with the previous finding that the protein expression of LIME is barely detectable in the thymus (Hur et al., 2003). Secondly, LIME is expressed in effector T cells, such as Th1, Th2, Th17 and CTLs, but not in naïve T cells, while the expression of LAT was consistent throughout peripheral T cell differentiation. Consistent with these findings, LIME is dispensable in the initial activation of naïve T cells. LAT$^{-/-}$ T cells show severe defects in calcium flux, Erk phosphorylation, and IL-2 production (Finco et al., 1998). However, LIME-deficiency resulted in only marginally reduced intracellular calcium mobilization and Erk phosphorylation upon TCR engagement. In addition, T cell proliferation, IL-2 production, and the expression levels of activation markers such as CD69 and CD25 were not altered in LIME$^{-/-}$ T cells. Moreover, cytokine production by effector T cells (IFN-γ for Th1 cells, IL-4 for Th2 cells, and IL-17 for Th17 cells) and the surface levels of Th1-type (CXCR3 and CCR5) or Th2-type (CCR3 and CCR4) chemokine receptors were also comparable in WT and LIME$^{-/-}$ T helper cells.

Thirdly, we have shown that LIME is required for inflammatory response. CHS and DTH responses, also known as hapten-specific hypersensitivity reactions, are regarded as antigen-specific T cell-mediated inflammatory responses primarily involving CD4^{+} or CD8^{+} T cell activation (Cher and Mosmann, 1987; Grabbe and Schwarz, 1998). These reactions are characterized by infiltration of effector T cells in the inflammatory lesion and the release of mediators from activated T cells. The T cells then activate local endothelial cells and recruit more leukocytes, which results in local inflammation and swelling. In the sensitization phase of CHS, the CD69^{high} and CD62L^{low} activated T cell population and *in vivo* expansion of CD4^{+} and CD8^{+} T cell population in draining lymph node were comparable in WT and LIME$^{-/-}$ mice (unpublished data), indicating that the reduced inflammation was not due to abnormal T cell priming. However, LIME$^{-/-}$ mice showed reduced inflammation in CHS as well as DTH responses, which was shown by the reduced swelling of tissue and diminished leukocyte infiltration in the hapten-rechallenged ear pinnae of LIME$^{-/-}$ mice. Also, the migration of LIME$^{-/-}$ effector T cells to the inflamma-

tory sites was consistently reduced. In contrast to the involvement of LIME in inflammation, no report has been published implicating the involvement of LAT in inflammation. As the expression of LAT is constitutive throughout all differentiation stages, the observed defect in inflammation in LIME$^{-/-}$ mice suggests that LAT is not able to compensate for the function of LIME in effector T cells.

We have shown that LIME$^{-/-}$ effector T cells have reduced ability to migrate to inflammatory site due to a defect in chemokine-dependent polarization and migration. Effector T cells migrated to inflammatory sites robustly secrete pro-inflammatory cytokines, which promote the production of inflammatory chemokines from epithelial cells and macrophages as well as effector T cell itself, thereby potentiating the recruitment of more leukocytes to the inflammatory sites and further inflammation (Odaka et al., 2007). Since the expression of LIME is limited to T and B cells (Ahn et al., 2006; Gregoire et al., 2007; Hur et al., 2003), the intrinsic migration capacity of other types of leukocytes is not expected to be affected by the absence of LIME. Therefore, it is likely that the reduced number of migrated LIME$^{-/-}$ effector T cells in the inflammatory sites affected the migration of other types of leukocytes into the inflammatory sites, which resulted in the significant reduction in the inflammation. Similarly, resistance to EAE in LIME$^{-/-}$ mice might be due to the effect of the reduced migration of LIME$^{-/-}$ MOG-reactive T cells across the blood-brain barrier.

In this report, we have demonstrated the involvement of LIME in chemokine-mediated migration and integrin-mediated adhesion of effector T cells, two functions which have not been reported for other TRAPs. *In vivo*, activated T cells proliferate and differentiate into effector T cells, and during this process, the expression of receptors for the inflammatory chemokines is upregulated and integrins are activated (Kinashi, 2005). At the same time, presumably, LIME expression is elevated and then the effector T cells become ready to receive chemokine signals. Subsequently, the effector T cells migrate in response to inflammatory chemokines and signals from the ECM, those leading to integrin-mediating adhesion. Interestingly, migration of peripheral naïve T cells toward the homeostatic chemokine CXCL12 was not altered in the absence of LIME, whereas migration toward the inflammatory chemokines CCL5 and CXCL10 was significantly reduced in LIME$^{-/-}$ Th1 cells compared with WT Th1 cells. Consistently, the *in vivo* homing ability of naïve T cells to the secondary lymphoid organs was comparable in WT and LIME$^{-/-}$ T cells, whereas antigen-experienced LIME$^{-/-}$ T cells were impaired in their ability to migrate into inflammatory sites in DNFB-treated mice. These results strongly indicate that enhanced expression of LIME in effector T cells is essential for the amplification of chemokine signals and that the signaling pathways activated by homeostatic chemokines are different from those activated by inflammatory chemokines.

Previously, it has been reported that CD4 cross-linking of Jurkat cell line or conjugation of primary human T cells with anti-CD4-coated beads drive dynamic reorganization of chemokine receptors, integrins and lipid rafts to the sites of CD4 engagement, and Lck is found to be critical in these processes and chemokine- and integrin-mediated migration (Fagerholm et al., 2002; Inngjerdingen et al., 2002; Nguyen et al., 2005). Since LIME is constitutively localized in plasma membrane lipid rafts, interact with and phosphorylated by Lck after cross-linking of CD4 coreceptor, LIME/Lck/CD4-containing lipid rafts may be accompanied by chemokine receptors and integrin (Brdickova et al., 2003; Hur et al., 2003). Several studies shows that Lck is required for downstream signaling of chemokine receptor, including CXCR3 and CCR5 (Dar and Knechtle, 2007; Wong et al., 2001), and this may occur through the interaction with CD4 (Staudinger et al., 2003). Therefore, by this accessibility, LIME may participate in the signaling pathways downstream of the chemokine receptors and integrins by linking Lck and other signaling molecules. In these regards, we have tested the activation of several signaling molecules in responses to CXCL10 in Th1 cells, and found that the activation of Rac1, Rap1, Pyk2, and Cas are affected by LIME deficiency. We have previously showed that LIME mediate TCR stimulation-dependent actin polymerization and integrin clustering through direct interaction with Vav-GEF and Gads, and subsequent activation of Rho family GTPase, Rac1 (Son et al., 2012). It is well known that the small GTPases Rap1 and Rac1 play pivotal roles in cell migration, trigering rapid changes in T cell adhesion and actin cytoskeletal remodeling in response to chemokine signaling as well as TCR engagement. During this processes, phosphorylation of p130Cas/CasL protein is required, which mediates membrane localization of Crk, an adaptor protein selectively promote activation of small GTPase Rap1 and Rac1 (Klemke et al., 1998). Indeed, previous study reported that p130Cas-Crk-C3G-Rap1 signaling axis in T cells were required for effector T cell trafficking into sites of inflammation, but not for migration to lymphoid organs (Huang et al., 2015). Since Pyk2 and p130Cas are also substrates of Lck kinase, and the phosphorylation of tyrosine residues in the cytoplasmic region of LIME mediates protein-protein interaction (Son et al., 2012), it is possible that LIME serves as a transmembrane adaptor for chemokine-mediated tyrosine phosphorylation of these signaling proteins at the plasma membrane proximal sites, and also as a signaling hub to exert inside-out signaling for integrin-mediated cell adhesion and migration. The observation that tyrosine phosphorylation of Pyk2 and Cas is dysregulated in LIME$^{-/-}$ effector T cells in response to chemokine stimulus supports this speculation. However, we failed to observe any differences in other chemokine signaling, such as MAPK (Figs. 6B and 6C) and Akt activation (unpublished data), between WT and LIME$^{-/-}$ Th1 cells. The exact mechanism for the integration of LIME in chemokine signaling remains to be further elucidated.

In conclusion, our data indicate that LIME is essential for chemokine-mediated migration of effector T cells to inflammation sites.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

I.P. conceived and performed experiment and wrote the manuscript. E.A. and M.S. performed experiments. Y.W.K. and Y.Y.K. were in charge of the construction of LIME-deficient mice and provided the mice and reagent. Y.Y. wrote the manuscript, provided idea and secured for funding as a head of project.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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