The Adaptor Protein Sh2d3c Is Critical for Marginal Zone B Cell Development and Function

Amin Al-Shami, Carrie Wilkins, Jeannette Crisostomo, Dhaya Seshasayee, Flavius Martin, Nianhua Xu, Adisak Suwanichkul, Stephen J. Anderson and Tamas Oravecz

*J Immunol* published online 26 May 2010

http://www.jimmunol.org/content/early/2010/05/26/jimmunol.1000096

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/05/26/jimmunol.1000096.DC1

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The Journal of Immunology is published twice each month by

The American Association of Immunologists, Inc.,

1451 Rockville Pike, Suite 650, Rockville, MD 20852

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Sh2d3c is an adaptor protein that has been implicated in T cell activation and shown to associate with different components of the integrin signaling pathway ex vivo. However, the in vivo significance of Sh2d3c expression in the regulation of the immune response and/or hematopoietic cell lineage development is not known. In this study, we show that expression of Sh2d3c is more critical for development and function of marginal zone B (MZB) cells than for T cell maturation. Mice deficient in Sh2d3c expression (Sh2d3c<sup>-/-</sup>) had a reduced number of MZB cells, and the residual MZB cells failed to properly capture polysaccharide Ags. Activation-induced proliferation, cytokine production, and migration of Sh2d3c<sup>−/−</sup> splenic B cells were also significantly reduced in vitro compared with wild-type (Sh2d3c<sup>+/+</sup>) cells. In contrast, T cell development and function were largely normal in Sh2d3c<sup>−/−</sup> mice. The thymi of Sh2d3c<sup>−/−</sup> mice showed no maturational abnormalities, the number of splenic T cells was only modestly reduced, and the T cells responded normally to in vitro polyclonal activation. The observed B cell deficiency in the Sh2d3c<sup>−/−</sup> mice led to diminished humoral immune response against thymus-independent type 2, but not thymus-dependent Ags, which highlights the primary role of Sh2d3c in regulating B cell development and function. The Journal of Immunology, 2010, 185: 000–000.

Materials and Methods

Generation of Sh2d3c<sup>−/−</sup> mice

The Sh2d3c<sup>−/−</sup> line was generated by homologous recombination as part of Lexicon and Genentech’s collaboration to knock out and analyze the function of 500 secreted and transmembrane proteins. The targeting vector

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The online version of this article contains supplemental material.
was derived using the λ KOS system (21). The λ KOS phage library was screened by a PCR primer pair overlapping exon 4 of the murine Sh2d3c gene (GenBank accession number NM-013781 [www.ncbi.nlm.nih.gov/Genbank]), Sh2d3c+/- [5'-CTT ACT TGT GCT TGT ACG AAC CJC] and Sh2d3c-2 [5'-GGC ACG TGA GCA CAG ATG CCC]). One genomic clone, pKOS-82, was isolated from the library and confirmed by sequence analysis. To generate the target vector, first gene-specific arms (5'-GCC AAT CTC GCT CTC CCC GTG CAG TTC TCC AAG GA-3') were appended by PCR to a yeast selection cassette containing the URA3 selectable marker. The yeast selection cassette and pKOS-82 were cotransformed into yeast, and clones that had undergone homologous recombination to replace the yeast cassette with an exogenous genomic region containing exons 4–6 were identified and confirmed by Southern blot analysis. To generate the target vector, first gene-specific arms (5'-GCC ACC AAC GAG AAC AGC ATT ACA GAC TAC CCC CT-3') were appended by PCR to a yeast selection cassette containing lacZ, the targeting selectable marker. The yeast selection cassette and pKOS-82 were cotransformed into 129/SvEvBrd embryonic stem (ES) cell selection cassette containing lacZ, the targeting selectable marker were isolated. After replacing the yeast cassette with the corresponding genomic fragment containing exons 4–6, targeting ES cells were isolated. G418/FIAU-resistant ES cell clones were isolated, and clones 2A1, 2A2, and 2F9 were injected into blastocysts, and clone 2A1 ultimately achieved germline transmission. Lex-2 represents control ES cell DNA. RT-PCR performed on RNA extracted from thymus, spleen, and lungs from Sh2d3c+/- and Sh2d3c-/- mice using specific primers. Primers for actin were used as a control. D, Immunoblot analysis of lysates from spleens extracted from Sh2d3c+/- and Sh2d3c-/- mice using anti-Sh2d3c Abs, followed by anti-GAPDH Abs.

Expression analysis of Sh2d3c by RT-PCR

To confirm disruption of the Sh2d3c gene in the knockout (KO) mice, total RNA were collected from lung, spleen, and thymus from three WT and three homozygous mice using a bead homogenizer (BioSpec Products, Bartlesville, OK) and TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Reverse transcription was performed to produce cDNA using 5 µg total RNA with SuperScript II (Invitrogen), random hexamer primers, according to the manufacturer's instructions. PCR amplification was performed using 1 µl of the RT product at initial denaturing step of 95°C for 2.5 min, followed by 35 cycles of 95°C (30 s), 59°C (30 s), and 70°C (1 min) with oligonucleotide primers complementary to exons 4 and 5 of Sh2d3c (5'-AGG AAC TGA AGC TCA GCA GCA CGG-3' and 5'-TAG GCT GGT GAG TGA GTC TCG G-3'). Primers to the mouse β actin gene (GenBank accession number M12481 [www.ncbi.nlm.nih.gov/Genbank]), 5'-GGC TGG CCG GGA CCT GAC GGA CTA CCT CAT-3' and 5'-GCC TAG AAG CAC TTT TGG TCC GAC ATG GAG-3') were also used as internal control for sample handling.

Immunizations, Ig, and trinitrophenyl measurements

Basal, anti-trinitrophenyl (TNP), and anti-OVA Ig concentrations in serum were measured by ELISA using Abs from Southern Biotechnology Associates (Birmingham, AL). Mice were immunized by i.p. injection of 100 µg TNP-Ficoll, TNP-LPS, or OVA in CFA, and Ag-specific Abs were captured on plates coated with TNP or OVA (BD Biosciences, San Jose, CA). TNP retention on MZB cells was assessed, as described previously (22),
with the exception of using PE-labeled streptavidin (BD Biosciences) for detection.

**Immunofluorescent histochemistry**

Spleens were rapidly frozen in Tissue-Tek OCT compound (Sakura Fine-technical, Torrance, CA) and sectioned. Sections were stained with Abs to Moma-1 (biotinylated; Abcam, Cambridge, MA), MARCO (unconjugated; AbD Serotec, Raleigh, NC), B220 (PE), IgM (FITC), or IgD (PE), and with secondary anti-rat Abs or streptavidin (FITC; all from BD Biosciences), as required. Slides were examined using Axiovert inverted fluorescent microscope (Zeiss, Thornwood, NY).

**BM transplantation**

Recipient mice were irradiated, as described before (23), with the following exception: 2 x 10^6 donor BM cells were injected 8 h after the second dose of irradiation. The average percentage of Neo^+ cells derived from donor Sh2d3c^+/+ BM was 93% in the reconstituted animals.

**FACS analysis**

These procedures were performed, as described previously (23); all Abs were from BD Biosciences. Indicated B cell subsets were sorted to 99% purity by using a FACSARia (BD Immunocytometry Systems, San Jose, CA).

**Cell migration, proliferation, and cytokine production assays**

Migration of splenocyte subsets was assessed after a 4-h incubation in 5-μm pore-size 96-well plates (NeuroProbe, Gaithersburg, MD), with 1 ng/ml murine stromal cell-derived factor (SDF)-1α or 100 ng/ml CXCL13 (PeproTech, Rocky Hill, NJ) in the bottom chamber. Cell proliferation was assessed, as described before (24), using mAbs to CD3 and CD28 at equal concentrations (as indicated), LPS (1 μg/ml), αIgM mAb (5 μg/ml), or a mixture of CD40 mAb and IL-4 (2.5 ng/ml and 5 ng/ml, respectively). B cell subsets were purified with an enrichment kit (StemCell Technologies, Vancouver, BC, Canada). The concentration of cytokines in cell culture supernatants was measured 48 h after activation using CBA mouse inflammation kits (BD Biosciences).

**Ca^2+ mobilization assay**

Measurements were made by using a Fluo-4 NW Ca^2+ assay kit (Invitrogen). B cell-enriched splenocytes (1 x 10^7) were activated with 10 μg/ml αIgM mAb, and maximal fluorescence was assessed by addition of 100 ng/ml ionomycin. Basal fluorescence measured at the first time point was subtracted from each subsequent measurement.

**Western blot analysis**

Enriched B cells from spleen (4 x 10^7 cells/ml) were stimulated with 10 μg/ml LPS, and Western blotting of lysed samples was performed using polyclonal Abs to Pyk2 and phosphorylated Pyk2 (Cell Signaling Technology, Beverly, MA), HRP-conjugated goat anti-rabbit Ab (Bethyl Laboratories, Montgomery, TX), and ECL detection system (GE Life Sciences, Pittsburgh, PA). Spleen lysates from Sh2d3c^+/+ and Sh2d3c^−/− mice were tested by anti-Sh2d3c (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GAPDH (Bethyl Laboratories) Abs.

**Statistical analyses**

Statistical significance of group differences was evaluated by unpaired, two-tailed, Student t test. A p value of <0.05 was considered significant.
Results

*Sh2d3c*<sup>−/−</sup> mice do not show gross abnormalities

We generated *Sh2d3c*<sup>−/−</sup> mice by targeted disruption of exons 4–6 of the murine homolog of the *Sh2d3c* gene (GenBank accession number NM_013781 [www.ncbi.nlm.nih.gov/Genbank/]) by homologous recombination in ES cells (21), followed by germline transmission of the mutant gene into chimeric, *Sh2d3c*<sup>+/+</sup>, and *Sh2d3c*<sup>−/−</sup> mice (Fig. 1A). Successful targeting of the *Sh2d3c* gene in the KO mice was confirmed by Southern hybridization analysis of ES cell (Fig. 1B), expression analysis of the gene transcript (Fig. 1C), and Western blot analysis of the Sh2d3c protein (Fig. 1D). Mating of *Sh2d3c*<sup>+/+</sup> mice generated pups of the three possible genotypes with ratios that fit well with normal Mendelian frequencies.

*Sh2d3c*<sup>−/−</sup> mice exhibited no substantial difference in growth rate and size. Likewise, comprehensive clinical diagnostic and pathologic analysis (described in detail in Ref. 25), including tests of inflammation, behavior, obesity, diabetes, bone, cell proliferation, and cardiovascular function, did not demonstrate significant differences in *Sh2d3c*<sup>−/−</sup> mice relative to their WT littermates (data not shown).

Absence of *Sh2d3c* leads to impaired MZB cell development

Hematologic and FACS analysis of lymphoid organs revealed decreased cellularity and markedly reduced representation of B220<sup>+</sup>CD21<sup>high</sup>CD23<sup>med/low</sup> MZB cells in the spleen of *Sh2d3c*<sup>−/−</sup> mice compared with WT littermates (Fig. 2). A decrease in the proportion of the IgM<sup>high</sup>CD21<sup>high</sup> T2/MZB cell population, which includes MZ precursor cells in addition to MZB cells in the spleen, was also readily apparent in the *Sh2d3c*<sup>−/−</sup> mice. Although the absolute number of FOB cells also appeared to be reduced, the difference did not reach statistical significance. Furthermore, no differences were observed in the immature T1 or mature B cell populations (Fig. 2B). Immunohistological examination of the spleen showed that the region of IgM<sup>high</sup>IgD<sup>low</sup> MZB cells that form a rim around the follicles was greatly diminished in the absence of *Sh2d3c* (Fig. 2C), which correlates with the flow cytometric analysis data. In contrast, the resident metallophilic macrophages that are important components of the marginal zone (26), identified by the marker Moma-1, were correctly located at the border between the marginal and follicular zones in both WT and mutant mice (Fig. 2C). Similarly, the distinct MARCO<sup>+</sup> marginal zone macrophages that are required for retaining MZB cells (27) were also detected at comparable levels in WT and *Sh2d3c*-deficient spleens (Fig. 2C). Of note, the blood, BM, and lymph nodes of *Sh2d3c*<sup>−/−</sup> mice did not demonstrate obvious B cell deficiencies, and the peritoneal cavity also contained normal number of cells of the B1 B cell phenotype (data not shown).

**FIGURE 3.** *Sh2d3c* regulates the TI-2 response. A, The basal concentration of the different Ig isotypes in the serum of the indicated mice was determined by ELISA. Data are presented as mean ± SEM. B, Anti-TNP Ab titers were determined by ELISA at the indicated time points after immunization of WT and KO mice with TNP-Ficoll. Serum dilutions were 1/1000 for IgG1 and 1/100 for the rest of the Ig subclasses. Data were obtained from 14–20 mice of each genotype pooled from at least three independent experiments giving similar results. Data are presented as mean ± SEM, *p* < 0.05; **p** < 0.005, *Sh2d3c*<sup>−/−</sup> versus WT. C, Retention of TNP by MZB cells is deficient in *Sh2d3c*<sup>−/−</sup> mice. Representative FACS histograms of cell surface anti-TNP and isotype-matching control mAb reactivities are shown as detected 30 min after TNP-Ficoll injection. D, Frozen sections from spleens of indicated mice injected with TNP-Ficoll and analyzed 30 min later. Red fluorescence identifies TNP accumulation in the marginal zone area (×10 magnification). MFI, mean fluorescence intensity.
findings indicate that Sh2d3c is critical for MZB cell development, but less so for the homeostasis of other B cell subsets. Transplantation of BM cells from Sh2d3c−/− mice to irradiated WT recipients recapitulated the MZB cell phenotype of the complete KOs, with the additional observation that the KO chimeras showed a modest reduction in the fraction of B220+ cells in the BM (Supplemental Fig. 1). These findings demonstrate that the defect in MZB cell formation in Sh2d3c-deficient mice is intrinsic to hematopoietic cells.

Sh2d3c−/− mice exhibit deficient humoral immune response to TI-2 Ags

MZB cells have been associated with the TI-2 Ab response (18); therefore, we immunized Sh2d3c−/− and Sh2d3c+/+ mice with TI-2 Ag TNP-Ficoll and measured the levels of TNP-specific Abs in the serum 7, 14, and 28 d after immunization. The basal serum concentrations of Ig subclasses were similar in unchallenged Sh2d3c−/− and Sh2d3c+/+ mice (Fig. 3A). On days 7 and 14 after immunization with TNP-Ficoll, there was a clear trend of reduced TNP-specific Ig in Sh2d3c−/− mice that was statistically significant for IgG1, IgG2b, and IgG3 on day 7 (Fig. 3B). By day 28 after immunization, the levels of TNP-specific IgM, IgG1, IgG2b, and IgG3 Abs all were significantly lower in Sh2d3c−/− mice compared with their Sh2d3c+/+ littersmates. The Ag-specific IgG2a response was also reduced in the KO mice, but the decrease did not achieve statistical significance. Importantly, the humoral response to OVA, a thymus-dependent Ag, and to TNP-LPS, a thymus-independent type 1 Ag, showed no significant differences at either

FIGURE 4. Sh2d3c is indispensable for LPS-induced MZB cell activation. A, Enriched B cells from spleen were cultured in the presence of the indicated stimulators for 48 h, and cell proliferation was assessed by [3H]TdR uptake for the last 10 h. Data were obtained from 20 mice of each genotype pooled from four independent experiments giving similar results. B, Enriched spleen B cells were stimulated with LPS, as above, and concentration of the indicated cytokines was measured in the cell culture supernatants at the end of the assay (n = 11 mice). C, Sorted FOB (B220+CD21medCD23high) and MZB (B220+CD21highCD23mid/low) cells were cultured with LPS, and cell proliferation was assessed, as above (n = 6 mice). Background signals (FOB and MZB cells without LPS) were between 30 and 100 cpm. Data are presented as mean ± SEM; numbers above bars indicate p values.

FIGURE 5. Sh2d3c is essential for chemokine-induced B cell migration. Splenocytes migrating to the indicated stimuli were collected, counted, and analyzed by FACS. Total splenocyte numbers (upper left panel) were normalized to the number of migrating cells in the WT vehicle control samples in each experiment. The rest of the bar graphs show the fraction of indicated cell populations migrating to the different stimuli. Data were obtained from 9–11 mice of each genotype pooled from three independent experiments giving similar results, using three replicates of each sample. Data are presented as mean ± SEM; numbers above bars indicate p values.

FIGURE 6. Pyk2 activation is diminished in Sh2d3c−/− splenic B cells. A and B, Enriched B cells from the spleens of indicated mice were activated with LPS. Lysates of these cells were analyzed for Pyk2 and phospho-Pyk2 expression at the indicated time points by Western blotting. A, Western blot of a representative experiment. B, Densitometry graph of data pooled from four independent experiments giving similar results.

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mice from three experiments). Data are presented as mean representative FACS plots show normal CD4/CD8 mAb-staining patterns of MZ macrophages and B cells, and retained on the cell surface in the absence of Sh2d3c. We explored the response of B cells from Sh2d3c-deficient mice with WT counterparts (Fig. 3). Furthermore, immunofluorescent histochecmistry revealed that in WT spleens TNP-Ficoll was localized exclusively in the marginal zone area (Fig. 3D), whereas Sh2d3c-deficient spleens showed weak and thin rim of positive staining that sometimes appeared to be in a dotted pattern, most likely reflecting the TNF-Ficoll bound to MZ macrophages.

Sh2d3c expression is essential for the robust LPS response of MZB cells

We explored the response of B cells from Sh2d3c−/− and Sh2d3c+/+ mice to in vitro stimulation by using a variety of polyclonal stimuli, including LPS. When treated with LPS, cell proliferation (Fig. 4A) as well as the production of the inflammatory cytokine IL-6 (Fig. 4B) were greatly reduced in splenic B cell cultures of Sh2d3c-deficient mice compared with WT littermates. In contrast, no differences were observed in the proliferation rate when the cells were stimulated with a mixture of CD40 mAb and IL-4 or with anti-IgM mAb (Fig. 4A). We also examined whether the absence of Sh2d3c would affect early signaling events, such as calcium mobilization via the BCR. Enriched B cells from Sh2d3c+/+ and Sh2d3c−/− mice did not differ in their kinetic response to anti-IgM–elicited calcium mobilization (Supplemental Fig. 3).

To test whether MZB cells were responsible for the observed deficiency in the LPS response of Sh2d3c−/− B cells, we repeated the LPS activation study with purified MZB and FOB cells. Consistent with previous reports (28), WT MZB cells proliferated more to LPS than WT FOB cells. However, Sh2d3c-deficient MZB cells proliferated poorly after LPS activation compared with WT MZB cells. The cell proliferation defect was restricted to MZB cells because purified FOB cells from Sh2d3c+/+ and Sh2d3c−/− mice proliferated similarly when activated with LPS (Fig. 4C). Of note, the deficient LPS response of Sh2d3c−/− MZB cells was not due to differences in TLR4 expression, as confirmed by FACS analysis (data not shown).

Sh2d3c−/− B cells are refractory to CXCL13-induced chemotactic response in vitro

Proper migratory response of B cell precursors is a prerequisite for normal MZB cell development (14, 18). Furthermore, Sh2d3c has been implicated in cell migration in COS cells (5). Therefore, we examined the ability of Sh2d3c−/− splenocytes to migrate in response to the chemokines SDF-1α and CXCL13 in vitro. SDF-1α is produced in the red pulp of the spleen and is involved in migration of B cells (29, 30), whereas CXCL13 is required for the localization of B cells in the spleen (31, 32). The motility of Sh2d3c-deficient splenocytes to both SDF-1α and CXCL13 was significantly reduced when compared with WT cells (Fig. 5). FACS analysis revealed that the deficiency in the migratory response was not limited to MZB cells. Both FOB and MZB cells were largely refractory to the CXCL13-induced migratory signal in the absence of Sh2d3c. In contrast, only Sh2d3c−/− FOB cells appeared to show deficient migratory response to SDF-1α, although the response of MZB cells was generally poor to this chemokine irrespective of Sh2d3c expression (Fig. 5). The surface density of CXCR4 and CXCR5, the receptors for SDF-1α and CXCL13, respectively, was comparable between WT and Sh2d3c-deficient cells (data not shown), ruling out a role for Sh2d3c in regulating the expression of these receptors.

Absence of Sh2d3c alters the kinetics of Pyk2 phosphorylation

Pyk2 is a signaling component of the integrin pathway that is involved in regulating cell morphology and cell migration to chemotactic signals (33). Deficiency in Pyk2 has been shown to result in a diminished number of MZB cells (22). Because Sh2d3c reportedly interacts with Pyk2 (6), it seemed plausible that the defect in MZB cell development in Sh2d3c−/− mice might be associated with an alteration in Pyk2 signaling. To test this hypothesis, and to provide a validation in primary B cells for the reports linking Sh2d3c and Pyk2 (6), we examined the phosphorylation of Pyk2 in B cell-enriched splenocytes of Sh2d3c−/− and Sh2d3c+/+ mice (Fig. 6). In WT B cells, LPS induced a time-dependent phosphorylation of Pyk2 that was observed as early as 1 min after activation and was maintained throughout the test period (60 min). In contrast, a weaker phospho-Pyk2 signal was detected in Sh2d3c−/− B cells, which was apparent only 5 min after LPS stimulation and continued to strengthen over the entire 60-min time course.

Sh2d3c is dispensable for normal T cell development

Previous reports have shown that overexpression of Sh2d3c increases TCR-induced IL-2 production in Jurkat T cells (6), thus impairing this adaptor protein in T cell function. However, the distribution of the major thymocyte subpopulations was normal in the Sh2d3c−/− mice (Fig. 7), and there were no substantial differences in the naive and memory T cell numbers in the blood or distribution of the same cell subsets in the spleen of Sh2d3c−/− versus Sh2d3c+/+ mice (data not shown). Furthermore, despite a modest reduction in numbers (Fig. 7), splenic T cells from
Sh2d3c−/− mice proliferated normally to activation via the CD3/CD28 pathway (Fig. 7) and produced similar levels of IL-2 (data not shown). Together, our data suggest that Sh2d3c plays a non-critical role in T cell development or function.

Discussion

Comprehensive physiological analysis of Sh2d3c-deficient mice demonstrated that Sh2d3c has an essential and relatively specific role in MZB cell development. The residual MZB cells in the KO mice also had impaired function, as demonstrated by decreased ability to retain and get stimulated by polysaccharide Ags, and to migrate in response to chemotactic signals and G protein-linked receptors (22, 39). B cells in Sh2d3c KO mice had decreased ability to migrate in response to chemotactic signals, which suggests a role for this protein in the localization of MZB cell precursors to the appropriate area of the spleen. It is possible that Sh2d3c regulates cell migration via its interacting partners, Pyk2 and Cas-L (5–7). The latter proteins have been shown to regulate integrin signaling (6), and the immune phenotype of both the Pyk2 and Cas-L-deficient mice is consistent with this proposed mechanism. Each of these three KO lines showed reduced MZB cell formation, significantly blunted T1-2 immune responses, and defective migration of splenic B cells (22, 40), which are also hallmarks of the Sh2d3c−/− mice phenotype. Furthermore, overexpression of Sh2d3c enhanced the phosphorylation and activation of Pyk2H in Jurkat cells (6), which is consistent with our results that Sh2d3c-deficient B cells showed weaker phosphorylation of Pyk2. It is important to note that the surface density of integrin receptors CD11a, CD18, CD49d, and CD29, which are involved in regulating B cell homing (40), was normal on Sh2d3c−/− splenocyte subsets (unpublished results), which rules out integrin expression as a primary mechanism of the deficient MZB cell generation in Sh2d3c−/− mice.

Earlier work indicated that in Jurkat T cells Sh2d3c was phosphorylated upon TCR engagement, and that overexpression of Sh2d3c increased JNK activation and boosted IL-2 production (6). Moreover, Cas-L (41) and Pyk2 (33, 42, 43), which associate with Sh2d3c, were also implicated in T cell activation and IL-2 production. Although there was a modest decrease in the number of splenic T cells in Sh2d3c−/− mice, associated with a general reduction in the cellularity of this organ, T cell subpopulations in the thymus and blood of Sh2d3c−/− mice did not show significant changes and splenic T cells responded normally to TCR-mediated activation in vitro. Together our data suggest that Sh2d3c does not play a critical role in T cell development or function. It is possible that the role of Sh2d3c in T-lineage cells is redundant and alternative pathways are in place to compensate for its absence, or that the signaling mechanism has been affected in cell lines, such as Jurkat.

In conclusion, our results reveal a nonredundant role for Sh2d3c in MZB cell development and function. We propose that Sh2d3c is required for proper localization of cells, particularly MZB cells in vivo, and support the concept that Sh2d3c is a component of a signaling pathway that also includes Pyk2 and Cas-L. Although our data show a clear and relatively selective role for Sh2d3c in MZB cell formation, the exact role of Sh2d3c in downstream signaling pathways of integrins or chemokine receptors is yet to be determined. The decrease in polysaccharide binding on MZB cells in Sh2d3c-deficient mice suggests a reduced function for complement receptors on these cells, but the underlying causes are not yet clear. Finally, in light of the apparent absence of a nonimmunological phenotype in Sh2d3c null mice, the significance of alternative splicing of Sh2d3c RNA and the relatively ubiquitous expression of one of the described Sh2d3c isoforms remains to be defined.

Acknowledgments

We thank the phenotypic analysis groups at Lexicon Pharmaceuticals for carrying out the comprehensive clinical diagnostic tests on the mutant mouse lines. We thank the Pathology Department for help in preparing slides from tissue sections.

Disclosures

The authors of this article are or have been employees of, and received stock options from, Lexicon Pharmaceuticals or Genentech.

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