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The Actin-Bundling Protein L-Plastin Is Essential for Marginal Zone B Cell Development

Elizabeth M. Todd, Lauren E. Deady, and Sharon Celeste Morley

B cell development is exquisitely sensitive to location within specialized niches in the bone marrow and spleen. Location within these niches is carefully orchestrated through chemotactic and adhesive cues. In this article, we demonstrate the requirement for the actin-bundling protein L-plastin (LPL) in B cell motility toward the chemokines CXCL12 and CXCL13 and the lipid chemottractant sphingosine-1-phosphate, which guide normal B cell development. Impaired motility of B cells in LPL−/− mice correlated with diminished splenic maturation of B cells, with a moderate (40%) loss of follicular B cells and a profound (>80%) loss of marginal zone B cells. Entry of LPL−/− B cells into the lymph nodes and bone marrow of mice was also impaired. Furthermore, LPL was required for the integrin-mediated enhancement of Transwell migration but was dispensable for integrin-mediated lymphocyte adhesion. These results suggest that LPL may participate in signaling that enables lymphocyte transmigration. In support of this hypothesis, the phosphorylation of Pyk-2, a tyrosine kinase that integrates chemotactic and adhesive cues, is diminished in LPL−/− B cells stimulated with chemokine. Finally, a well-characterized role of marginal zone B cells is the generation of a rapid humoral response to polysaccharide Ags. LPL−/− mice exhibited a defective Ab response to Streptococcus pneumoniae, indicating a functional consequence of defective marginal zone B cell development in LPL−/− mice. The Journal of Immunology, 2011, 187: 3015–3025.

Lymphocyte motility depends on tightly regulated rearrangements in the actin cytoskeleton (1, 2). Actin-binding proteins coordinate the rapid cytoskeletal changes required for motility, including the polymerization of actin, the generation of actin-based structures such as lamellipodia, and the polarization of lymphocytes (3). L-plastin (LPL), an actin-bundling protein uniquely expressed in hematopoietic cells, is required for normal motility of T lymphocytes and for full T cell activation (4, 5). LPL binds two F-actin helices to create tightly cross-linked bundles of parallel actin filaments and thus stabilizes larger-order actin structures (6). LPL is the only member of the plastin subclass of actin-bundling proteins expressed in hematopoietic cells. LPL has no defined function in signal transduction beyond its capacity to bundle actin filaments, and the mechanism by which LPL functions in lymphocyte motility remains unclear. Furthermore, although LPL is required for the adhesion-dependent respiratory burst in neutrophils, LPL is dispensable for neutrophil motility (7). The requirement for LPL in motility of hematopoietically derived cells is therefore cell specific. To determine whether LPL is required for motility of B lymphocytes and to further elucidate the mechanism by which LPL functions in lymphocyte motility, we investigated the dependence of B cell motility and B cell development on LPL.

B cell motility during B cell maturation is guided by chemokines and the lipid chemottractant sphingosine-1-phosphate (S1P). Chemokines, small protein chemoattractants, decorate the stromal network of fibroblasts and epithelial cells that create the cellular framework of secondary lymphoid organs through which lymphocytes continually recirculate (8). Chemokine receptors are seven-transmembrane G protein-coupled proteins that enable the lymphocytes on which they are expressed to navigate lymphoid compartments. Chemokines and their receptors thus determine both underlying lymphoid architecture and lymphocyte movement through and localization within this architecture. CXCR4, the chemokine receptor for CXCL12, and CXCR5, the receptor for CXCL13, are critical for B cell development (9, 10).

B cell development begins in the bone marrow and is completed in the spleen (reviewed in Ref. 11). In the bone marrow, CXCL12 and CXCR4 retain the earliest B cell precursors within the appropriate bone marrow niches required for B cell maturation (12). Following successful rearrangement of the Ig locus to generate and express IgM, immature B cells emigrate from the bone marrow, guided by receptors for S1P (13–15). Entering the spleen through the bloodstream, these immature B cells form the population of transitional B cells. CXCL13 and CXCR5 guide B cells to the B cell compartment within splenic and lymph node tissue. In the absence of CXCR5 or CXCL13, lymphoid architecture is disturbed, devoid of clearly defined B and T cell zones or B cell follicles (9, 16). Transitional B cells move through the red pulp, cross the barrier of cells lining the marginal sinus of the spleen, and enter the white pulp. In the white pulp, transitional B cells generate IgD and develop into either follicular (FO) or marginal zone (MZ) B cells.

Chemotactic cues guide the development of MZ B cells, because MZ B cell development requires the appropriate localization of maturing B cells at the border of the follicle and the red pulp (11). MZ B cells are among the first immune cells to capture...
blood-borne Ags and can generate rapid humoral responses to T-independent Ags. Absence of MZ B cells reduces the humoral response mounted to polysaccharide Ags (17). MZ B cells also traffic into the follicle to stimulate an adaptive response (18). Development of splenic MZ B cells is especially sensitive to location within the appropriate lymphoid architecture (19–20). Deficiencies of proteins such as Dock2, Dock8, Rap1B, and Pyk2, required for chemotaxis and adhesion, can disrupt the localization and maturation of MZ B cells (17, 21–24).

In this article, we present the requirement for LPL in B cell motility and MZ B cell development. Developing B cells isolated from the bone marrow of LPL−/− mice demonstrated diminished motility toward CXCL12 and S1P. Despite reduced movement toward CXCL12, bone marrow development of early B cell precursors was not impacted in LPL−/− mice. However, there was a 40% decrease in the number of mature B cells isolated from the spleens of LPL−/− mice, and MZ B cells were nearly absent. Splenic architecture was not disrupted in LPL−/− mice, and defective MZ B cell maturation was intrinsic to LPL−/− B cells, suggesting that LPL−/− B cells could not migrate to the correct splenic niches to complete MZ B cell maturation. Functionally, LPL−/− mice demonstrated a reduced humoral response to i.v. challenge with the polysaccharide Ag heat-killed Streptococcus pneumoniae, as would be predicted from a deficit of MZ B cells. MZ B cell development thus requires LPL.

Materials and Methods

Mice

LPL−/− mice were used between the ages of 5 and 14 wk (7). Age- and gender-matched wild-type (WT) mice were bred and maintained in the same specific pathogen-free housing as LPL−/− mice at Washington University School of Medicine (St. Louis, MO). All experiments were performed in accordance with protocols approved by the Washington University Animal Studies Committee.

Cell isolation

For determination of cell counts, two axillary and two inguinal lymph nodes were isolated from each mouse. Single-cell suspensions were generated from lymph nodes and spleen by gentle disruption of the tissue with a 3-ml pipette tip. Lymph node cells and splenocytes were isolated from both femurs and tibias of each mouse unless otherwise noted.

Flow cytometry

Directly conjugated Abs were commercially available: anti-IgM-FITC (clone II/41), anti-B220-PerCP/Cy5.5 (clone RA3), anti-IgD-PE (clone 11-26), anti-CD23-PE (clone B34), anti-CD11d-biotin (clone B1), streptavidin-allophycocyanin, AA4.1-allophycocyanin (clone AA4.1), anti-CD11b-PE/Cy7 (clone M1/70), anti-CD5-biotin (clone 53-7.3), anti-CD45.1-allophycocyanin/Cy7 (clone A20), anti-CD45.2-Pe/Cy7 (clone M5/104), anti-CD43-PE (clone 1B11), anti–Ly51-biotin (clone 6C3), anti–CD45.2-PE/Cy7 (clone 104), anti–CD24-PE/Cy7 (clone M1/69), anti–IgM-FITC (clone H1.2F3), and anti–CD86-PE (clone GL1) (eBioscience, San Diego, CA); and anti–CD23-PE (clone B3B4), anti–CD1d-biotin (clone 1B1), streptavidin-PE/Cy5, streptavidin-AF488 (BioLegend). Secondaries used were Streptavidin-Dylight 594, Anti-MOPC31C-FITC, or goat anti-mouse IgG-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa Fluor 488 anti-CD23, CD1d-biotin (clone RMM-1), anti–CD45.1, and anti–CD45.2 by flow cytometry.

Adhesion assays

Adhesion assays were performed as previously described with minor modifications (22, 27–29). Flat-bottom 96-well Immulon plates were coated overnight with Fc-VCAM-1 (1 or 3 μg/ml) with BSA or with CXCL12 (500 ng/ml) in PBS at 4˚C. Plates were washed with PBS and then blocked with 1% BSA in PBS at 37˚C for 1 h. Splenocytes from WT or LPL−/− mice were incubated in complete 10% media (IMDM plus 10% FBS and 10 ng/ml HEPES) in a cell culture flask for 30 min at 37˚C to remove adherent cells. Nonadherent splenocytes were removed from the flasks, subjected to RBC lysis, and resuspended in warmed serum-free media (RPMI 1640 medium with 10 mM HEPES and 0.5% BSA) and rested for at least 1 h at 37˚C. Cells (5 × 10^5/well) were plated onto the blocked plate, briefly centrifuged to settle cells (30–50 g × 20 s), and incubated at 37˚C for 5 min. Experimental wells were washed with warm serum-free media eight times. Adherent cells were then detached by incubation for 20 min on ice with cold RPMI 1640 medium with 10% FBS and EDTA. The number of "input" cells was determined from control wells coated with BSA in which cells were plated but not subjected to washing and detachment. Cells recovered from each well were counted and analyzed for expression of B220, CD23, and CD21/35 by flow cytometry. The percentage of adherent cells was determined by dividing the number of cells, gated as indicated, by the total number of equivalently gated input cells.

Uptregulation of activation markers and proliferation

B220+ cells isolated from WT or LPL−/− splenocytes were incubated overnight with plate-bound anti-IgM. Uptregulation of CD69 and CD86 on B cells was assessed by flow cytometry.

For proliferation assays, B220+ cells isolated from WT or LPL−/− splenocytes using negative selection (Miltenyi Biotec) were labeled with CFSE (Intronigent, Carlsbad, CA) and assayed for proliferation by measuring CFSE dilution by flow cytometry.

Spleens from naive WT and LPL−/− mice were embedded in OCT (Sakura Finetek, Torrance, CA), frozen with 2-methylbutane cooled in liquid nitrogen, sectioned, and stored at −20˚C before staining. For staining, the 8-μm sections were rehydrated with PBS, blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA) in 1:10 tween 20, and stained with anti–MO-MAC-1 (Serotec, Raleigh, NC), anti–B220-PE, anti–IgD, anti–IgM (eBioscience), anti–IgM-biotin, or anti–Thy-1.2-AF488 (BioLegend). Secondaries were used Streptavidin-Dylight 594, 1:50 dilution by flow cytometry.

Spleens from naive WT and LPL−/− mice were embedded in OCT (Sakura Finetek, Torrance, CA), frozen with 2-methylbutane cooled in liquid nitrogen, sectioned, fixed with acetone, and stored at −20˚C before staining. For staining, the 8-μm sections were rehydrated with PBS, blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA) in 1:10 tween 20, and stained with anti–MO-MAC-1 (Serotec, Raleigh, NC), anti–B220-PE, anti–IgD, anti–IgM (eBioscience), anti–IgM-biotin, or anti–Thy-1.2-AF488 (BioLegend). Secondaries were used Streptavidin-Dylight 594, 1:50 dilution by flow cytometry.
Sweptaxin-Dlyght 488 (BioLegend), or Alexa Fluor 594 anti-rat IgG (H+L) (Invitrogen). Sections were mounted and visualized on a Zeiss Axioskop using a Zeiss Plan-Neofluar ×10 or ×20 objective or an Olympus BX60 using an Olympus U Plan Fl ×20 objective. Images were acquired using a Zeiss AxiosCam with AxiosVision software. Color levels (brightness and contrast) were adjusted in Adobe Photoshop.

**ImmunobLOTS**

Purified B220+ cells (Miltenyi Biotec) from WT or LPL−/− spleens were stimulated with CXCL12 (100 ng/ml) or the F(ab′)2 fragment of goat anti-mouse IgM (10 μg/ml; Jackson Immunoresearch Laboratories) for the indicated time. Cells were lysed in Tris/NaCl/EDTA buffer with protease inhibitors added. Postnuclear lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with the indicated primary Abs (Cell Signaling, Danvers, MA). Goat anti-rabbit- Alexa Fluor 680 (Invitrogen) and goat anti-mouse-IRDye800 (Rockland Immunchemicals, Gilbertsville, PA) Abs were used to detect membrane-bound Abs. Signal was visualized and quantitated using LiCOR Odyssey imager and software (Li-Cor, Lincoln, NE). To compare the degree to which phosphorylation of each protein was stimulated in WT and LPL−/− B cells, the ratio of each phospho-specific protein to the total protein level in stimulated cells was determined and then normalized to the ratio of the phospho-specific protein to the total level of protein in unstimulated cells.

**Determination of IgM response to i.v. S. pneumoniae**

Mice were injected i.v. with 1 × 10⁸ CFU heat-killed S. pneumoniae (InvivoGen, San Diego, CA) in 100 μl HBSS. Mice were bled at the indicated time points following immunization. Sera were plated at an initial dilution of 1:30 and diluted serially 1:3 in Immulon II plates (Fisher Scientific, Pittsburgh, PA) coated with 5 μg/ml phosphorylcholine-BSA (Biosearch Technologies, Novato, CA) and blocked with PBS/0.1% Tween/1% BSA. Bound serum IgM was detected by anti-mouse IgM-HRP (Biosearch Technologies, Novato, CA) and blocked with PBS/0.1% Tween/1% BSA. Bound Abs were detected with goat anti–mouse-IgM conjugated to Alexa Fluor 680 (Invitrogen) and goat anti–rabbit-IRDye800 (Rockland Immunchemicals, Gilbertsville, PA) Abs used to detect membrane-bound Abs. Signal was visualized and quantitated using LiCOR Odyssey imager and software (Li-Cor, Lincoln, NE). To compare the degree to which phosphorylation of each protein was stimulated in WT and LPL−/− B cells, the ratio of each phospho-specific protein to the total protein level in stimulated cells was determined and then normalized to the ratio of the phospho-specific protein to the total level of protein in unstimulated cells.

**Statistics**

The Mann–Whitney U test was used to determine statistical significance, with p < 0.05 considered significant. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA).

**Results**

**LPL required for motility, but not development, of B cells in the bone marrow**

Chemokine-inducible motility of thymocytes and T cells depends on the actin-binding protein LPL (4). We hypothesized that B cell motility would also require LPL. B220+ B cells were isolated from the bone marrow of WT and LPL−/− mice and defined as pro-B, pre-B, immature, and mature B cells based on expression of B220, IgM, and CD43 (Supplemental Fig. 1A). Pro-B, pre-B, immature B, and mature B cells isolated from LPL−/− mice all demonstrated reduced motility toward CXCL12, despite normal expression of CXCR4 (Fig. 1A, Supplemental Fig. 1B). Increasing the concentration of CXCL12 was insufficient to overcome the diminished motility of LPL−/− B cells (Supplemental Fig. 1C). Immature LPL−/− B cells (defined as Hardy Fraction E, see below) also demonstrated reduced motility to the lipid chemokine (SIP), indicating that motility induced by multiple chemotactant receptors requires LPL (Fig. 1B). Interactions between CXCL12 and CXCR4 are essential for B cell production in the bone marrow (10), and interactions between SIP and at least two receptors, S1P1 and S1P3, regulate bone marrow egress of immature B cells (13–15). To determine whether diminished motility toward CXCL12 or SIP would impact bone marrow development of B cells in LPL−/− mice, we used the Hardy method to delineate subsets of developing B cells using the markers B220, CD43, Ly51, CD24, IgM and IgD to determine fractions A–F (FrA–FrF) (Fig. 1C) (30). Despite the requirement for LPL in CXCL12- and SIP-stimulated motility, bone marrow development of B cells proceeded normally in LPL−/− mice (Fig. 1C, 1D). In addition, turnover of bone marrow subsets in LPL−/− mice, assessed using BrdU incorporation, appeared normal (Fig. 1E), in contrast to the reduced turnover of immature B cells observed in mice deficient for S1P1 (15). Thus, chemokine-induced motility appeared to be dispensable for B cell development, indicating that other functions of CXCR4 and receptors for S1P must be required for B cell maturation in the bone marrow.

**Defective maturation of MZ B cells in LPL−/− mice**

Following egress from the bone marrow, immature B cells traffic to the spleen, forming the subset of T1 splenic B cells that then undergo further maturation (22). Spleens from LPL−/− mice contained fewer lymphocytes overall (Fig. 2A) and fewer B220+ cells (Fig. 2B). The percentage of IgMhighIgDlow B cells was maintained in LPL−/− mice, whereas the percentages of IgMhigh IgDhigh and IgMlowIgDlow populations were diminished (Fig. 2C, Supplemental Fig. 2A). Numerically, there was a loss of the more mature IgMhighIgDhigh and IgMlowIgDlow populations, relative to the slight reduction in the number of IgMhighIgDlow B cells (Fig. 2C). The IgMhighIgDlow population of B220+ B cells contains both immature transitional B cells and MZ B cells. To better define the subsets of splenic B220+ cells, we analyzed the expression of the surface markers CD21/23 and CD23. FO B cells have been defined as CD23low/CD21/23low, MZ B cells as CD23low/CD21/23high, and “newly forming” cells as CD23low/CD21/23low (23). Numbers of FO cells were reduced by ~40%, and numbers of MZ B cells were reduced by ~80% in the spleens of LPL−/− mice (Fig. 2D). The numbers of newly forming B cells were equivalent in the spleens of WT and LPL−/− mice. Four-color analysis of the expression of IgM, CD21/23, CD23, and B220, performed on a sample subset, confirmed that the number of early T1 splenic B cells, defined as B220+CD23low/CD11bIgMlowCD21/23low, was maintained in the spleens of LPL−/− mice, whereas more mature populations of FO, T2, and MZ precursor (T2-MZP) and MZ B cells were lost (Supplemental Fig. 2B, 2C). The maintenance of T1 B cells was confirmed by AA4.1 expression (Supplemental Fig. 2D), and loss of MZ B cells was confirmed by CD1d expression (Supplemental Fig. 2E). Maintenance of the immature B220+ splenocyte population is consistent with the observation that bone marrow development is normal in LPL−/− mice and suggests little defect in trafficking of immature B cells into the spleen. Defective B cell maturation in LPL−/− mice is therefore specific to the splenic compartment.

To determine whether the defective MZ B cell development in LPL−/− mice was cell intrinsic or extrinsic, we generated mixed bone marrow chimeras (Fig. 2E). Analysis of bone marrow subsets in chimeric mice revealed no significant block in bone marrow maturation of B cells derived from LPL−/− bone marrow (data not shown), although some variability in engraftment into the WT recipients resulted in a statistically insignificant reduction of immature B cells derived from LPL−/− bone marrow (Fig. 2E). Analysis of splenic B220+ cells from the competitive, mixed bone marrow chimeras revealed no significant differences in the number or percentage of immature, CD23low/CD21/23low, MZ B cells derived from WT or LPL−/− bone marrow (Fig. 2E, Supplemental Fig. 2F), and the statistically insignificant decrease seen is likely due to the variable engraftment also observed in the bone marrow subset. However, there was a pronounced and significant reduction in the number of IgMhighIgDlow B cells, whereas more mature populations of FO, T2, and MZ precursor (T2-MZP) and MZ B cells were lost (Supplemental Fig. 2B, 2C). The maintenance of T1 B cells was confirmed by AA4.1 expression (Supplemental Fig. 2D), and loss of MZ B cells was confirmed by CD1d expression (Supplemental Fig. 2E). Maintenance of the immature B220+ splenocyte population is consistent with the observation that bone marrow development is normal in LPL−/− mice and suggests little defect in trafficking of immature B cells into the spleen. Defective B cell maturation in LPL−/− mice is therefore specific to the splenic compartment.

**Conclusion**

In conclusion, CXCL12 and S1P receptors are both required for B cell maturation in the bone marrow, with CXCL12 playing a more significant role. While CXCL12 has been shown to be critical for normal B cell development (24), the results of this study demonstrate that CXCL12 is not sufficient for normal B cell development in the absence of S1P. The mechanisms by which CXCL12 and S1P cooperate to promote B cell development remain to be elucidated, but these findings highlight the importance of understanding the interplay between chemokines and G protein-coupled receptors in the regulation of B cell development.
Interestingly, the loss of FO and MZ B cells from LPL−/−-derived marrow in the mixed bone marrow chimeras does not appear to be as profound as the losses observed in the LPL−/− mice. For instance, the percentage of MZ B cells derived from LPL−/− bone marrow, compared with the percentage of MZ B cells derived from WT bone marrow, was reduced by ∼50% (Supplemental Fig. 2F), which was not as great as the 80% reduction in MZ B cells seen when comparing WT and LPL−/− mice (Fig. 2D). The partial correction of FO and MZ B cell development observed in the competitive chimeras may indicate that the splenic B cell maturation defect is partially cell extrinsic in the LPL−/− mouse. However, the significant reduction of MZ B cells derived from LPL−/− bone marrow in a competitive chimeric assay indicated that the major requirement for LPL in MZ B cell maturation is cell intrinsic.

A loss of both FO and MZ B cells in the competitive bone marrow chimeric mice suggested a block in early splenic maturation. Consistent with this suggestion, turnover of splenic B cell subsets in LPL−/− mice, assessed using BrdU incorporation, revealed a mild, though not statistically significant, reduction in the turnover of T1 cells in LPL−/− mice (Fig. 2F). Turnover of other splenic subsets appeared unaffected (Fig. 2F). Reduced turnover of T1 B cells in LPL−/− mice would explain the reduction in numbers of both FO and MZ B cells. These data indicate that after maturation, B cells can exit the spleen and populate the peripheral lymph nodes in LPL−/− mice, there was no shift toward a more immature population in the blood.

Diminished splenic B cell development in LPL−/− mice did not result in, or result from, disturbed splenic architecture.

Increased numbers of B cells in the blood of LPL−/− mice

To determine whether the loss of mature B cells from the spleen affected the number of peripheral B cells in other sites, the total numbers of B cells from blood and peripheral lymph nodes in LPL−/− mice were assessed. Total and B220+ lymphocytes from the blood of LPL−/− mice were greater than those of WT mice (Fig. 4A). The percentages of IgMlowIgDlow, IgMhighIgDhigh, and IgMhiIgDlow cells were similar between WT and LPL−/− mice (Fig. 4B), whereas the total number of mature and immature subsets of B cells were increased in the blood of LPL−/− mice (Fig. 4C). Thus, although more B cells circulate in the bloodstream of LPL−/− mice, their distribution did not result in a shift toward a more immature population in the blood.
**FIGURE 2.** Reduced splenic maturation of B cells in LPL<sup>−/−</sup> mice, with profound loss of MZ B cells. A, Total numbers of splenocytes isolated from WT or LPL<sup>−/−</sup> mice. B, Flow cytometric analysis of B220 expression on splenocytes isolated from WT (filled gray histogram) or LPL<sup>−/−</sup> (solid line) mice. Bar graph depicts number of B220<sup>+</sup> splenocytes isolated from WT or LPL<sup>−/−</sup> mice. C, Flow cytometric analysis of IgM and IgD expression on all splenocytes isolated from WT or LPL<sup>−/−</sup> mice. D, Flow cytometric analysis of CD21/35 and CD23 expression on B220<sup>+</sup> splenocytes from WT or LPL<sup>−/−</sup> mice. FO cells identified as CD21/35<sup>int</sup>CD23<sup>high</sup> and MZ cells identified as CD21/35<sup>high</sup>CD23<sup>low</sup>. Bar graphs depict number of CD21/35<sup>low</sup>CD23<sup>low</sup>, FO, and MZ splenocytes from WT or LPL<sup>−/−</sup> mice. A–D, All quantitative data and WT (gray bars, n = 2) and LPL<sup>−/−</sup> (filled bars, n = 2) data shown as mean ± SEM; p value determined by Mann–Whitney U test. E, Numbers of cells derived from WT (gray bars) or LPL<sup>−/−</sup> (filled bars) bone marrow in competitive bone marrow chimeric mice that were either immature bone marrow B cells (B220<sup>int</sup>IgM<sup>+</sup>; see Supplemental Fig. 1A), or B220<sup>+</sup> splenocyte subsets, the newly forming CD21/35<sup>low</sup>CD23<sup>low</sup> B cells, FO B cells, or MZ B cells (n = 5 chimeric mice from three independent experiments; data shown as mean ± SEM; p value determined by Mann–Whitney U test). F, BrdU incorporation into splenic B220<sup>+</sup> cell subsets isolated from WT (gray bars) or LPL<sup>−/−</sup> (filled bars) mice. Data shown as mean ± SEM (n = 9 WT and 10 LPL<sup>−/−</sup> mice, from five independent experiments).

**LPL<sup>−/−</sup> B cells are defective in entry into lymph nodes and bone marrow**

The increased number of B220<sup>+</sup> cells in the blood of LPL<sup>−/−</sup> mice suggested that LPL<sup>−/−</sup> B cells may be deficient in the ability to exit the blood and enter peripheral sites. We tested for deficient lymphoid organ entry by cotransferring CFSE-labeled WT and DDAO-labeled LPL<sup>−/−</sup> B220<sup>+</sup> cells into WT mice and enumerating transferred cells in the blood, spleen, lymph nodes, and bone marrow of recipient mice (Fig. 5A). An increased ratio of transferred LPL<sup>−/−</sup> to WT cells in the blood and a reduced ratio of...
LPL−/− to WT cells in the lymph nodes and bone marrow of recipient mice indicated that LPL−/− B cells are relatively, though not absolutely, defective in the ability to enter lymphoid organs (Fig. 5A). LPL−/− B cells were able to enter the spleen normally, consistent with findings that there are normal numbers of immature B cells in the spleens of LPL−/− mice (Fig. 2C, 2D). LPL is thus required for normal B lymphocyte entry into lymph nodes and bone marrow but not for entry into the spleen.

Integrin binding did not enhance the Transwell migration of LPL−/− B cells

 Trafficking of B cells is regulated in part by the chemokine receptors CXCR4, CXCR5, and S1P1 and by the integrin VLA-4 (9, 16, 19, 33). We assessed the CXCL12- and CXCL13-directed motility of splenic B cells from LPL−/− mice in the presence or absence of VCAM-1, which is the ligand for VLA-4 (Fig. 5B). Integrin binding augments the ability of cells to migrate across a barrier (25), and coating the upper surface of Transwell inserts with VCAM-1 dramatically increased the percentage of WT cells that crossed the insert (Fig. 5B). When analyzing the migration of all B220+ cells, we found a 50% decrease in the percentage of LPL−/− B cells that migrated to the chemokines CXCL12 and CXCL13 in the absence of integrin ligation, despite normal expression of CXCR3 and CXCR4 (Supplemental Fig. 3A, 3B). Integrin binding had a negligible effect on the transmigration of LPL−/− B220+ cells (Fig. 5B). Separating the migrating cells into populations of newly forming CD21/35−CD23−, FO, and MZ B cells revealed that both newly forming and FO B cells from LPL−/− mice demonstrated significant defects in motility in the presence or absence of integrin ligand, whereas MZ B cells appeared less deficient in the absence of integrin ligation (Supplemental Fig 3C). However, MZ B cells still demonstrated a requirement for LPL in chemokine-mediated motility in the presence of integrin ligand (Supplemental Fig 3C). Thus, LPL is required for the integrin-induced increase in chemokine-mediated transmigration.

MZ B cells are dependent on S1P for localization within the MZ (19). We therefore tested whether MZ B cell migration to S1P would require LPL. LPL−/− MZ B cells demonstrated a diminished chemotactic response to S1P (Fig. 5C), indicating that LPL is required for normal motility toward S1P in mature as well as immature B cells.

Intriguingly, LPL was not required for the chemokine-enhanced adhesion to integrins. Chemokine stimulation can activate integrin-mediated adhesion in the presence of shear forces (34, 35). We tested the dependence of chemokine-activation of integrins upon LPL by using a static plate-bound adhesion assay (Fig. 5D).

It is thought that the extensive washing in this assay provides the shear forces necessary to generate chemokine-mediated integrin activation (36). LPL−/− B cells adhered to plates coated with VCAM-1 and CXCL12 similarly to WT B cells (Fig. 5D, 5E).

Analysis of the results presented in Fig. 5D and 5E used the additional markers CD21/35 and CD23 revealed no difference in the adhesion of WT and LPL−/− splenic B cell subsets (Supplemental Fig. 3D, 3E), indicating that LPL was not required for adhesion in any splenic subset. LPL is thus dispensable for integrin-mediated adhesion but is required for the integrin-mediated enhancement of Transwell migration in response to chemokine stimulation.

**Diminished Pyk-2 phosphorylation in LPL−/− B cells following chemokine stimulation**

Many downstream signaling effectors are required for chemokine-mediated motility, including the small GTPase Rac, and the kinases ERK, Akt, and Pyk-2 (reviewed in Ref. 37). To determine whether the activation of signaling molecules depended on LPL, we used phospho-specific AbS in immunoblot assays of cell lysates from WT and LPL−/− B cells stimulated with either anti-IgM or with CXCL12 (Fig. 6A, 6B). The total cellular concentration of Pyk-2 was decreased in comparison with the levels of the kinase ERK (Fig. 6A, 6B); immunoblots of the same membrane are shown in Fig. 6A, 6B). Generation of postnuclear lysates separates a detergent-insoluble fraction which can contain cytoskeletal elements. Immunoblot did not reveal increased partitioning of Pyk-2 into the detergent-insoluble fraction in LPL−/− B cells (data not shown), indicating that total protein expression of Pyk-2 is diminished in the absence of LPL. Furthermore, stimulation of LPL−/− B cells with CXCL12 did not result in the modest but consistent 1.5-fold increase in phosphorylation of Pyk-2 observed in CXCL12-stimulated WT B cells (Fig. 6A). Thus, both the total level and chemokine-induced phosphorylation of Pyk-2 were diminished in LPL−/− B cells. Although phosphorylation of the mediator ERK at times appeared to be slightly diminished in stimulated LPL−/− B cells, no significant reduction of ERK activation was observed in replicate experiments (Fig. 6B). Similarly, stimulation of phosphorylation of p38 was not significantly diminished in LPL−/− B cells (data not shown). Thus, the requirement of LPL for maintenance of Pyk-2 protein levels and activation-dependent phosphorylation appeared to be specific for Pyk-2.

To determine whether LPL is required for activation signaling through IgM, splenocytes from WT and LPL−/− mice were stimulated overnight on plate-bound IgM. B cells from LPL−/− mice
upregulated CD69 and CD86 to a slightly greater extent than B cells from WT mice (Fig. 6C). Splenic B cells isolated from LPL<sup>−/−</sup> mice also underwent proliferation in response to stimulation through soluble anti-IgM and IL-4, although the percentage of proliferating cells was slightly reduced compared with that of WT B cells (Fig. 6D). Thus, proximal IgM signaling appeared largely intact in LPL<sup>−/−</sup>B cells, although there may be a subtle alteration in signaling outcomes.

**Discussion**

In this article, we demonstrate the requirement for the actin-bundling protein LPL in splenic maturation of B cells. LPL<sup>−/−</sup> mice have significantly reduced numbers of splenic FO and MZ B cells, despite normal bone marrow development and apparently normal splenic entry of immature B cells. Furthermore, B cell entry into the lymph nodes and bone marrow was partially impaired by LPL deficiency. Splenic B cell development and lymphoid organ entry are both regulated by chemokine receptor signaling. Normal chemokine-mediated motility of B cells required LPL. Furthermore, the addition of an integrin ligand to the Transwell assay did not promote the transmigration of LPL<sup>−/−</sup> cells as it did for WT cells. Interestingly, MZ B cells that did develop in LPL<sup>−/−</sup> mice appeared less dependent on LPL for phosphocholine IgM was reduced in challenged LPL<sup>−/−</sup> mice (Fig. 7). Thus, LPL<sup>−/−</sup> mice demonstrate a functional immunodeficiency predicted by the loss of MZ B cells.

Reduced immunity of LPL<sup>−/−</sup> mice to S. pneumoniae

MZ B cells are critical to the generation of a rapid humoral response to blood-borne, polysaccharide organisms (17). Given that MZ B cells are greatly diminished in LPL<sup>−/−</sup> mice, we predicted a functional deficit of LPL<sup>−/−</sup> mice in responding to blood-borne, T-independent, polysaccharide Ags. We tested this prediction through measuring Ab titers generated against the pneumococcal cell wall component, phosphocholine, in response to i.v. injection of heat-killed S. pneumoniae. The increase in anti-phosphorylcholine IgM was reduced in challenged LPL<sup>−/−</sup> mice (Fig. 7). Thus, LPL<sup>−/−</sup> mice demonstrate a functional immunodeficiency predicted by the loss of MZ B cells.
CXCL12- and CXCL13-induced motility than did newly forming and FO splenic B cell subsets. A diminished requirement for LPL in chemokine-mediated motility in MZ B cells from LPL−/− mice may represent selection bias in that only those developing B cells able to migrate in the absence of LPL were selected to populate the MZ B cell niche. MZ B cells did, however, require LPL for migration toward S1P, a chemotactant critical for MZ B cell development (19). Also, like newly forming and FO splenic B cells, MZ B cells required LPL for normal chemokine-mediated transmigration in the presence of integrin ligand. LPL was not required for chemokine-mediated activation of integrin adhesion in any splenic B cell subset. The activity of LPL in motility therefore seems to be similar to that of Wiskott–Aldrich Syndrome protein in that deficiency of these proteins impairs motility without impacting adhesion (38). The observation that integrin ligation does not enhance the transmigration of LPL−/− cells, despite normal adhesion, suggests a critical role for LPL in the convergence of chemokine and integrin contributions to the transmigration of lymphocytes.

Chemokine receptor signaling pathways that regulate lymphocyte motility and adhesion have been reviewed extensively (3, 39). In brief, chemokactins bind to G protein-coupled seven-transmembrane receptors. Binding triggers calcium flux, activates src-family kinases and other kinases such as ERK, Akt, p38, and Pyk-2, and recruits small GTP-binding proteins such as Rho, Rac, and Rap. Activation of the small GTP-binding proteins requires the recruitment of guanine nucleotide exchange factors such as Dock2 and Dock8. The plethora of activated signaling molecules combines to trigger changes in cell shape, polarity, and adhesion, which promote cellular motility. Changes in shape, polarity, and adhesion require dynamic rearrangements in the actin cytoskeleton that are enabled by multiple actin-binding proteins such as Wiskott–Aldrich Syndrome protein and coromin. Specific deficiencies in these proteins lead to diminished chemotaxis and impaired B cell development (17, 22–25, 40, 41). Although many individual elements of chemokine signaling have been identified, a thorough understanding of interactions between different components has remained elusive. A comparison of B cell development in LPL−/− mice to B cell development in other mice genetically deficient for molecules required for chemotaxis, adhesion, or transmigration should further elucidate signaling pathways unique to each process.

Early bone marrow development of B cells depends on CXCL12 and its receptor CXCR4. CXCR4 and CXCL12 localize B cell precursors to specific bone marrow niches. Despite deficient motility toward CXCL12, early B cell development in the bone marrow of LPL−/− mice appeared unaffected, as assessed by flow cytometric analysis of B cell subsets in WT and LPL−/− mice, analysis of competitive bone marrow chimeras, and BrdU incorporation to determine turnover of bone marrow subsets. Both integrin-mediated adhesion and chemotaxis were reduced in Rap1B−/− B cells, and early bone marrow development was impaired in Rap1B−/− mice (23). The normal B cell development we observed in the bone marrow of LPL−/− mice argues that CXCR4-induced motility or transmigration is dispensable for early bone marrow development, whereas CXCR4-induced adhesion is required. This argument correlates with prior observations that CXCR4 signaling upregulates the adhesiveness of pro- and pre-B cells for VCAM-1 (12). Thus, the dominant function of CXCR4 within the bone marrow compartment for early B cell development may be the promotion of adhesion.

After progressing to the immature B cell stage, developing B cells move from the bone marrow to the spleen via the bloodstream. Normal emigration of immature B cells from the bone marrow in LPL−/− mice is suggested by normal turnover of B cells subsets (Fig. 1E), because diminished emigration of immature B cells has
been shown to result in reduced turnover of the immature B cell subset (15). Normal emigration of immature B cells appears to occur in LPL$^{-/-}$ mice despite a requirement for LPL in S1P-mediated motility, again suggesting that a downstream function of the chemoattractant receptor other than induction of motility, such as the promotion of changes in adhesion or effects upon signaling through CXCR4, is required for maturation and egress of bone marrow B220$^+$ cells (13, 42). Alternatively, the residual motility of LPL$^{-/-}$ bone marrow B220$^+$ cells may be sufficient to permit normal bone marrow maturation and egress.

Normal splenic entry by LPL$^{-/-}$ immature B cells is implied by the normal number of immature B cells in the spleens of LPL$^{-/-}$ mice (Fig. 2D) and directly demonstrated through cotransfer experiments (Fig. 5A). These results suggest that chemokine-mediated transmigration is not required for splenic entry. This conclusion is supported by the analysis of mice deficient for both Rac1 and Rac2 in which immature B cells are defective in both chemotaxis and adhesion. Immature Rac1/Rac2$^{-/-}$ B cells also enter the spleen normally, demonstrating that neither the movement of immature B cells from the bone marrow to the blood nor from the blood into the spleen requires intact chemotaxis (25).

B cell development in LPL$^{-/-}$ mice proceeds unimpeded until the immature B cells enter the spleen. After splenic entry, developing B cells cross the marginal sinus lining cells into the splenic white pulp, where a majority mature into FO B cells and a small number differentiate into MZ B cells. MZ B cells require additional chemotactic and adhesive cues to remain appropriately localized in the MZ (11). Deficiencies of various regulators of chemotaxis or adhesion impair different stages of splenic B cell development, suggesting a high degree of complexity in the regulation of B cell movement through splenic compartments during maturation. Rac1/Rac2$^{-/-}$ B cells cannot enter the splenic white pulp, and are therefore suspended in an early transitional stage (25). B cell development in LPL$^{-/-}$ mice proceeds unimpeded until the immature B cells enter the spleen.

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development but not maturation of T1, FO, and T2 B cells. These different models of deficiency suggest a maturation process in which there are no barriers to restrict splenic entry of immature B cells, but at least two barriers regulate maturation of MZ B cells. First, transitional B cells must migrate into the splenic white pulp to mature into FO and T2 cells (25). Second, MZ precursor or MZ B cells must cross out of the white pulp and reside in the splenic MZ (33). LPL deficiency partially blocks FO B cell development and severely, though not completely, blocks MZ B cell development. Furthermore, B cell transfer experiments suggested slightly reduced turnover of the earliest T1 splenic subset, consistent with diminished maturation of this stage (Fig. 2F). The simplest explanation is that the combined chemotactic and integrin signals enabling lymphocyte transmigration across barriers are partially dependent upon LPL. Thus LPL deficiency partially inhibits both in entering the white pulp, resulting in the decrease in FO B cell maturation, and further inhibited in migrating to the MZ, resulting in the loss of MZ B cells.

A requirement for LPL in lymphocyte transmigration also explains the competitive defect in entry of LPL−/− B cells into lymph nodes and bone marrow. Lymph node entry occurs via high endothelial venules. Lymphatic endothelial cells present a variety of adhesion and chemokine cues, including CD62L ligand, CCL21, and the lymphatic vessel endothelial hyaluronan receptor 1 (43, 44). Mature FO B cells recirculate to the bone marrow and reside in perisinusoidal niches (45). Lymphocytes must respond to the appropriate chemotactic and adhesive cues, then cross the cellular barriers into the lymph nodes or perisinusoidal niches. Lymphocytes from mice deficient for proteins required for chemotaxis or adhesion, such as talin1, Dock2, or CasL, are impaired in lymph node entry (40, 44, 46). We found increased B cells in the blood of LPL−/− mice (Fig. 4A), and LPL−/− B cells were at a competitive disadvantage in entering both lymph nodes and the bone marrow in cotransfer assays (Fig. 5A). The observation that there is no decrease in the number of B cells present in the lymph nodes or bone marrow of LPL−/− mice may be due to an inability of LPL−/− B cells to egress from these sites, although this possibility has not yet been formally tested.

We have further advanced our understanding of the mechanism by which LPL promotes B lymphocyte transmigration through the observation that Pyk-2 levels are decreased in LPL−/− B cells, relative to the expression of the critical kinase ERK. We also found that phosphorylation of Pyk-2 upon stimulation of CXCR4, and to a lesser extent, IgM, is diminished in LPL−/− B cells. The tyrosine kinase Pyk-2 is homologous to focal adhesion kinase and Pyk-2 is activated in response to a variety of adhesions, chemotactic, and AgR stimuli (17, 47–50). Phosphorylation of tyrosine residue 402 of Pyk-2 enables kinase activation and the recruitment of src family kinases (50). Pyk-2 deficiency results in diminished chemotaxis and impaired MZ B cell development (17). Interestingly, Pyk-2 phosphorylation was not inhibited in LPL−/− PMNs stimulated through integrin adhesion, indicating a different role for LPL in neutrophils than in lymphocytes (7). LPL deficiency did not appear to significantly impact the activation of the kinase ERK, indicating that the effect of LPL deficiency on Pyk-2 activity is specific. The mechanism by which the activity of the actin bundling like LPL regulates the level and phosphorylation of the kinase Pyk-2 remains an area of active investigation. As Pyk-2 is known to be required for normal lymphocyte motility, the diminished levels and activation of Pyk-2 in LPL−/− B cells offers a molecular explanation for the motility deficit of LPL−/− lymphocytes.

Although LPL is clearly required for chemokine-mediated motility and transmigration, LPL is dispensable for several outcomes of chemokine signaling. As discussed, the chemokine-mediated increase in integrin adhesion is intact in LPL−/− cells (Fig. 5D, 5E). ERK activation downstream of chemokine stimulation is intact in LPL−/− B cells (Fig. 6B). Also, T and B cell zones are clearly separated in LPL−/− splenic sections, indicating that the functions of chemokine receptors required for maintaining T and B cell zones do not depend on LPL (Fig. 3A). Increasing the concentration of chemokine did not enhance motility of LPL−/− B cells (Supplemental Fig 1C), suggesting that LPL deficiency does not reduce the sensitivity of developing B cells to chemokine. Finally, Transwell experiments indicate that the baseline motility of LPL−/− B cells is reduced as well as chemokine-mediated motility, such that the fold-increase over control in migration can appear similar between WT and LPL−/− B cells. Combined, these results indicate that LPL is required specifically for the recruitment of the cellular machinery needed for motility but not for the initial receptor engagement of chemokine or other chemokine-mediated signaling or functions.

Assessment of IgM signaling in LPL−/− B cells did not reveal a severe block in anti-IgM-mediated upregulation of activation markers or proliferation, indicating that IgM signaling in LPL−/− B cells is largely intact. There was a slight increase in the expression of activation markers on anti-IgM-stimulated LPL−/− B cells and a slight decrease in the anti-IgM-mediated proliferation of LPL−/− B cells, suggesting a subtle alteration in the outcome of IgM signaling in LPL−/− B cells. It is possible that this slight decrease in anti-IgM-mediated proliferation contributed to the diminished BrdU incorporation of T1 B cells in LPL−/− mice. We did not find statistically significant increases in phosphorylation of Pyk-2, ERK, or p38 following IgM engagement on LPL−/− B cells. However, the possibility that the reduction of total Pyk-2 levels in LPL−/− B cells altered the outcome of IgM signaling has not been excluded.

By virtue of their localization at the border of the splenic MZ with the splenic red pulp, MZ B cells are among the first immune cells to contact blood-borne Ags (21). MZ B cells are critical to the rapid response to T-independent, polysaccharide Ags (17, 51). Immunity to the Gram-positive bacterium S. pneumoniae depends on the ability to generate Abs to polysaccharide Ags. A reduced humoral response to i.v. injection with heat-killed S. pneumoniae in LPL−/− mice correlated with diminished numbers of MZ B cells (Fig. 7). Whether a deficient humoral response to S. pneumoniae leads to increased susceptibility of LPL−/− mice to pneumococcal infection is under study.

In summary, the actin-bundling protein LPL is required for B lymphocyte motility toward chemokines and for the integrin-associated enhancement of chemokine-induced Transwell migration. LPL is dispensable for integrin-mediated adhesion. Deficiency of LPL results in diminished protein levels and activation of the tyrosine kinase Pyk-2, which is required for normal lymphocyte migration. Defective motility of LPL−/− B cells results in diminished splenic maturation of B cells, with a significant defect in maturation of MZ B cells. A loss of MZ B cells leads to an inability to respond to blood-borne polysaccharide Ags such as S. pneumoniae. LPL is a critical regulator of B lymphocyte motility and thus of splenic B lymphocyte development.

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Disclosures

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