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Crk-Associated Substrate Lymphocyte Type Is Required for Lymphocyte Trafficking and Marginal Zone B Cell Maintenance

Sachiko Seo,* Takashi Asai,* Toshiki Saito,* Takahiro Suzuki,† Yasuyuki Morishita,‡ Tetsuya Nakamoto,* Motoshi Ichikawa,* Go Yamamoto,* Masahito Kawazu,* Tetsuya Yamagata,* Ryuichi Sakai,‡ Kinuko Mitani,§ Seishi Ogawa,* Mineo Kurokawa,2* Shigeru Chiba,* and Hisamaru Hirai*

The lymphocyte-specific Cas family protein Cas-L (Crk-associated substrate lymphocyte type) has been implicated to function in lymphocyte movement, mediated mainly by integrin signaling. However, its physiological role is poorly understood. In this study we analyzed the function of Cas-L in lymphocytes using gene-targeted mice. The mutant mice showed a deficit of marginal zone B (MZB) cells and a decrease of cell number in secondary lymphoid organs. An insufficient chemotactic response and perturbed cell adhesion were observed in Cas-L-deficient lymphocytes, suggesting that the aberrant localization was responsible for the deficit of MZB cells. Moreover, we found that lymphocyte trafficking was altered in Cas-L-deficient mice, which gave a potential reason for contraction of secondary lymphoid tissues. Thus, Cas-L affects homeostasis of MZB cells and peripheral lymphoid organs, which is considered to be relevant to impaired lymphocyte migration and adhesion. The Journal of Immunology, 2005, 175: 3492–3501.

The Crk-associated substrate (Cas) lymphocyte-specific protein Cas-L, also known as human enhancer of filaments 1 (1), was originally identified as a 105-kDa protein that is tyrosine-phosphorylated by the ligation of β1 integrin in peripheral T cells (2). Cas-L is a docking protein in focal adhesion and consists of an N-terminal Src homology (SH)3 domain, a substrate domain containing multiple tyrosine motifs for SH2-binding sites, a serine-rich region, and a C-terminal dimerization motif (2). Owing to its homology with p130Cas (3), Cas-L is recognized as a member of the Cas family. Cas is expressed ubiquitously and plays a crucial role in integrin-mediated signaling. Our group demonstrated that the lack of Cas protein resulted in fetal death because of perturbed organogenesis (4). In contrast, Cas-L is predominantly expressed in lymphocytes and epithelial cells (1, 2), which implies that it has distinct functions from Cas.

Integrins are a family of adhesion receptors composed of α and β subunits. They are involved in cell-cell and cell-matrix interactions and induce various biological signals for cell adhesion, migration, apoptosis, proliferation, and differentiation (5–8). Among the integrin family, β1 integrin constitutes the largest group that mediate cell attachment via fibronectin or VCAM-1. Previous reports showed that Cas-L is tyrosine-phosphorylated by binding to focal adhesion kinase or Pyk-2 in the SH3 domain upon engagement of β1 integrin (9, 10). Subsequently, the phosphorylated Cas-L regulates several signals involved in cell motility (11–13) and cell adhesion (14) as a downstream effector of focal adhesion kinase. In addition, Cas-L functions as a signal transducer of TCR (12, 15, 16), BCR (17), and G protein-coupled calcitonin receptor (18). The biological functions of Cas-L in lymphocytes, however, remain to be determined.

Early B cell development arises in bone marrow as immature B cells expressing surface IgM emigrate to the spleen (19). In the spleen, immature B cells can differentiate into follicular B (FOB) cells and marginal zone B (MZB) cells characterized by IgMhighIgDlowCD21intCD23low and IgMhighIgDbrightCD21brightCD23low, respectively (20). A number of studies using gene-targeted mice have demonstrated loss of MZB cells in the mutant mice, and this defect of MZB cells was explained by two major mechanisms: failure of MZB cell development and impaired localization. MZB cell development is presumably related to BCR or Notch signal, as suggested from studies of mice lacking Aiolos, Lyn, Notch2, or RBP-J (21–24). The hypothesis of perturbed localization was derived from observations of altered lymphocyte motility in mice lacking Pyk-2, DOCK2, or Lsc (25–27). Despite several investigations, however, the precise mechanism of MZB cell development and localization remains unclear. In particular, no relevant mouse model that recapitulates a defect of integrin-mediated MZB cell retention has been obtained so far.

Lymphocyte trafficking is a multistep process mediated by chemokines and adhesion molecules (28). Lymphocytes express several kinds of chemokine receptors and integrin receptors. The chemokine CXCL12, previously called stromal cell-derived factor

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3 Abbreviations used in this paper: Cas, Crk-associated substrate; Cas-L, Cas lymphocyte-type; MZB, marginal zone B; FOB, follicular B; ES, embryonic stem; EGFP, enhanced GFP; TNP, 2,4,6-trinitrophenyl; KLH, keyhole limpet hemocyanin; SH, Src homology.
1. is integral for mature B cell movement and is expressed in secondary lymphoid tissues, the red pulp in spleen, and the medullary cord in lymph nodes (29, 30). CXCL13, known as B lymphocyte chemotactant, plays a crucial role in proper localization of B cells in peripheral lymphoid organs (31). With regard to adhesion molecules, previous studies have indicated that the integrin receptors LFA-1 (αβ2) and VLA-4 (αβ1) are involved in lymphocyte homing to peripheral lymph nodes or the splenic white pulp (32–34).

To elucidate the physiological function of Cas-L, we generated Cas-L-deficient mice using gene-targeting strategy. The mutant mice showed reduced numbers of lymphocytes in secondary lymphoid organs and an almost complete loss of MZB cells in the spleen. We demonstrated that Cas-L regulates responses to chemokines and adhesion molecules, and that its deficiency may be related to aberrant peripheral lymphoid organization including MZB cell maintenance.

Materials and Methods

*Generation of Cas-L−/− mice*

A genomic mouse C57BL/6 library was screened with a 300-bp Cas-L probe that included the SH3 region of Cas-L. A 15-kb clone identified with this probe was subcloned in pBluescript and all of the genomic sequence was defined. A targeting vector was constructed using the following procedure. The enhanced GFP (pEGFP, Clontech) was directly combined with the Cas-L genome at the site of HindIII within exon 2, and a neomycin resistance cassette was infused to the vector. Electroporation was performed to insert the targeting vector into TT2 embryonic stem (ES) cells. Clones that underwent homologous recombination were selected in the presence of G418 and confirmed by PCR with a primer set containing the probe that included the SH3 region of Cas-L. The enhanced GFP (pEGFP, Clontech) was directly combined with the Cas-L genome and cut at 5′-GACTACGAGCCTTCCACACTA-3′ (Clontech). Prepared cells were stained with relevant mAbs. Analyses were performed using mainly 8- to 12-wk-old sex-matched littermates.

*Western blot analysis*

To confirm the deletion of Cas-L protein, thymin from wild-type and Cas-L−/− mice were homogenized in cold RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris (pH 8.0) and 5 mM EDTA-3Na). Cell lysates were incubated with anti-Cas Ab (BD Transduction Laboratories), which cross-reacts to Cas-L, and protein G-Sepharose (Amersham Biosciences) overnight at 4°C. The products of immunoprecipitation were segregated by 7.5% SDS-PAGE. Electrophoretic blotting was conducted with anti-goat human en- hancer of filamentation 1 mAb, which developed against a peptide mapping at the N terminus (Santa Cruz Biotechnology).

*Cell counts and flow cytometric analysis*

Cells from each tissue were washed twice with PBS after hemolysis and enumerated. Peripheral blood was obtained by puncturing the retro-orbital sinus. To avoid any nonspecific binding of Ly5, Fc block was performed by using anti-CD16/32 Abs (BD Pharmingen) to eliminate the number of MZB cells in the spleen. We demonstrated that Cas-L regulates responses to chemokines and adhesion molecules, and that its deficiency may be related to aberrant peripheral lymphoid organization including MZB cell maintenance.

*Immunohistochemistry*

Spleens were frozen in Tissue-Tek OCT compound (Sakura Finetechanical) and cut at 5 µm. Sections were stained with FITC anti-mouse IgD and biotin anti-mouse IgM (BD Pharmingen) diluted to 1/100. IgM expression was detected using Alexa Fluor 594-conjugated streptavidin (Molecular Probes) diluted to 1/250. Confocal laser scanning microscope (Bio-Rad) was used.

*Reconstitution of MZB cells*

Bone marrow cells (Ly5.2−) were derived from both sides of the femurs, and mononuclear cells were collected using Histopaque (Sigma-Aldrich). Mononuclear cells (1 × 106) in PBS with 10% FCS were i.v. injected into sublethally irradiated (950 rad) 8- to 10-wk-old female wild-type mice with Ly5.1 (C57BL/6). The recipients were sacrificed after 12 wk. The cells of donor origin, separated based on their cell surface expression of Ly5.1 or Ly5.2, were investigated for the presence of MZB cells using FACScalibur. Fc block was performed by using anti-CD16/32 Abs (BD Pharmingen) to avoid any nonspecific binding of Ly5.

*Measurement of intracellular Ca2+ concentration*

Splen B cells were purified as described and loaded with 3 µM Indo-1 (Molecular Probes) in RPMI 1640 medium with 1% FCS at 37°C for 45 min. After a rinse of Indo-1, the cells were stained with PerCP anti-mouse B220 (BD Pharmingen) to confirm the B cell fraction. Prepared cells were adjusted to 1 × 106 cells/ml and warmed to 37°C for 5 min. The basal Ca2+ concentration was recorded for 20 s. The increase in intracellular free Ca2+ after stimulation by 10 µg/ml anti-IgM F(ab′)2 (Jackson ImmunoResearch Laboratories) or with the combination of 3 µg/ml anti-IgM F(ab′)2, 5 µg/ml anti-CD40 (BD Pharmingen), 10 ng/ml recombinant mouse IL-4 (Genzyme/Techne), and 20 µg/ml LPS (Sigma-Aldrich) for 52 h. [3H]Thymidine was added at 1 µCi/well and incubation was done for an additional 8 h.

*Immunization and measurement of serum Ig titers*

Seven- to 8-wk-old mice were immunized i.p. with 10 µg of 2,4,6-trinitrophenyl keyhole limpet hemocyanin (TNP-KLH; Biosearch Technologies) mixed with alum, given as a booster injection every 3 wk. Spleen cell suspensions were collected from the mice and pooled to obtain 106 cells/ml and washed to yield 108 CFU of Streptococcus pneumoniae (T9664a, Jackson ImmunoResearch Laboratories; 3:1 ratio) mixed with alum, given as a booster injection every 3 wk. Serum was collected on day 7 and the anti-phosphorylcholine-specific Ig levels were measured by ELISA using phosphorylcholine-BSA (Biosearch Technologies). Nonparametric tests were performed on each Ig level.

*Migration and adhesion assays*

For chemotaxis assays, 5-µm pore-sized transwells (Costar) were used. Splenocytes were stained with CD21, CD23, B220, and Thy1.2. The prepared cells (1.5 × 106) in 100 µl of RPMI 1640 medium were added to the insert, and the bottom chamber was supplied with 450 µl of RPMI 1640 with 10% FCS, 2% BSA, CCL21 (Genzyme/Techne) at various concentrations. Migration of the cells to the lower chamber was allowed for 3 h at 37°C. The cells prepared from wild-type or Cas-L−/− mice were counted and the number of FOB or T cells was calculated as a control for FACS-Calibur. Subsequently, migrated cells were counted and the ratio of FOB or T cells to control cells was calculated. For adhesion assays, erythrocyte-depleted splenocytes on tissue culture plates were incubated at 37°C for 50 min to remove adherent macrophages and stained with CD21, CD23, B220, and Thy1.2. The ratio of FOB or T cells to control cells was calculated.

*Lymphocyte trafficking assay*

Splenocytes were harvested from Cas-L−/− mice and mixed with TNP-BSA (LSL). Ten- to 12-wk-old mice were immunized i.p. with 4 × 107 CFU of Streptococcus pneumoniae strain (436A, a kind gift of H. Ito, University of Kagoshima (Kagoshima, Japan). Serum was collected on day 7 and the anti-phosphorylcholine-specific Ig levels were measured by ELISA using phosphorylcholine-BSA (Biosearch Technologies). Nonparametric tests were performed on each Ig level.
cells was evaluated by detecting BCECF-AM-positive cells among donor lymphocytes that colonized the spleen, lymph nodes, and peripheral blood.

Statistical analyses

Values of \( p \) for differences between groups were determined by Student’s \( t \) test using Microsoft Excel software. Statistical analyses of measured serum Ig level were performed by Mann-Whitney \( U \) test.

Results

Generation of Cas-L-deficient mice

To generate Cas-L-deficient mice, the 1.2-kb Cas-L genomic region that contains exon 2 encoding the N-terminal SH3 domain in the Cas-L protein was replaced with EGFP and a neomycin resistance cassette by homologous recombination in ES cells. EGFP was introduced into the exon to generate a fusion protein (Fig. 1A). Correct integration of the targeting vector into the Cas-L genomic locus was identified by PCR and Southern blot analysis. Two ES cell lines with successful homologous recombination were used to generate chimeric mice and mutant mouse lines were established through germ-line transmission. The mutant loci were confirmed by Southern blot analysis of DNA isolated from the tail (Fig. 1B). Western blot analysis of cell lysates from the thymus showed a loss of Cas-L protein in mutant mice, although the possibility remains that truncated Cas-L proteins lacking the exon 2-dependent sequence were not detected (Fig. 1C). Cas-L mutant (Cas-L\(^{-/-}\)) mice were born with the expected Mendelian frequency and were apparently indistinguishable from wild-type littermates.

Reduced lymphocytes in peripheral lymphoid organs from Cas-L\(^{-/-}\) mice

Because Cas-L is preferentially expressed in lymphocytes (2), we first examined peripheral lymphocyte populations in Cas-L\(^{-/-}\) mice. Cas-L\(^{-/-}\) mice had normal numbers of peripheral blood cells, which showed no morphological abnormality (Fig. 2A). No obvious alteration in the B or T cell populations was observed. Subsequent analyses of splenocytes showed a significant reduction of the total lymphocyte number, to \(~50\%\) of that in wild-type mice (Fig. 2B). Both B cell and T cell numbers in the spleen were significantly diminished in the absence of Cas-L. The ratio of T cells to B cells in the spleen was increased, which showed that B cells were more affected than T cells (data not shown). The populations of T and B cells in the lymph nodes were also investigated. The total number of lymphocytes and the numbers of T and B cells in Cas-L\(^{-/-}\) were significantly reduced compared with those in wild-type mice (Fig. 2C). T cell subsets in the peripheral blood and secondary lymphoid tissues were assessed by measuring the cell surface expression of CD4 and CD8. The results showed a normal ratio of CD4 to CD8 in the mutant mice (data not shown). To examine whether these reductions result from abnormal hematopoiesis in the bone marrow, we analyzed the total cell number and
B cell population in the bone marrow based on the criteria of Hardy et al. (35). The findings revealed no difference in the number of B cell subsets, suggesting that early B cell development was intact in the mutant mice (Table I). As for the thymus, no abnormality was detected in CD4 or CD8 single positive cell populations (data not shown). Taken together, these results indicate that Cas-L affects normal homeostasis in peripheral lymphoid compartments.

**MZB cell defect in Cas-L-deficient mice**

Because initial analyses showed a striking reduction in the number of B cells in the spleen of Cas-L−/− mice, late B cell development in the spleen was investigated. We examined the cell surface expression of CD21 and CD23 to discriminate among newly formed B cells (B220⁺CD21lowCD23low), FOB cells (B220⁺CD21int CD23high), and MZB cells (B220⁺CD21highCD23low). Cas-L−/− mice showed a marked decrease in the MZB cell population and a complementary increase in the percentage of FOB cells (Fig. 3A). The absolute numbers of both MZB cells and FOB cells were decreased (Table I). We next analyzed the splenic B cell compartment in Cas-L−/− mice by measuring other B cell markers, including IgM and IgD. The B220⁺IgM⁺IgDlow population, which includes MZB cells (21), was also reduced in Cas-L−/− mice compared with wild-type mice (Fig. 3B, Fraction III type cells, and Table I). To confirm the defect of MZB cells in Cas-L−/− mice, the expression of CD1d, which is a distinctive marker of MZB cells (36), was analyzed in B220⁺IgM⁺IgDlow cells. Decreased CD1d⁺ cell population was found in Cas-L−/− mice compared with wild-type mice (Fig. 3C). Consistent with the results from flow cytometric analyses, histological examination of the spleen showed that the IgM⁺IgDlow MZB cell number was reduced in the absence of Cas-L (Fig. 3D).

MZ is composed of several kinds of cells in addition to MZB cells such as stromal cells and macrophages. The MZB cell defect could result from either cell autonomous impairment or an incomplete microenvironment that supports B cell differentiation. To determine which possibility is more likely in Cas-L−/− mice, we examined the reconstitution of MZB cells after reciprocal cell transfer between wild-type mice and Cas-L−/− mice. The transfer of Cas-L-deficient bone marrow into sublethally irradiated congenic wild-type hosts did not result in the reconstitution of MZB cells (Fig. 4, middle panel). In contrast, the transfer of bone marrow cells from wild-type mice into sublethally irradiated Cas-L−/− recipients resulted in the generation of normal MZB cell populations (Fig. 4, right panel). Thus, these results demonstrated that Cas-L is integral for the maintenance of MZB cells and that the defect of MZB cells in these mice is intrinsic to Cas-L−/− B cells.

**BCR-mediated signaling and immune responses in the absence of Cas-L**

Among a number of reports that analyzed the mechanism of the MZB cell deficit, some reports have indicated that the absence of MZB cells arises from enhanced BCR signaling (21, 23). Cas-L is also suggested to be involved in BCR-mediated signal transduction (17). Therefore the defect of MZB cells in Cas-L−/− mice might result from aberrant BCR signaling. To test this issue, Ca²⁺ mobilization assays were performed. The results showed that the extent of Ca²⁺ flux in Cas-L−/− B cells was slightly decreased compared with that in wild-type B cells (Fig. 5A). We next investigated the mitogenic responses of Cas-L−/− splenic B cells. The proliferations of Cas-L−/− B cells in response to graded concentrations of anti-IgM were comparable to those of the control cells (Fig. 5B).

Moreover, no significant differences were observed in the proliferation resulting from the combination of anti-IgM, anti-CD40, or IL-4 (Fig. 5C).

In addition, we analyzed the involvement of Cas-L in the humoral immune response. The measurements of Ig isotype levels in the serum from nonimmunized Cas-L−/− mice showed a decrease in IgG2a compared with the serum from wild-type mice (Fig. 5D). Subsequently, we immunized mice against TNP-conjugated KLH, which is a T cell-dependent Ag. The concentrations of TNP-specific Abs in Cas-L−/− mice revealed a normal response to the T cell-dependent Ag (Fig. 5E). Because MZB cells are indicated to be involved in the T cell-independent Ab response against phosphorylcholine (37), we next examined the levels of anti-phosphorylcholine Abs after immunization with S. pneumoniae strain R36A. The slightly reduced level of phosphorylcholine-specific IgM was detected in Cas-L−/− mice (Fig. 5F), which is compatible with the decreased number of MZB cell, although other Ig isotype levels in Cas-L−/− mice were comparable to those in wild-type mice (data not shown). Taking these findings together, the defect of MZB cells in Cas-L−/− mice is unlikely to be caused mainly by enhanced BCR signaling.

### Table I. B cell populations in Cas-L-deficient mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cell Type</th>
<th>Cas-L+/+</th>
<th>Cas-L−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>Total B cells</td>
<td>37.4 ± 3.0 (×10⁶/ml)</td>
<td>15.0 ± 4.1* (×10⁶/ml)</td>
</tr>
<tr>
<td></td>
<td>NFB cells</td>
<td>3.0 ± 0.8</td>
<td>1.2 ± 0.5**</td>
</tr>
<tr>
<td></td>
<td>FOB cells</td>
<td>25.6 ± 7.5</td>
<td>11.9 ± 4.9*</td>
</tr>
<tr>
<td></td>
<td>MZB cells</td>
<td>1.63 ± 0.2</td>
<td>0.2 ± 0.05**</td>
</tr>
<tr>
<td></td>
<td>Fraction I</td>
<td>14.1 ± 4.5</td>
<td>6.3 ± 1.9**</td>
</tr>
<tr>
<td></td>
<td>Fraction II</td>
<td>7.1 ± 1.8</td>
<td>3.6 ± 1.6**</td>
</tr>
<tr>
<td></td>
<td>Fraction III</td>
<td>6.8 ± 1.7</td>
<td>2.1 ± 0.9**</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Total B cells</td>
<td>22.3 ± 6.2 (×10⁶/ml)</td>
<td>25.1 ± 8.2 (×10⁶/ml)</td>
</tr>
<tr>
<td></td>
<td>Fraction A–C</td>
<td>2.6 ± 0.7</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Fraction D</td>
<td>9.1 ± 3.7</td>
<td>9.7 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>Fraction E</td>
<td>3.2 ± 1.6</td>
<td>2.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Fraction F</td>
<td>3.5 ± 1.8</td>
<td>4.5 ± 2.1</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>Total B cells</td>
<td>9.9 ± 4.5 (×10⁶/ml)</td>
<td>5.0 ± 1.1* (×10⁶/ml)</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>Total B cells</td>
<td>10.5 ± 5.4 (×10⁶/ml)</td>
<td>11.8 ± 2.9 (×10⁶/ml)</td>
</tr>
</tbody>
</table>

*Cells from the spleen, bone marrow, lymph nodes, and peripheral blood were stained with appropriate B cell markers, and each cell population was analyzed by FACS. FOB, NFB, and MZB represent follicular B, newly formed B, and marginal zone B cells, respectively. Fraction I, II, and III were defined by IgM⁺IgD⁺, IgM⁺IgD⁻, and IgM⁻IgD⁻, respectively. Fractions A-D were determined using the criteria of Hardy. Data are mean ± SD derived from five mice except for the subset analysis for the spleen, where data are derived from six mice. * p < 0.05 and ** p < 0.01 by Student’s t test.
Aberrant migration and adhesion in Cas-L-deficient FOB cells

Previous reports had also indicated that the loss of MZB cells can result from altered lymphocyte motility, which presumably causes mal-localization (25–27). Because in vitro assays had shown that Cas-L was involved in cell movement, the loss of MZB cells in Cas-L−/− mice might be relevant to the impairment of cell migration. To address this possibility, chemotaxis assays were performed using splenocytes derived from wild-type and Cas-L−/− mice. To preclude the effect of the decrease of MZB cells in Cas-L−/− mice, we analyzed FOB cells, which account for 80% of splenic B cells and include MZB cell precursors. We examined their migration in response to CXCL12 and CXCL13, which induce a strong chemotactic reaction in peripheral B cells (29, 38). The results showed that the chemotaxis of Cas-L−/− FOB cells was decreased in a dose-dependent fashion (Fig. 6, A and B).

Considering lymphocyte localization, integrins are indispensable molecules and Cas-L plays a crucial role in the integrin-mediated pathway. Among the integrin ligands, VCAM-1 and ICAM-1 are essential for the retention of MZB cells (39). Therefore we investigated the response of Cas-L−/− FOB cells to VCAM-1 and ICAM-1 using adhesion assays. As shown in Fig. 6, C and D, FOB cells from Cas-L−/− mice showed attenuated responses to both VCAM-1 and ICAM-1 compared with those from wild-type mice.

We then performed flow cytometric analyses to evaluate the expression level of the relevant receptor for each ligand. Measurements of surface chemokine receptors (CXCR4 and CXCR5, receptors of CXCL12 and CXCL13, respectively) and integrin receptors (integrin α4 and β1, subunits of the receptor for VCAM-1, and αE and β7, subunits of the receptor for ICAM-1) on splenic B cells or FOB cells from wild-type mice and Cas-L−/− mice showed no significant difference in their expression levels (data not shown). Taken together, Cas-L−/− FOB cells show perturbed migration and adhesion, which may be associated with the defect of MZB cells.

Cas-L affects lymphocyte trafficking in secondary lymphoid organs

In Cas-L−/− mice, another significant finding is the reduced number of lymphocytes in peripheral lymphoid organs. Our findings that migration and adhesion of splenic B cells were impaired in Cas-L−/− mice suggest that the aberrant lymphocyte movement may be responsible for this reduction. Previous studies indicated that chemokines (CXCL12, CXCL13, and CCL19/CCL21) and the
integrin family (VLA-4 and LFA-1, receptors of VCAM-1 and ICAM-1, respectively) have pivotal roles in lymphocyte trafficking in spleen and lymph nodes (30–34). Therefore we first examined chemotaxis and adhesiveness of splenic T cells to determine whether T cells from Cas-L−/− mice also show the same inadequate migration and adhesion as Cas-L−/− B cells. As shown in Fig. 7, A and B, the chemotaxis of Cas-L−/− T cells to CXCL12 and CCL21, both of which are known to be important chemokines for peripheral T cells (40, 41), was significantly attenuated. The adhesion assay revealed impaired adhesiveness to both VCAM-1 and B220+ cells (right panel). Data are representatives of three independent experiments using at least two mice in each experiment.

FIGURE 4. MZB cell deficit in Cas-L−/− mice is lymphocyte autonomous. Bone marrow cells from wild-type (WT Ly5.2+) or Cas-L−/− 10-wk-old mice (KO Ly5.2+) were transferred into lethally irradiated congenic wild-type mice (Ly5.1+) (left and middle panels). Bone marrow cells from congenic wild-type mice (Ly5.1+) were reciprocally injected into Cas-L−/− mice (right panel). Twelve weeks later, splenocytes of donor origin were stained for Ly5.1 (or Ly5.2), B220, CD21, and CD23 and analyzed by FACS. Numbers indicate the percentage of gated cells for Ly5.2+ and B220+ cells (left and middle panels) or for Ly5.1+ and B220+ cells (right panel). Data are representatives of three independent experiments using at least two mice in each experiment.

FIGURE 5. Immune responses in Cas-L-deficient mice. A, BCR-mediated signaling in the absence of Cas-L. Wild-type and Cas-L−/− B cells were loaded with Indo-1. The panel shows the changes of intracellular calcium concentration ([Ca2+]i) in B220-gated cells after stimulation with anti-IgM F(ab′)2 (10 μg/ml). Data are expressed as the ratio to the beginning concentration level. A representative of at least three independent experiments is shown. B and C, BCR- and LPS-mediated proliferation of splenic B cells. Proliferation of wild-type or Cas-L−/− B cells was examined by measuring [3H]thymidine incorporation after stimulation with the indicated doses of anti-IgM F(ab′)2 (B). The assay was also performed with the combination of anti-IgM F(ab′)2 (3 μg/ml), anti-CD40 (5 μg/ml), mouse IL-4 (10 ng/ml), and LPS (20 μg/ml). The panel is representative of at least three independent experiments. D, Serum Ig concentrations in nonimmunized mice. Serum Ig levels in 8- to 9-wk-old wild-type and Cas-L−/− mice were measured by ELISA. Each point represents the value obtained from one mouse. *, p < 0.05 by Mann-Whitney U test. E, Humoral responses to T cell-dependent (TD) Ag. Seven- to 8-wk-old mice were immunized with TNP-KLH. Secondary immunization was given at day 21. TNP-specific Abs were measured in serum collected at day 28 after the initial immunization. F, Immune response against phosphorylcholine (PC). Ten- to 12-wk-old mice were immunized with S. pneumoniae strain R36A. Serum titers of anti-phosphorylcholine-specific IgM were analyzed 7 days after immunization. Data in E and F were derived from six mice per genotype. *, p < 0.05 by Mann-Whitney U test.
and ICAM-1 in Cas-L−/− T cells compared with control cells (Fig. 7, C and D).

Given the impaired migration and adhesion of Cas-L−/− B and T cells, we performed trafficking assays in vivo to determine whether lymphocytes in Cas-L−/− mice might show altered cell movement in spleen and lymph nodes. Splenocytes isolated from wild-type and Cas-L−/− mice (Ly5.2+), which were labeled with BCECF only for Cas-L−/− cells, were mixed equally and injected
into congenic wild-type mice (Ly5.1+). Forty-eight hours after injection, splenocytes were harvested and host cells were separated from donor cells using Ly5.1. Cas-L−/− cells were discriminated from wild-type cells by the marker BCECF (Fig. 8A). Peripheral lymph nodes and peripheral blood were also analyzed in the same way. The number of homing Cas-L−/− cells showed a modest but significant decrease in both the spleen and lymph nodes (Fig. 8, A and B). In contrast, the ratios of total lymphocytes and B cells in peripheral blood derived from Cas-L−/− mice were elevated, although the change in the T cell ratio was not statistically significant (Fig. 8C). Thus, these results suggest the possibility that the reduction in the number of lymphocytes in spleen and lymph nodes of Cas-L−/− mice is due to altered lymphocyte trafficking.

Discussion
Cas-L-deficient mice have defective MZB cells, although other subsets of splenic B cells show an almost normal pattern. On the basis of previous reports, we addressed two possible reasons for this defect: distorted localization of MZB cells and impairment of MZB cell differentiation.

The first hypothesis is suggested by data indicating alteration of the migratory ability in gene-targeted mice lacking Pyk-2 or DOCK2 (25, 27). These proteins function as important mediators of G protein-coupled chemokine receptor signal transduction. A previous study showed that pertussis toxin, a G protein inhibitor, causes the disappearance of MZB cells, suggesting that aberrant chemokine receptor signaling could give rise to defective MZB cells (27). In Cas-L-deficient mice, a decreased chemotactic response is also detected, although no obvious correlation between Cas-L and chemokine receptors has yet been reported. Taken together with our demonstration that Cas-L regulates not only integrin-mediated but also chemokine-mediated cell motility, Cas-L could have a function in the signal pathway from G protein-coupled chemokine receptor.

Gene-disrupted mice lacking DOCK2, a key molecule of chemokine-mediated Rac activation, showed complete loss of chemotactic responses, which would result in the defect of MZB cells. In Cas-L-deficient mice, however, the response to chemokines is reduced to ~50% of that seen in wild-type mice. Therefore, it would appear that abnormal chemotaxis is not enough to explain the marked reduction of MZB cells in Cas-L−/− mice. Although previous studies have stressed altered chemotactic activity to explain the absence of MZB cells, cell lodgment to the lymphoid-specific region is controlled not only by migration but also by adhesion. Significant in this regard is our observation that Cas-L−/− B cells have the impaired ability to adhere to VCAM-1 and ICAM-1, both of which are known to be indispensable molecules for the adhesion of MZB cells. In the current study, we first demonstrated that the

![FIGURE 8. Aberrant lymphocyte trafficking in Cas-L−/− mice. BCECF-labeled splenocytes from Cas-L−/− mice (KO) were mixed with unlabeled splenocytes from wild-type (WT) mice. Both cells (Ly5.2+) were injected into congenic recipient mice (Ly5.1+). Forty-eight hours later, spleens, lymph nodes, and peripheral blood were harvested. Ly5.2+ cells in the spleen were selected and the ratio of Cas-L−/− cells (BCECF+ cells) and wild-type cells (BCECF− cells) detected in the transferred cells were presented (A). Data are mean ± SD from three independent experiments using two mice in each case (B). The assays were also performed for lymph nodes (C) and peripheral blood (D). *p < 0.05 and **p < 0.01 by Student’s t test.](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org)
defect of MZB cells may result from abnormalities in both migration and adhesion. These findings are also consistent with the previous reports that Cas-L functions in the pathway mediated by β1 integrin, a receptor subset for VCAM-1. In contrast, no previous studies have clarified a correlation between Cas-L and LFA-1, which was suggested by our observation that Cas-L is required for the signaling from LFA-1.

Although our findings suggest that Cas-L is indispensable for lymphocyte localization in MZB, they do not preclude the possibility that MZB cell development is perturbed in Cas-L−/− mice. Previous studies have indicated the involvement of signaling from BCR and Notch2 in MZB cell differentiation, both of which are membrane-bound receptors distinctly expressed on B cells. The former leads to a hypothesis that enhanced BCR signaling causes loss of MZB cells, although this remains disputable (42, 43). In this regard, Cas-L is suggested to commit to BCR signaling through association with Lyn, which negatively regulates BCR signaling by mediating inhibitory signals from CD22 and FcyRIIB (44, 45). Interestingly, previous studies reported that the absence of Lyn or CD22 resulted in loss of MZB cells with hypersensitive BCR signaling (23, 46). From these findings, we expected Cas-L−/− B cells to show enhanced BCR signal intensity. Our data, however, showed no evidence of BCR signal enhancement. Recent studies have suggested that the Notch2 signal pathway plays a pivotal role in determining MZB cell differentiation fate (22, 24). The results of our trafficking assay suggested that this contraction would have resulted from aberrant lymphocyte movement. We also examined other possibilities to explain contraction of secondary lymphoid organs: maturation arrest, insufficient proliferation, and altered cell turnover. With regard to the first possibility, flow cytometric analyses of bone marrow and thymus showed no significant abnormalities in B or T cell development that could account for this reduction (Table I and data not shown). Second, the lymphocyte proliferation assay upon BCR and LPS stimulation revealed no obvious difference between wild-type and Cas-L−/− mice (Fig. 5, B and C). Therefore the scenario of insufficient propagation of lymphocytes is unlikely. To test the third possibility, altered cell turnover, we performed an in vivo BrdU labeling assay (data not shown). The frequencies of BrdU-labeled cells in Cas-L−/− mice remained comparable to those in wild-type mice. Furthermore, no remarkable augmentation of apoptotic cells in the Cas-L-deficient spleen was detected in histological evaluation with TUNEL staining or flow cytometric analyses using annexin V assay (data not shown). Taking these findings together, it might be difficult to attribute the decreased number of peripheral lymphocytes to altered cell turnover. Therefore it would appear that lymphocyte reduction in secondary lymphoid organs is mainly due to altered lymphocyte trafficking. Because reduction of cell population is most striking in MZB cells of the spleen, we also analyzed a BCR-mediated response or cell turnover in the MZB cells. Although Cas-L−/− MZB cells showed slightly reduced Ca2+ flux in the Ca2+ mobilization assays or decreased apoptotic cells in the annexin V assay, no significant differences were obtained (data not shown). However, because the number of MZB cells in Cas-L−/− mice is very small, a subtle alteration, if any, might not have been detectable.

In summary, using gene-targeted mice, we have demonstrated that Cas-L is integral for both cell migration and adhesion, a lack of which may contribute to a defect of MZB cells in the spleen and contraction of secondary lymphoid organs.

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Disclosures

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References


