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J Immunol  published online 12 November 2010
http://www.jimmunol.org/content/early/2010/11/12/jimmunol.1001424

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Actin-Bundling Protein L-Plastin Regulates T Cell Activation

Chen Wang,* Sharon Celeste Morley,† David Donermeyer,‡ Ivan Peng,‡ Wyne P. Lee,‡ Jason Devoss,‡ Dimitry M. Danilenko,‡ Zhonghua Lin,‡ Juan Zhang,‡ Jie Zhou,§ Paul M. Allen,*1 and Eric J. Brown*1

Engagement of TCRs induces actin rearrangements, which are critical for T cell activation. T cell responses require new actin polymerization, but the significance of higher-order actin structures, such as microfilament bundles, is unknown. To determine the role of the actin-bundling protein leukocyte-plastin (L-plastin; LPL) in this process, T cells from LPL−/− mice were studied. LPL−/− T cells were markedly defective in TCR-mediated cytokine production and proliferation. LPL−/− T cells also spread inefficiently on surfaces with immobilized TCR ligands and formed smaller immunological synapses with APCs, likely due to defective formation of lamellipodia. LPL−/− mice showed delayed rejection of skin allografts after release from immunosuppression. Moreover, LPL−/− mice developed much more severe neurologic symptoms in experimental autoimmune encephalomyelitis, which correlated with impaired T cell responses to Ag, manifested by reduced proliferation and production of IFN-γ and IL-17. Thus, LPL-dependent actin bundling facilitates the formation of lamellipodia and normal immunological synapses and thereby enables T cell activation. The Journal of Immunology, 2010, 185: 000–000.

T cell receptor-mediated T cell activation induces immediate rearrangements in the actin cytoskeleton that are critical to TCR signaling. TCR engagement induces transient actin polymerization and accumulation of actin filaments (F-actin) at the interface formed between T cells and APCs (1, 2). This is a site where receptors, integrins, and signaling molecules are specifically segregated to form a structure known as the immunological synapse (3, 4). Inhibition of actin polymerization or induction of its depolymerization disrupts formation of the immunological synapse and T cell activation (5, 6). Actin rearrangement may generate structures that optimize T cell interactions with APCs and create a submembrane scaffold to organize signaling cascades required for T cell activation (7, 8).

Actin monomers can be polymerized into highly branched actin webs or nonbranched long arrays through the action of various proteins of the formin family, such as FMNL1 and diaphanous (Dia1 and Dia2), form normal lamellipodia and immunological synapses but fail to reorient the microtubule organization center (MTOC) toward the immunological synapse (16). This reorientation is important for directional cytotoxicity and cytokine secretion (17–19). These studies suggest that different actin structures participate in different aspects of T cell activation and only a fully reorganized actin cytoskeleton is optimal for full T cell activation.

The structure of the actin network is also regulated through the actions of a variety of actin cross-linking proteins. For instance, actin-bundling proteins simultaneously bind two actin filaments to form parallel arrays of filaments called actin bundles. Plastins are important actin-bundling proteins, characterized by an N-terminal plastin binding domain (20). These NPFs suggest both types of structures contribute to T cell activation (7–11). Highly branched actin webs are usually found in sheet-like structures called lamellipodia, and their formation is promoted by NPFs, such as Wiskott-Aldrich syndrome protein (WASP), a WAVE/SCAR family member (WAVE2), and hematopoietic lineage cell-specific protein 1, acting through the actin-related–protein complex ARP2/3. Depletion of these proteins individually results in poor actin polymerization and weakens the ability of T cells to form immunological synapses with APCs (12–15). In contrast, T cells lacking the nonbranched actin arrays formed by proteins of the formin family, such as FMNL1 and diaphanous proteins (Dia1 and Dia2), form normal lamellipodia and immunological synapses but fail to reorient the microtubule organization center (MTOC) toward the immunological synapse (16).

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1001424

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Received for publication April 30, 2010. Accepted for publication October 13, 2010.

This work was supported by Genentech (to C.W., I.P., W.P.L., J.D.M.D., Z.L., J.Z., and E.J.B.), National Institutes of Health Grants AI-24157 (to P.M.A.) and K08AI081751-01 (to S.C.M.), and the Pediatric Infectious Diseases Society-St. Jude’s Award for Basic Research (to S.C.M.). S.C.M. is a Scholar of the Child Health Research Center of Excellence in Developmental Biology at Washington University School of Medicine (K12-HD01487).

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Abbreviations used in this paper: DRC, differential interference contrast; EAE, experimental autoimmune encephalomyelitis; Hb, hemoglobin; hIgG, hamster IgG; IS, immunological synapse; LAT, linker for activation of T cells; LPL, leukocyte-plastin; MMF, mycoplasmaefolate; MOG, myelin oligodendrocyte glycoprotein; MTOC, microtubule organization center; NPF, nucleation and elongation promoting factor; PFA, paraformaldehyde; PKC, protein kinase C; WT, wild type.
of a defect in cellular polarization (27). LPL accumulates at the immunological synapse in human peripheral blood T cells conjugated to superantigen-loaded APCs, suggesting a role in T cell activation as well (28). However, it is unknown whether LPL is needed for TCR-mediated cytokine production and cell proliferation. Using an LPL−/− mouse, we report in this study that LPL is a positive regulator of TCR-mediated activation both in vitro and in vivo through its effects on TCR ligation-induced actin rearrangement.

Materials and Methods

**Mice**

LPL−/− mice were generated as described (26) and were back-crossed to B6 mice for more than seven generations at Genentech (South San Francisco, CA). BALB/c mice were from The Jackson Laboratory-West (Sacramento, CA). WASP−/− mice were those studied previously (13). n3. L2 TCR transgenic LPL−/− mice were generated at Washington University (St. Louis, MO) (27). Animals used in this study were maintained in accordance with American Association of Laboratory Animal Care guidelines. All experimental animal studies were conducted under the approval of the Institutional Animal Care and Use Committees of Genentech Lab Animal Research.

**Abs**

Anti-CD11a mAb (mM17) was generated by Genentech. Anti-CD3 mAb (clone 500A2), anti-CD28 mAb (clone 37.51), and anti-IL-2 mAb (clone JES6-5H4) were from BD Biosciences (San Diego, CA). Anti–phosphotyrosine (clone 4G10) was from Millipore (Lake Placid, NY). Anti-ERK, phospho-ERK, AKT, phospho-AKT, and phospho-LAT Abs were from Cell Signaling (Danvers, MA). Phalloidin-Alexa Fluor 488 and fluorescence conjugated secondary Abs were from Invitrogen (Eugene, OR). Anti-protein kinase C (PKC)-8 Ab was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pericentrin was from Covance (Berkeley, CA).

**Activation of primary T cells**

Pooled spleen and lymph nodes were forced through a 70-μm cell strainer to generate a single-cell suspension. Cells (2×10⁶) were cultured in RPMI 1640 complete medium (10% FBS, 10 mM HEPES, 50 mM β-mercaptoethanol with antibiotics) in the presence of stimuli. Cells were stimulated with plate-bound anti-CD3 at indicated doses, in the presence or absence of recombinant IL-2 (10 ng/ml; R&D Systems, Minneapolis, MN). For PMA stimulation, PMA (50 ng/ml) and ionomycin (200 ng/ml) were added directly. Cell proliferation was measured as metabolic activity with the MTT assay at the end of the incubation (R&D Systems). Cytokines in the medium were measured by ELISA kits (eBioscience, San Diego, CA). For Ag-specific activation of n3.1L2 CD4+ cells, cell numbers were normalized by n3.1L2 cells and stimulated with irradiated splenocytes in the presence of increasing concentrations of hemoglobin (Hb) peptide (aa 64–76).

**MLRs**

CD3+, CD4+, or CD8+ T cells or cells isolated from peripheral lymph nodes from mice of C57BL/6j background were stimulated with irradiated splenocytes from BALB/c mice for 72 h. Cellular proliferation was measured by incorporation of [3H]thymidine in the last 8 h.

**OVA immunization of mice and T cell restimulation ex vivo**

Age-matching mice (6–8 wk old) were immunized with 100 μg OVA (Sigma-Aldrich, St. Louis, MO) in 30 μl Imject Alum solution (Thermo Scientific, Rockford, IL) by footpad injection. On day 7, draining popliteal lymph node was collected and forced through a cell strainer to generate a single-cell suspension. Cells (2×10⁶) were cultured in RPMI 1640 complete medium with increasing doses of OVA for 72 h. Cellular proliferation was measured by incorporation of [3H]thymidine in the last 8 h of the incubation.

**Quantitative RT-PCR**

Primary CD4+ cells were stimulated with plate-bound anti-CD3. Total RNA was extracted from cells with RNeasy kit (Qiagen, Valencia, CA) and reverse-transcribed with random oligonucleotide primers to cDNA. Quantitative PCR was performed on a 7500 Real Time PCR system with mouse IL-2 TaqMan primers (Applied Biosystems, Foster City, CA). GAPDH level was used to normalize the IL-2 mRNA level.

**Intracellular staining**

Primary CD4+ cells were stimulated in the presence of Golgi-endoplasmic reticulum blocker monensin for 5.5 h. Cells were stained with anti-CD4–allophycocyanin, fixed with 3.7% paraformaldehyde (PFA), permeabilized with 0.1% saponin (eBioscience), stained with anti–IL-2–PE, and analyzed by FACS (FACSCalibur; BD Biosciences, San Jose, CA).

**Immunoblotting**

Beads of 4-μm (Interface Dynamics) were coated with anti-CD3 mAb or control IgG, blocked with PBS/1% BSA, washed, resuspended in PBS, and cooled on ice. Precoupled CD4+ cells were mixed 1:2 with the beads, centrifuged at 300 × g for 5 min, and incubated at 37°C for indicated times. The reaction was stopped by addition of SDS-PAGE sample buffer. Cell lysates were separated by SDS-PAGE, electrophoresed onto polyvinylidene difluoride membrane, and immunoblotted with indicated Abs.

**Calcium influx**

Calcium influx was imaged as previously described in Ref. 29 with modification. Primary CD4+ cells were labeled with 5 μg/ml fura 2-AM and mixed with anti-CD3-coated beads in an oval well glass chamber in imaging buffer (HBSS/0.5% BSA, 2 mM CaCl₂, 1 mM MgCl₂). Imaging was performed with a Zeiss Axiosvert 100TV station (Oberkochen, Germany) equipped with a CoolSNAP_HQ CCD camera (Roper Scientific, Ottobrunn, Germany) at 37°C. Cells were excited alternatively at 340 nm and 380 nm, and emitted light at 510 nm was collected at 10-s intervals for 10 min. Metamorph software (Universal Imaging, Downingtown, PA) was used for image acquisition and analysis. Emission ratio at 340 nm/380 nm was coded 0–255 and represents the relative calcium level. Time zero was defined as the time when the value of 340/380 increased abruptly from background.

**Spreading assay**

T cell spreading on anti-CD3-coated surface was assayed as previously described in Ref. 30 with modification. For lower-resolution imaging, CD4+ cells were seeded into a well of a 48-well plate coated with 5 μg/ml anti-CD3 mAb and incubated at 37°C until directly fixed with 2% PFA at indicated times. Phase-contrast images were taken with an Olympus DSU ×40, numerical aperture 0.7, Tokyo, Japan). Higher-resolution time-lapse imaging was carried out with a Nikon Eclipse Ti with Perfect Focus (Tokyo, Japan). CD4+ cells were added to a glass chamber coated with 5 μg/ml anti-CD3 Ab, and phase-contrast images were collected at 10-s intervals for 30 min (×60, numerical aperture 1.4). Spreading cells were counted manually; cells showing more than eight thin spike-like protrusions in more than half of the images obtained during the first 5 min after landing to the glass were counted as having “filopodial spikes.” The percentage of cells with filopodial spikes was quantitated. For assays of actin cytoskeleton, CD4+ cells were added to cover glasses coated with 5 μg/ml anti-CD3 mAb that contained 100 μM imaging buffer, and the cells were allowed to adhere and spread at 37°C for the indicated time. Cells were fixed with 2% PFA, permeabilized with 0.2% Triton X-100, and stained with phalloidin-Alexa Fluor 488. Fluorescence microscopy was performed with a Nikon Eclipse Ti. Cell area and shape factor (defined as 4πAp², where Ap = perimeter) based on fluorescence were automatically measured and calculated by Metamorph software based on the cell boundary defined by fluorescence above threshold. Cells with area greater than one third of average area of wild type (wt) spreading cells were selected.

**Conjugate formation**

To form T cell-APC conjugates, CH27 cells were pulsed with Hb peptide at indicated concentrations at 37°C for 3 h. n3.1L2 CD4+ cells and Hb-pulsed CH27 cells were labeled with succinimidyl esters of Alexa Fluor 633 and 488 (Invitrogen), respectively, according to the manufacturer’s manual. The two cells were mixed in a 1:1 ratio, spun briefly, and incubated at 37°C for 30 min. Cells were fixed with 1% PFA and immediately analyzed by FACS.

**Confocal immunofluorescence microscopy**

For synapse analysis, conjugates were formed as above with n3.1L2 CD4+ cells and CH27 cells pulsed with 1 μM Hb peptide. Cells were settled on poly-L-lysine–coated cover glass, fixed, permeabilized, and stained with...
phalloidin-Alexa Fluor 488 and anti–PKC- \( \mu \)Ab followed by Alexa Fluor 555-secondary Ab. Imaging was performed with a Zeiss LSM 510 META or Leica SPE confocal microscope (Wetzlar, Germany), with \( Z \) stacks of 0.3 \( \mu \)m (\( \times 63 \), numerical aperture 1.4). The dimension of the immunological synapse was defined as the maximal length of F-actin accumulation across the synapse through all slices. For cytokine vesicle and MTOC polarization, the conjugates were incubated for 4 h at 37 °C, fixed, permeabilized, and stained with rabbit anti-pericentrin and rat anti-mouse IL-2 Abs, followed by secondary Abs conjugated with Alexa Fluor 555 and 488, respectively.

Skin graft rejection

Full-thickness tail skin grafts (10 mm diameter) were collected from donors and transplanted to the dorsal thorax of recipient mice using 6-0 nylon. A protective bandage was applied to the skin graft after transplantation. For recipient mice that were treated with immune suppressors: 1 mg anti-CD11a (mM17) was injected in 100 \( \mu \)l saline per mouse on day 2 followed by 0.5 mg per mouse i.p. every other day for 2 wk; mycophenolate mofetil (MMF, Roche, Basel, Switzerland) at 50 mg/kg was injected i.p. daily in 200 \( \mu \)l saline starting on day 1 for 2 wk. Recipient mice were monitored daily after the skin graft surgery, and graft rejection was scored daily after bandages were removed on day 8. Rejection was defined as a complete loss of live graft tissues.

Experimental autoimmune encephalomyelitis

Eight- to fourteen-week-old female mice were immunized s.c. with 200 \( \mu \)l emulsion containing 300 \( \mu \)g myelin oligodendrocyte glycoprotein (MOG) peptide (aa 35–55) and 800 \( \mu \)g Mycobacterium tuberculosis in CFA (Difco Lab, Detroit, MI) on day 0, followed by i.p. injection of 200 ng pertussis toxin (List Biological Lab, Campbell, CA) on day 0 and day 2, as previously described (31). Clinical signs were scored using the following grading system: 0 = no overt signs of disease; 1 = limp tail or hind limb weakness; 2 = limp tail and hind limb weakness; 3 = partial hind limb paralysis; 4 = complete hind limb paralysis; 5 = moribund or complete hind limb paralysis with moderate to severe forelimb paralysis.

Histology and immunohistochemistry

On day 28 postimmunization, mice were euthanized. Spinal cord and brain were removed, fixed in 10% neutral buffered formalin, paraffin embedded, and sectioned and stained with H&E for inflammation and with Luxol fast blue staining for evaluation of demyelination. To score inflammation and demyelination in spinal cord, four sections each from the cervical, thoracic, and lumbar levels (for a total of 12 sections) were scored as follows: 0 = no significant findings; 1 = minimal; 2 = mild; 3 = moderate; 4 = marked. The mean histologic score for inflammation and demyelination was calculated for each mouse. For immunohistochemistry, formalin-fixed, paraffin-embedded sections were stained with the mAb F4/80 (Serotec, Raleigh, NC) to detect macrophages or with a goat anti-mouse CD4 (R&D Systems). Positive staining was visualized using a biotin immunoperoxidase system (Vector Laboratories, Burlingame, CA).

Statistical analysis

Statistical difference between different groups was analyzed by JMP software (SAS, Cary, NC). The \( p \) values for survival time of skin grafts and...
We first examined T cell maturation in LPL−/− mice in which decreased LPL expression resulted in diminished TCR Abs (5). These findings are also consistent with a very recent report in which decreased cell death.

Results
LPL−/− T cells are defective in TCR-mediated activation

We first examined T cell maturation in LPL−/− mice by measuring expression of several surface markers on peripheral CD4+ T cells. LPL−/− and wt CD4+ T cells showed similar expression of CD3 and contained comparable proportions of memory cells (CD62Llow CD44high) and natural regulatory cells (CD25high) (Fig. 1A). This suggests that LPL−/− and wt CD4+ cells are phenotypically similar.

To explore how LPL deficiency affects TCR-mediated CD4+ T cell activation, we measured cell proliferation in response to plate-bound anti-CD3. To test if LPL plays a distinct role from WASP, we measured proliferation in response to immobilized Ags but not to cross-linked soluble anti-CD3 (data not shown). Ligation of costimulatory receptors by CD3 (data not shown). Ligation of costimulatory receptors by anti-CD28 only partially rescued the IL-2 production defects (Fig. 1C). As has been reported for WASP−/− cells (14), the proliferation defect of LPL−/− cells was less severe at a higher dose of anti-CD3 (Fig. 1C). Viability of nonstimulated cells and activation-induced apoptosis were not affected by LPL deficiency (data not shown), suggesting that impaired proliferation in LPL−/− T cells is not due to increased cell death.

Our observation that exogenous IL-2 can partially rescue the proliferation defect in LPL−/− CD4+ T cells prompted us to investigate their ability to produce IL-2. As shown in Fig. 1D, LPL−/− as well as WASP−/− cells showed a marked defect in IL-2 production. Similar to CD4+ cells, primary LPL−/− CD8+ T cells also produced less IL-2 when stimulated with plate-bound anti-CD3 (data not shown). Ligation of costimulatory receptors by anti-CD28 only partially rescued the IL-2 production defects (Fig. 1E). In contrast, LPL−/− CD4+ cells showed no defect in IL-2 production when stimulated with soluble anti-CD3 plus anti-CD28 or with PMA plus ionomycin (data not shown), demonstrating there was no intrinsic IL-2 synthesis or secretion disability in these cells. This result is consistent with earlier findings that disruption of F-actin with F-actin depolymerization drugs impaired T cell responses to immobilized Ags but not to cross-linked soluble anti-TCR Abs (5). These findings are also consistent with a very recent report in which decreased LPL expression resulted in diminished T cell proliferation in response to stimulation with APCs loaded with superantigen but not in response to Ab stimulation (32).

We did not see a difference in TCR ligation-induced proliferation or IL-2 production between wt and LPL−/− heterozygous CD4+ cells (data not shown), suggesting that one allele of LPL is sufficient to exert its normal function. The defects in LPL−/− cells were not restricted to mice of the B6 background, as they were observed in BALB/c background as well (data not shown).

FIGURE 2. LPL−/− T cells are defective in Ag-specific responses. A, n3L2 CD4+ cell responses to Hb peptide. Cells were stimulated with irradiated splenocytes in the presence of Hb peptide and IL-2 secreted was measured at 24 h. Shown are means of duplicates, a representative of three independent experiments. B, T cell responses to immunized OVA. On day 7 postimmunization, cells isolated from draining lymph nodes were restimulated with OVA, and proliferation was measured by incorporation of [3H]thymidine. Shown are means ± SD of five mice per group. C, MLR. CD3+ T cells in B6 background were stimulated with irradiated BALB/c splenocytes, and proliferation was measured by incorporation of [3H]thymidine. Shown are means ± SD of triplicates.

FIGURE 3. LPL−/− CD4+ cells are defective in TCR ligation-induced IL-2 mRNA and protein. A, IL-2 mRNA level. CD4+ cells were stimulated with plate-bound anti-CD3 at low (2 μg/ml) or high (10 μg/ml) doses. At 4 or 24 h, the IL-2 mRNA level was measured by quantitative RT-PCR and standardized to GAPDH levels. Error bars represent means ± SD of triplicates, a representative of three independent experiments. B, Intracellular staining of IL-2 protein. CD4+ cells were stimulated by plate-bound anti-CD3 (10 μg/ml) or PMA plus ionomycin for 5.5 h in the presence of monensin. The IL-2 protein was measured by intracellular staining assayed by FACS. Quantitation shows percentage of IL-2+ cells out of CD4+ cells after anti-CD3 stimulation and averaged 1.6 ± 0.3% in wt compared with 0.8 ± 0.03% in LPL−/− CD4+ cells from three independent experiments.
**LPL**−/− T cells are defective in Ag-specific responses

We then tested Ag-specific responses by using T cells expressing transgenic TCR n3.L2, which recognizes the d allele of Hb peptide (aa 64–76) bound to I-Ek. Primary LPL−/− n3.L2 CD4+ cells produced less IL-2 when stimulated with Hb peptide Ag at all concentrations tested (Fig. 2A). TNF-α and IFN-γ production was similarly reduced (data not shown). To determine whether there are Ag-specific activation defects in nontransgenic LPL−/− mice, we immunized mice with OVA in alum and then measured T cell proliferation after restimulation with OVA ex vivo. The LPL−/− cells responded more poorly than wt cells at all OVA concentrations tested (Fig. 2B). To measure responses of LPL−/− T cells to more physiological stimuli, MLRs were performed. LPL−/− CD3+ T cells displayed a significant proliferation defect in response to alloantigens (Fig. 2C). Dependence of proliferation on LPL remained when purified CD8+ or CD4+ cells were used as responder cells (data not shown). These data indicate that LPL is important for T cell activation in response to various TCR ligands.

**LPL is required for normal upregulation of IL-2 mRNA in CD4+ cells after TCR ligation**

To begin to understand the mechanism by which LPL influences T cell activation, we measured the level of IL-2 mRNA and intracellular protein in primary CD4+ cells stimulated with plate-bound anti-CD3. The IL-2 mRNA level in wt cells was up to twice as much as that in the LPL−/− cells as early as 4 h, using either low (2 μg/ml) or high (10 μg/ml) plate-bound anti-CD3 stimulation (Fig. 3A, left panel), a difference that was increased on longer incubation (Fig. 3A, right panel). This difference was paralleled by the percentage of CD4+ cells that stained for intracellular IL-2 (Fig. 3B). The small number of IL-2+ cells after anti-CD3 stimulation likely reflects the fact that naive cells were used, as PMA and ionomycin stimulation gave robust IL-2 staining (Fig. 3B). As expected, LPL deficiency did not affect the number of IL-2+ CD4+ cells after stimulation by PMA plus ionomycin.

**LPL is not required for early TCR signaling**

Disruption of actin polymerization attenuates early TCR signaling in both the Jurkat T cell line and naive murine CD4+ cells (33, 34). To test if LPL deficiency affects early TCR signaling, we examined multiple biochemical events that occur shortly after T cells are stimulated with anti-CD3 immobilized on beads. As shown in Fig. 4A, the tyrosine phosphorylation profile in LPL−/− cells after TCR engagement displayed a very similar pattern and kinetics to those of wt cells. This similarity remained when cells were stimulated with plate-bound anti-CD3 at low or high dose (data not shown). Further analysis showed that phosphorylation of ZAP70 and linker for activation of T cells (LAT), key events in the assembly of the tyrosine kinase signaling cascade, were normal (Fig. 4B). Although the full range of calcium influx induced by immobilized anti-CD3 requires actin polymerization (5), it is not affected by LPL deficiency (Fig. 4C), even though LPL was required for IL-2 production in response to T cell activation by the anti-CD3–coated beads used in this experiment (Fig. 4D). Lack of a requirement for LPL in TCR- or chemokine-mediated calcium signals has been reported very recently (32, 35). Activation of downstream kinases AKT and ERK (Fig. 4B, 4D) was comparable in wt and LPL−/− cells, further suggesting that early TCR signaling is intact in the absence of LPL. Consistent with this, up-regulation of expression of CD69 and CD25 after T cell stimulation were unaffected by LPL deficiency (Ref. 27 and data not shown), suggesting the signals required for IL-2 and CD69 or CD25 are regulated differentially by LPL.

**FIGURE 4.** Proximal TCR signaling is normal in LPL−/− CD4+ cells stimulated by immobilized anti-CD3. A, B, and D, Immunoblotting. CD4+ cells were mixed with anti-CD3–coated beads at 37˚C for indicated time. Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with Abs against phospho-tyrosine or GAPDH as loading control (A), phosphorylated forms of ZAP-70, LAT, and AKT (B), and total or phospho-ERK (D). C, Calcium flux. Fura 2-AM–labeled CD4+ cells were mixed with anti-CD3–coated beads. Time-lapse images were collected at emission 510 nm/excitation 340 or 380 nm, 10 s/frame, for 10 min. The average ratio of emission 340 nm/380 nm (coded 0–255) of multiple cells (LPL−/−, n = 18; wt, n = 22) is plotted over time. E, IL-2 produced by CD4+ cells stimulated with hamster IgG (hIgG)– or anti-CD3–coated beads. Shown are average of duplicates, a representative of two experiments.

LPL \( ^{-/-} \) CD4\(^+\) cells exhibit abnormal actin dynamics and cell spreading on anti-CD3–coated surface

F-actin is important for T cell activation through regulating cell spreading and directing the formation of lamellipodia on the surface presenting TCR ligands (30, 34, 36). We compared the spreading of wt and LPL \( ^{-/-} \) CD4\(^+\) cells on anti-CD3–coated surfaces via phase-contrast microscopy. Spreading cells appeared phase dark (indicated by arrows). Original magnification \( \times 40 \). Scale bar, 5 μm. B. Percentage of spreading cells quantitated after incubations of 5 and 30 min. Shown are means ± SEM of four fields from two independent experiments (each field with \( > 100 \) total cells). *C. CD4\(^+\) cells were seeded onto glass chamber wells coated with anti-CD3. Phase-contrast images were captured at 10-s intervals for 30 min at 37°C. Shown are single frames at the indicated time points. Time zero is defined as the point when the cell started to attach to the glass. Original magnification \( \times 60 \). D. Percentage of cells with \( > 8 \) filopodia-like spikes in more than half of the images acquired in the first 5 min of spreading. Spreading cells (\( n = 20 \) per group) were chosen randomly from six videos from two independent experiments. E. Actin cytoskeletal structure of spreading CD4\(^+\) cells. Cells were seeded onto anti-CD3 mAb-coated cover glass for indicated times, fixed and permeabilized, stained for F-actin by phalloidin-Alexa Fluor 488, and analyzed by wide-field fluorescence microscopy. Shown are representative cells from two independent experiments. Original magnification \( \times 60 \). F and G. Change of cell area and shape of spreading cells. Cell area (F) and shape factor (G) were analyzed and are shown as mean ± SEM of multiple cells (\( n > 30 \) per group per time point) from different fields. *p < 0.05.
actin ring, which nonetheless failed to coalesce to the same extent as in wt cells during the 60-min assay. Additionally, there was no significant increase in cell area of LPL^{−/−} cells at all time points tested (Fig. 5F). The irregularity of cell shape caused by actin structure arrangement can be characterized by a parameter called the shape factor, which ranges from 0 to 1 (37). Shape factors closer to 1 represent cells with a rounder shape; whereas lower shape factors describe a greater irregularity of the edge. The time course of changes in the shape factor of wt cells corresponded with the morphological changes visualized by fluorescence microscopy. At 2 and 5 min after contact with the coverslip, wt cells have spread and extended smooth lamellipodia, which is represented by the shape factors close to 0.8 (Fig. 5E, 5F). Wt cells then retracted the lamellipodia after ~15 min, leaving behind narrow projections (Fig. 5E). These narrow projections result in a more irregular edge and a correspondingly lower shape factor around 0.7 (Fig. 5G). Wt cells maintained this retracted morphology through the 60-min time point. The different kinetics and morphology of LPL^{−/−} T cells during the spreading assay are also captured by the shape factor. At 2 and 5 min, LPL^{−/−} T cells have extended mostly thin filopodia rather than smooth lamellipodia and therefore have a decreased shape factor compared with that of wt cells. However, the
delayed extension of smaller lamellipodia is represented by the increase in shape factor observed at 15 min in LPL−/− cells, the time at which wt cells have already undergone retraction. The LPL−/− cells do not maintain this morphology and are smaller (Fig. 5F) and much spikier by 60 min (Fig. 5E), as is represented by the shape factor of almost 0.5 at this time point (Fig. 5G). For both wt and LPL−/− cells, shape change was specifically induced by TCR ligation and depended on F-actin polymerization, as the cells treated with an actin depolymerizing agent (latrunculin B) or attached to poly-1-lysine–coated cover glass showed only round cells with weak phalloidin staining (data not shown). The dynamic actin rearrangement upon TCR ligation has been reported to involve several actin-polymerizing proteins that are important for forming a lamellipodium with highly branched actin webs (12, 15, 16). Thus, although actin bundling has not been known previously to have a role in this process, LPL is required for the normal lamellipodia formation induced by TCR engagement.

LPL is required for F-actin accumulation at the immunological synapse but not for MTOC polarization

The abnormality of cell spreading onto anti-CD3 surface suggested LPL deficiency might affect Ag-specific interactions with APCs. To test this possibility, we examined conjugate formation between n3.L2 CD4+ cells and Hb peptide-loaded CH27 cells as APCs by FACS (Fig. 6A). As quantitated in Fig. 6B, n3.L2 LPL−/− T cells exhibited a modest defect in forming conjugates with APCs at all peptide concentrations tested, suggesting that LPL facilitates T cell interaction with APCs. To explore further how the interaction with APCs was affected in LPL−/− cells, we examined the structure of the immunological synapses more carefully by confocal immunofluorescence microscopy. Conjugated LPL+/− and LPL−/− CD4+ cells both displayed accumulations of F-actin and a critical downstream effector, PKC-θ, at the synapse (Fig. 6C). However, conjugated LPL−/− cells appeared to form smaller synapses as measured by F-actin accumulation at the T-APC interface, which extended to 5.9 μm on average in LPL+⁄− cells compared with 4.9 μm in LPL−⁄− cells (Fig. 6D). Because actin-driven lamellae can form proximal to, but not as part of, the interface, we also measured interface diameter by differential interference contrast. This measurement again showed that LPL−⁄− CD4+ cells form a smaller synapse than that formed by heterozygous LPL+/− cells (Fig. 6D). This result is consistent with the spreading defects of LPL−⁄− cells on an anti-CD3–coated surface and suggests that LPL is important in extending the contact area with APCs and therefore helps to maintain the signal for Ag-dependent activation.

Wabantz et al. (28) previously proposed that LPL is involved in protein secretion. Because it is technically challenging to analyze directly IL-2 secretion per se, we tested if reorientation of the MTOC and accumulation of IL-2 vesicles were affected in LPL−⁄− cells by examining conjugates formed by n3.L2 CD4+ cells and Hb Ag-loaded APCs using pericentrin as a marker for the MTOC. LPL−⁄− cells exhibited normal MTOC reorientation toward the synapse (Fig. 6E). However, the percentage of CD4+ cells in conjugates with visible IL-2 staining was significantly lower in LPL−⁄− T cells, with 10% wt T cells being IL-2+ compared with 4% LPL−⁄− cells (Fig. 6F). This 2-fold decrease is consistent with FACs measurements of IL-2 in nontransgenic CD4+ cells stimulated with plate-bound anti-CD3 (Fig. 3B). In cells expressing IL-2, LPL−/− cells showed similar colocalization of IL-2 vesicles with the MTOC. For IL-2+ conjugates, both wt and LPL−/− had 90% overlap of these IL-2 vesicles with the MTOC (Fig. 6G). These data indicate that conjugates formed with LPL-deficient T cells are less efficient in producing IL-2 but maintain the ability to polarize the MTOC and IL-2 vesicles.

LPL−⁄− mice exhibit delayed allograft rejection after immunosuppression

Because LPL−⁄− T cells displayed activation defects in response to alloantigen in vitro (Fig. 2C), we tested if LPL also played a role in skin allograft rejection. Rejection of transplanted full-thickness tail skin grafts occurred with equivalent kinetics in wt and LPL−⁄− recipient mice (Fig. 7A). Even so, lymphocytes from LPL−⁄− recipients taken after rejection still exhibited a significant proliferation defect in the MLR ex vivo (Fig. 7B), suggesting that the proliferation defect was not severe enough to alter the course of allograft rejection. This result is analogous to those obtained with mice lacking key costimulatory molecules, including mice doubly deficient in CD28 and CD40 (38). To determine whether a role for LPL in vivo might become apparent when pharmacologic immunosuppression was used, skin graft recipients were immunosuppressed by a 2-wk treatment with anti-CD11a plus MMF, a regimen that inhibits acute allograft rejection (Fig. 7C). Two days after the end of treatment, the skin grafts on wt recipients started to display signs of necrosis and were completely rejected by day 31 overall posttransplantation. In contrast, complete graft rejection was delayed a further 8 d in LPL−⁄− mice.

![Figure 7](http://www.jimmunol.org/)
Thus, LPL deficiency combined with short-term immunosuppression significantly prolongs allograft survival.

**LPL**<sup>−/−</sup> mice develop less severe EAE

To determine whether LPL is required for pathologic effects of T cell activation, we tested its function in EAE. LPL<sup>−/−</sup> mice both showed a delay in exhibiting symptoms and had significantly lower clinical scores compared with wt mice (Fig. 8A). The disease incidence also was lower in LPL<sup>−/−</sup> mice, with approximately one third of the LPL<sup>−/−</sup> mice free of disease during the entire period of monitoring (Fig. 8B). Histologically, the brain and spinal cord in LPL<sup>−/−</sup> mice showed significantly less demyelination, which correlated with significantly decreased inflammation as assessed by leukocyte infiltration, including a decrease in both macrophage and CD4<sup>+</sup> T cell infiltration (Fig. 8C, 8D). To determine whether the less severe disease in LPL<sup>−/−</sup> mice resulted from impaired immune responses, we restimulated cells from the draining lymph nodes with MOG peptide and measured proliferation. LPL<sup>−/−</sup> lymphocytes proliferated less efficiently than those of wt mice (Fig. 8E). Normal differentiation of CD4<sup>+</sup> cells to Th1 and Th17 effectors are critical for the disease initiation and development (39), so we measured the hallmark cytokines secreted by these subsets, IFN-γ and IL-17, respectively. Both cytokines were markedly decreased in LPL<sup>−/−</sup> cells (Fig. 8F, 8G). These T cell activation defects were similarly detected at several different time points between day 10 and 4 wk postimmunization (data not shown). The above data demonstrate that LPL has an important role in the proliferation and differentiation of autoimmune T cells.

**Discussion**

Actin rearrangement is critical for normal T cell activation, and the roles for several molecules involved in regulation of actin poly...
merization have been elucidated. Rather than a direct involvement in actin polymerization, LPL plays a role in aggregating actin filaments into parallel bundles. Our study demonstrates that LPL has an important role in T cell activation and participates in TCR-mediated formation of lamellipodia, which are structurally similar to the actin ring at the periphery of the immunological synapse (8). As a result, LPL−/− T cells form smaller immunological synapses with APCs. A shrunken synapse and less stable interaction with APCs have also been demonstrated with human peripheral T cells treated with LPL small interfering RNA by Wabnitz et al. (32). This suggests that the role of LPL in T cell activation results from its ability to stabilize and spread the area of contact between a T cell and an APC.

The actin structure supported by LPL seems important for proliferation and cytokine expression but not for early tyrosine phosphorylation events or calcium entry. This implies that LPL plays a different role from previously reported actin- regulatory proteins that are important for calcium influx in T cells (12, 14). The importance for T cell activation, but not for early calcium influx, has been observed at least with one other actin-regulatory protein, WASP-interacting protein (WIP) (40). Although WIP is thought to participate in formation of a branched actin network dependent on Arp2/3, rather than in actin bundling, it is possible that LPL and WIP nonredundantly regulate formation of common actin structures that help sustain signaling required for full T cell activation. In support of this point of view, we did observe a minor defect in activation of transcription factors important for IL-2 transcription, such as NFAT and AP-1, in LPL−/− cells at a late time point after TCR ligation. It is likely these small effects at several steps caused by LPL deficiency accumulate into the major defect in activation as we have demonstrated.

Wabnitz et al. (28) previously proposed that LPL is required for protein trafficking but not synthesis of activation markers in T cells based on experiments in which an unphosphorylatable mutant of LPL was overexpressed as a dominant negative. In our system, analyzing murine T cells deficient for LPL, we found that expression of CD25 and CD69 upon TCR ligation is not dependent on LPL. Instead, we found that LPL is required for normal cytokine production, as early as the mRNA level. Although we cannot exclude the possibility that cytokine secretion is also affected, the normal reorientation of the MTOC and accumulation of IL-2 vesicles at the immunological synapse in the absence of LPL suggest these steps directly upstream of secretion are not affected. The discrepancy in findings most likely arises from the use of different systems to analyze the role of LPL in TCR signaling. While Wabnitz et al. (28) examined the effects of LPL phosphorylation, our mice lacked LPL entirely. Although phosphorylated LPL has been proposed to enhance its F-actin binding in vitro (41), it is not required for localization to the immunological synapse (28). Further study is needed to determine whether phosphorylation of LPL affects its interaction with F-actin in T cells or its function in TCR signaling. Nonetheless, the importance for LPL in overall T cell activation is supported more recently by Wabnitz et al. (32) demonstrating that knockdown of LPL impairs TCR-mediated T cell proliferation, similar to what we have found in this study.

LPL−/− T cells are defective in egress from the thymus (27), but peripheral LPL−/− CD4+ T cells that do egress apparently mature normally, suggesting that the T cell motility and TCR-mediated activation defects are two independent phenotypes dependent on LPL. Nonetheless, both defects in activation and motility may stem from an importance of LPL in lamellipodium formation or stabilization, as lamellipodium not only guides directional cell migration but also enhances T cell sensitivity to TCR ligands (8, 42, 43). The involvement of LPL in lamellipodium formation in T cells is significant because biochemical studies demonstrate that the major role for plasmins is in cross-linking actin filaments to form parallel bundles, which was not believed previously to be required for formation of lamellipodia (21, 22, 42). This suggests that a higher level of actin organization is necessary for lamellipodium formation or stability and that plasmin contributes to this.

These in vitro roles for LPL clearly are important for T cell function in vivo. Because normal MLR responses required LPL, we examined allograft survival in LPL−/− mice. Although uninhibited immune responses led to allograft rejection with equal rapidity regardless of LPL, brief immunosuppression revealed a role for LPL in graft rejection. We hypothesize that rapid rejection may be mediated by preexisting T cells without much need for proliferation, as even when the kinetics of rejection were unaffected, draining LPL−/− lymph node T cells were still hypoproliferative. After brief immunosuppression to overcome this acute rejection, a role for LPL was revealed. The importance of LPL in the development of EAE is very evident, with less pathology and fewer symptoms in the knockout mice. Importantly, LPL−/− CD4+ cells were extremely defective in polarization into IFN-γ- and IL-17-secreting cells. Because both Th1 and Th17 cells are thought to be essential for development of disease (39), the contribution of LPL to T cell activation appears to be a critical element in this autoimmune disease. Although we cannot rule out that the role for LPL in this circumstance also involves T cell migration to the site of action, it is extremely likely that the proliferation and cytokine defects of the Ag-responsive T cells contribute significantly to the failure of LPL−/− mice to develop disease. These in vivo defects may not be solely dependent on the T cell abnormalities because LPL is also expressed in other leukocytes. Nonetheless, we believe that because both skin graft rejection and EAE are highly dependent on T cells, it is likely that the T cell activation defects in LPL−/− mice contribute significantly to the abnormalities.

In summary, we have shown that LPL is required for normal T cell proliferation and cytokine production in vitro and functions in allograft rejection and EAE in vivo. The mechanism for its importance is that LPL is required for normal lamellipodia formation or stability, a previously unappreciated role for actin bundling, and is manifest as a defect in cytokine expression, downstream of normal initial tyrosine kinase signaling, ERK, and AKT activation. This work reveals a connection between the actin cytoskeleton and T cell activation, mediated through effects on actin bundling, a poorly studied aspect of the actin rearrangements that accompany TCR activation.

Acknowledgments
We thank Matthew Krummel and Scott B. Snapper for the generous gift of animals and cell lines. We thank Wenjun Ouyang, Sanjeev Mariathasan, Shilpa Joshi, and Delu Zhou for critical comments on the manuscript; Meredith Sagolla, Jeffrey Eastham-Anderson, Kassidy Huynh, and Laszlo Komives for assistance with microscopy; Mercedes Balazs and Hieu Nguyen for suggestions on animal models and data analysis; and Hua Chen, Ping Wu, Hiroshi Morisaki, Janet Chan, Meijuan Zhou, and Eric Suto for assistance with experiments.

Disclosures
C.W., I.P., W.P.L., J.D., D.M.D., Z.L., J.Z., and E.J.B. are current employees of or contractors for Genentech, Inc. and are fully supported by Genentech, Inc.

References
Corrections


In the legend for Fig. 8, “C, Histology and immunohistochemistry of spinal cord sections of wt (a, c, e, g) or LPL−/− (b, d, f, h)…” should read “C, Histology and immunohistochemistry of spinal cord sections of LPL−/− (a, c, e, g) and wt (b, d, f, h)…”.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1190027