Delta-like 1 is essential for the maintenance of marginal zone B cells in normal mice but not in autoimmune mice

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Abstract

Notch2 and Delta-like 1 (Dll1) have been implicated in the development of marginal zone B (MZB) cells. In the present study, we characterized the expression and function of mouse Notch receptors and ligands in the spleen by using newly generated mAbs. Although Notch2 was expressed on both B and T cells in the spleen, the highest expression was observed on precursors of marginal zone B and MZB cells. Dll1 was expressed on macrophage and erythroblasts in the red pulp, but not on B cells or marginal zone macrophage. Administration of a blocking mAb against Dll1 not only blocked the development of MZB cells in juvenile mice but also gradually depleted the pre-established MZB cells in adult mice, indicating a critical role for Dll1 in the maintenance of MZB cells in the spleen of normal mice. Interestingly, Dll1 was not necessary for the maintenance of MZB cells in lupus-prone (NZB x NZW) F1 mice particularly after the onset of the disease, suggesting that the Dll1 independence may be a feature of dysregulated MZB cells producing auto-antibodies.

Introduction

The Notch genes encode transmembrane receptors that are highly conserved from invertebrates to mammals. Four mammalian Notch receptors have been identified, designated as Notch1, Notch2, Notch3 and Notch4. Notch receptors are activated upon interaction with four ligands, named Delta-like 1 (Dll1), Delta-like 4 (Dll4), Jagged1 and Jagged2. These ligands are also transmembrane proteins with conserved structure and signal through Notch receptors by a common mechanism (1). The specific binding of Notch with its ligands via direct contact between cells results in the proteolysis of Notch and movement of intracellular region of Notch into the nucleus, where making a complex with the DNA-binding protein RBP-J acting as an ortholog of Drosophila Su(H) (1, 2). This translocation is an essential part of the signal transduction process. While Notch signaling is controlled at multiple levels (2), it has been revealed that the cellular trafficking of both Notch ligand and Notch receptor plays a critical role in regulating the signaling activity (3).

Notch signaling is involved in many developmental processes and lineage decisions in fetal and post-natal organogenesis, as well as in adult self-renewing organs (4). Recent studies using conditional gene inactivation have shown that the Notch2 signal in B cells is essential for the generation of marginal zone B (MZB) cells at the branching point of MZB and conventional follicular B (FoB) cells in the spleen (5). In that case, MINT was shown to be a signal modifier in the cytoplasm (6). Similarly, conditional inactivation of Dll1 led to a selective loss of MZB cells in the spleen (7). Based on these findings, it has been suggested that Notch2 and Dll1 interaction regulates the MZB cell development in the spleen.

The splenic structure that separates the white pulp from the red pulp was designated the marginal zone (MZ). The MZ is primarily made of MZB cells, specialized macrophage such as marginal metallophilic macrophages (MMMs) and marginal zone macrophages (MZMs) and reticular cells. In rodents, MZB cells are now recognized as a distinct naive...
Dll1 is essential for the maintenance of MZB cells

B cell lineage, separate from FoB cells and B1 cells (8). B lymphopoiesis occurs in the bone marrow and yields newly formed or transitional B cells that emigrate to the spleen (9). Two types of transitional mature B cell precursors exist in the spleen (10). Type-1 transitional B (T1B) cells, which are recent immigrants from the bone marrow, develop into type-2 transitional B (T2B) cells in the spleen. Recent studies have indicated that these transitional B cells can further differentiate into FoB cells and MZB precursor cells (5, 11). A small proportion of memory B cells may also populate the MZ in rodents (10, 12). B cell receptor, B cell activating factor belonging to tumor necrosis factor (TNF) family and Notch2 signals play an essential role in the development of MZB cells (9, 13–15), but precise mechanisms remain elusive. For the retention of MZB cells in the MZ, interactions of integrins leukocyte function-associated antigen (LFA)-1 (αbβ2) and VLA-4 (α4β1) on MZB cells with their ligands are important in addition to the effects of chemokines (16).

MZB cells have long been considered a critical sentinel of host defense against blood-borne pathogens, which is primarily directed against T-independent multivalent antigens. However, a broader role for MZB cells in both T-independent and T-dependent immune responses has been also demonstrated (17–21). Furthermore, several recent studies have claimed a potential role for MZB cells in the development of auto-antibodies in systemic autoimmune (22, 23). CD1high B cells have been reported to produce large amounts of IgM anti-DNA antibodies in lupus-prone (NZB × NZW) F1 (BWF1) mice (24), which apparently have an increased number of MZB cells (25).

In this study, we characterized the expression of Notch receptors and ligands in the spleen of normal mice and lupus-prone BWF1 mice by utilizing a newly generated panel of specific mAbs. We also examined the effect of a blocking mAb againstDll1 on the development and maintenance of MZB cells. Interestingly, the maintenance of MZB cells in BWF1 mice was independent ofDll1 especially after the disease onset.

Methods

Mice

Female C57BL/6 (B6) mice were purchased from Charles River (Oriental Yeast, Tokyo, Japan) and female BWF1 mice were obtained from Sankyo Labo Service (Tokyo, Japan). All animal procedures described in this study were performed in accordance with the guidelines for animal experiments of Juntendo University School of Medicine.

Generation of mAbs

CHO cells expressing murineDll1, Jagged1 or Jagged2 were prepared as described previously (26). A CHO line expressing murineDll4 was also established (our unpublished data). Armenian hamsters (Oriental Yeast) were immunized by intra-peritoneal (i.p.) injection ofDll1-, Jagged1- or Jagged2-expressing CHO cells or recombinant mouseDll4 (R&D Systems, Minneapolis, MN, USA) three times at 7-day intervals. Three days after the final immunization, the splenocytes were fused with P3U1 myeloma cells. After HAT selection, antibodies that reacted withDll1-,Dll4-, Jagged1- or Jagged2-transfected CHO cells, but not with untransfected CHO cells, were screened by flow cytometry. Each mAb was cloned by limiting dilution.

To generate the mAb that react with mouse Notch1, Notch2, Notch3 and Notch4, Armenian hamsters were immunized by i.p. injection of mouse Notch1-Fc, Notch2-Fc, Notch3-Fc or Notch4-Fc fusion proteins (27) three times at 7-day intervals. The hybridoma cells were prepared as described above, and antibodies that reacted with mouse Notch1-, Notch2-, Notch3- or Notch4-transfected CHO cells, but not with untransfected CHO cells, were screened and cloned. All these mAbs were purified from ascites produced in pristan-primed ICR nude mice by the caprylic acid and ammonium sulfate precipitation method (28) and labeled with biotin for flow cytometric analysis.

Other antibodies

FITC-labeled mAbs against mouse CD3 (145-2C11), CD11b (M1/70), CD11c (HL3), CD62L (MEL-14) or CD71 (R172/17), PE-labeled mAbs against mouse CD11b (M1/70), CD23 (B3B4), Gr-1 (RB6-8C5) or TER119, non-labeled mAb against mouse CD1d (1B1) and APC- or PE-conjugated streptavidin were obtained from eBioscience, San Diego, CA, USA. CD1d was labeled with Pacific Blue succimidyl ester (Molecular Probes, Carlsbad, CA, USA) in our laboratory. FITC-labeled mAbs against mouse CD21 (7G6), PE-labeled mAb against mouse CD40 (3/23) and PerCP-labeled mAb against mouse B220 (RA3-6B2) were from BD Bioscience, San Jose, CA, USA. FITC-labeled mAbs against mouse CD68 (FA-11) or MOMA-1 and non-labeled mAb against mouse MARCO (ED31) were from Serotec, Oxford, UK. FITC-labeled mAb against mouse F4/80 (CI:A3-1) was from CALTAG, Carlsbad, CA, USA. Non-labeled mAbs against iCAM-1 (YN1/1.7) and VCAM-1 (MK/2) were prepared as described previously (29, 30).

Treatment with mAbs

Mice were i.p. injected with the indicated dose of anti-mouseDll1 mAb (HMD1-5), control Armenia hamster IgG (eBioscience), anti-LFA-1 mAb (KBA) (31) plus anti-VLA-4 mAb (PS/2) (32) or control rat IgG (Jackson Immunoresearch, West Grove, PA, USA).

Flow cytometry

Multi-color staining was conducted using combinations of the indicated mAbs. Briefly, 10^6 cells of spleen, bone marrow or peripheral blood were first incubated with Fc Block (BD Bioscience) to block non-specific binding to FcγR and then with optimized dilutions of biotinylated mAbs for 15 min on ice. After washing three times with 2% FCS and 0.1% sodium azide in PBS, the cells were incubated for 15 min on ice with optimized dilutions of FITC-, PE-, PerCP-, Toricolor-labeled mAbs or streptavidin and washed three times. The cells were analyzed on FACSAria or FACScan (BD Bioscience). The data were analyzed with CellQuest (BD Bioscience).

Immunohistological staining

Tissue samples of spleen were frozen in compound (SAKURA, Tokyo, Japan) and were cut into 3-μm sections.
After being air-dried, sections were fixed in acetone for 10 min. For staining Dll1, sections were blocked with 100 μg ml⁻¹ of control hamster IgG for 1 h after blocking internal biotin. Biotin-labeled HMD1-5 was used at 2.5 μg ml⁻¹ and incubated overnight at 4°C. For detection, Tyramide Signal Amplification Kit (Perkin Elmer, Waltham, MA, USA) was used. For staining MARCO, ICAM-1 and VCAM-1, sections were blocked with 10% normal goat serum for 10 min, and Alexa488-labeled goat anti-rat IgG (Molecular Probes) was used as secondary antibody. For staining with FITC-labeled mAbs to CD11b, CD68, B220, MOMA-1 or F4/80, sections were blocked with 10% normal rabbit serum for 10 min, and Alexa488-labeled rabbit anti-FITC (Molecular Probes) was used as secondary antibody. Images were acquired on confocal microscope (Carl Zeiss, Jena, Germany).

Cell sorting
Spleen cells were incubated with optimized dilution of biotin-labeled HMD1-5 for 15 min on ice. After washing twice with 2 mM EDTA in PBS, the cells were incubated with streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4°C and washed once. The cells were re-suspended in 0.5% BSA and 2 mM EDTA in PBS and sorted on AutoMACS (Miltenyi Biotec).

Reverse transcription–PCR
Total RNA was isolated using STAT60 (Tel-Test, Friendswood, TX, USA) and was reverse transcribed to cDNA with oligo-dT and SuperScript RT (Invitrogen, Carlsbad, CA, USA). PCR consisted of 35 cycles of 45 s at 94°C, 1 min at 58°C and...
The primers were as follows: Dil1 sense, 5′-ACCTTCTTTGAGGTCCCTCAAG-3′ and Dil1 anti-sense, 5′-AGAGTCTGTATGGAGGGCTTC-3′; beta-actin sense, 5′-GTGGGCCGCTCTAGGCACCAA-3′ and beta-actin anti-sense, 5′-CTCTTTGATGTCACGCACGCACGATTTC-3′. These beta-actin primers react with mouse and Chinese hamster cDNAs. PCR products were separated by electrophoresis on 2.0% agarose gels with 0.5 μg ml⁻¹ ethidium bromide and detected by UV.

**Reporter assay for Notch2 activation**

The reporter assay was performed as described previously (33). Briefly, Notch2-transfected CHO cells were transfected with a TP1 (the RBP-Jκ-responsive promoter) -luciferase reporter plasmid and then cultured with 20 μg ml⁻¹ of HMD1-5 or control hamster IgG for 24 h in a plate that was coated with 5 μg ml⁻¹ of Dil1-Fc fusion protein (Dil1-Fc) (26) or human IgG-Fc fragment (Athens Research & Technology, GA, USA). Luciferase activity in the mixture of the cells was then measured using a luminometer.

**Preparation and stimulation of peritoneal macrophages**

Peritoneal macrophages were obtained from B6 mice that received i.p. 2 ml of 4% thioglycolate (Sigma, St Louis, MO, USA) 4 days before. Peritoneal exudate cells (PECs) were harvested by peritoneal lavage with ice-cold PBS and depleted of non-adherent cells after 1 h culture on a plastic dish and then stimulated with 1 μg ml⁻¹ LPS (Sigma), 20 ng ml⁻¹ IFN-γ, 20 ng ml⁻¹ TNF-α (BD Bioscience) or 5 μg ml⁻¹ anti-CD40 (HM40-3) (34) at 37°C for 36 h.
Phlebotomy
Blood (0.5 ml) was withdrawn from B6 mice by venipuncture. Seven days later, RBCs in the spleen were analyzed by flow cytometry.

Results

Generation of anti-Dll1 mAb
To further investigate the role of Dll1 in the maintenance of MZB cells and to determine the protein expression of Notch receptors and ligands, we established mAbs specific for mouse Notch1, Notch2, Notch3, Notch4, Jagged1, Jagged2, Dll1 and Dll4 (Fig. 1A and B). A mAb that reacts with mouse Dll1 (HMD1-5) blocked the binding of Notch2-Fc fusion protein to Dll1-expressing CHO cells and Notch2 signaling triggered by Dll1-Fc fusion protein (Fig. 1C and D). Reverse transcription–PCR demonstrated that Dll1 mRNA was expressed exclusively in the HMD1-5-positive fraction but not in the negative fraction of splenocytes (Fig. 1E).

Expression of Notch receptors on B cells
Recent studies with conditional knock out (KO) mice have suggested that Notch2 on B cells interacts with Dll1 to specify the MZB cell fate. However, the expression of Notch2 and Dll1 on the surface of splenocytes has not been identified. The MZB cell population (B220CD11chiCD23lo/hi) appears in the spleen of normal B6 mice around 3 weeks of age (Fig. 5A). By using the mAbs we generated (Fig. 1A), we here examined the expression of Notch receptors on B cells before (1 week old), during (6 weeks old) and after (16 weeks old) the MZB generation. B cells had higher Notch2 expression than T cells (Fig. 2A). Notably, Notch2 expression level was significantly higher on MZB and precursors of marginal zone B (pre-MZB) (B220CD11chiCD23hi) cells than on T1B (B220CD11cCD21 CD23lo) and T2B/FoB (B220CD11cCD21 CD23hi) cells (Fig. 2B). There were no obvious differences in Notch2 expression levels on whole B cells among 1-, 6- and 16-week-old mice. Notch2 was also expressed preferentially on B cells during maturation in the bone marrow, especially on B220lo B cells (data not shown).

Expression of Notch ligands on splenocytes
We next examined the expression of Notch ligands in the spleen of 6-week-old mice using the mAbs we generated (Fig. 1B). Dll1 was expressed on a substantial part of macrophages (CD11bhiCD68+ Gr-1−/CD11c+) and a lower expression of Dll1 was observed on CD11c+ dendritic cells (Fig. 3A), but not on lymphocytes including CD3+ T cells, B220+ B cells and NK1.1+ NK cells (data not shown). The expression of

Fig. 3. Expression of Notch ligands on splenocytes. (A) Expression of Notch ligands on splenic macrophages (Mø: CD11bhiCD11c+) and DCs (CD11bhiCD11c+) from 6-week-old B6 mice was analyzed by flow cytometry. Filled histograms indicate the staining with control hamster IgG and open histograms indicate the staining with HMD1-5 (Dll1), HMD4-2 (Dll4), HMJ1-29 (Jagged1) or HMJ2-1 (Jagged2). Representative ratios of mean fluorescence intensity (MFI) (Notch ligand MFI divided by control MFI) are indicated on histograms. (B) Immunohistological analysis of Dll1-expressing cells in the spleen of 6-week-old mice. Spleen sections were stained with HMD1-5 (red) and mAbs against MARCO, CD11b, CD68, F4/80, B220, ICAM-1 or VCAM-1 (green). Magnification: MARCO, ×100; the others, ×400. (C) Expression of Dll1 on erythrocytes (TER119+CD71hi) and erythroblasts (TER119+CD71lo) in the spleen before (control) or after phlebotomy was analyzed by flow cytometry. Filled histograms indicate the staining with control hamster IgG and open histograms indicate the staining with HMD1-5.
Jagged2 was similar to the Dll1 expression (Fig. 3A). Immunohistochemical staining showed that Dll1 was expressed on CD11b+ and F4/80+ macrophages in the red pulp but not on MOMA-1+ MMM or MARCO+ MZM (Fig. 3B). Not all the Dll1-positive cells in the red pulp expressed CD68, suggesting that other cells such as stromal cells expressed Dll1 as recently reported (35). Interestingly, these Dll1-expressing cells at the red pulp frequently contacted with B220+ B cells (Fig. 3B). As reported previously (16), interactions of integrins on MZB cells with their ligands, VCAM-1 and ICAM-1, are important for the retention of MZB cells in the MZ. However, the Dll1-expressing cells did not co-express VCAM-1 or ICAM-1 (Fig. 3B). Moreover, Dll1 was also expressed on hemolysis-sensitive cells, which was identified as TER119+CD71+ erythroblasts enriched in the spleen of phlebotomized mice (Fig. 3C). The expression of Dll1 on macrophages and dendritic cells (DCs) in the spleen of 1-, 3-, 6- and 16-week-old mice was similar, and Dll1 was detected in the red pulp of 1-week-old mice while MMM was not organized yet (data not shown).

Fig. 4. Expression of Dll1 on monocytes and macrophages. (A) Expression of Dll1 on peripheral blood (PB) monocytes from 11-week-old B6 mice was analyzed by flow cytometry. PB monocytes detected as CD11bhiCD11c−Gr-1− cells were subdivided into inflammatory (CD11bhiCD62L−Gr-1+) and resident (CD11bhiCD62L−Gr-1−) monocytes. Filled histograms indicate the staining with control hamster IgG and open histograms indicate the staining with HMD1-5. (B) Expression of CD40 on Dll1-positive (D1+) or negative (D1−) PB monocytes and splenic (SP) macrophages. PB monocytes or SP macrophages were detected as CD11bhiGr-1− cells. Filled histograms indicate the staining with control rat IgG and open histograms indicate the staining with anti-CD40. Representative ratios of mean fluorescence intensity (MFI) (CD40 MFI divided by control MFI) are indicated on histograms. (C) Expression of Dll1 on peritoneal macrophages. Freshly prepared PEC (fresh) or cultured PEC with LPS, IFN-γ, TNF-α, anti-CD40 or without stimuli (−) were analyzed by flow cytometry.
Dll1 expression on peripheral monocytes and macrophages

Macrophages derive from bone marrow progenitor cells, circulate in the blood as monocytes and differentiate into macrophages in the spleen. Recently, two subsets of circulating monocytes have been identified in mice (36). One is termed as resident monocytes (CD11b<sup>hi</sup>CD62L<sup>-</sup>C<sub>Gr-1</sub><sup>-</sup>) that prefer to become resident macrophages and the other is termed as inflammatory monocytes (CD11b<sup>hi</sup>CD62L<sup>+</sup>C<sub>Gr-1</sub><sup>-</sup>) that migrate to sites of inflammation. Dll1 was expressed on a substantial part of resident monocytes but not on inflammatory monocytes (Fig. 4A). To further characterize the monocytes that expressed Dll1, expression of several markers on monocytes was investigated. As represented in Fig. 4(B), CD40 expression on Dll1-negative macrophages was higher than that on Dll1-expressing macrophages in the spleen. There was no clear distinction between Dll1-positive and -negative monocytes/macrophages on the expression of CD80, CD86, CD14 and MHC class II (data not shown).

PECs, a representative of inflammatory macrophages, did not express Dll1. The treatment of PEC with IFN-γ induced the expression of Dll1 (Fig. 4C). LPS stimulation also induced Dll1 expression (Fig. 4C) but did not enhance the effect of IFN-γ (data not shown). A similar level of induction was observed after anti-CD40 or TNF-α stimulation as well (Fig. 4C). No induction was observed after stimulation with other cytokines, such as IL-4, IL-6, IL-10 or IL-13 (data not shown).

Dll1 is necessary for the maintenance of MZB cells

As shown in Fig. 5(A), the ratio of MZB cells to total B cells increased gradually from 3 weeks to 10 weeks after birth.
Similar results were obtained in two independent experiments. The number of MZB cells was reduced to 0.73 \times 10^6 at 3 weeks and was kept at 8 weeks after injection (Fig. 5B). The reduction of MZB cell population reached a maximal level in 4 weeks and was decreased at day 3. The reduction was partly restored 8 weeks later. These results indicate that Dll1 is involved in the maintenance of MZB cells as well as in the development of MZB cells.

Effect of anti-Dll1 mAb on the maintenance of pre-MZB cells.

We next investigated the effect of HMD1-5 on pre-MZB cells. Although studies of Notch2-deficient mice have shown that Notch2 contributed to the development of pre-MZB cells, the role of Dll1 in the development and maintenance of pre-MZB cells has not been determined yet. Pre-MZB cells were defined as CD1dhiCD21hiCD23hi cells. Taken together, these results indicate that Dll1 is also required for the maintenance of pre-MZB cells in the spleen.

Dil1 is not essential for the maintenance of MZB cells in BWF1 mice after disease onset

MZB precursors are decreased by anti-Dll1 mAb treatment

MZB cells are predicted to participate in auto-antibody production. To explore the involvement of Dil1 in autoimmune diseases, we examined MZB cells in lupus-prone BWF1 mice. As compared with B6 mice (Fig. 2A), Notch2hi population was increased in B220+ CD1d+ CD21hi CD23+ cells in B220+ B cells (5, 10). When HMD1-5 was administrated in 16-week-old mice, pre-MZB cells were decreased to approximately half of the control in 3 days (the number of pre-MZB was 1.5 ± 0.37 × 10^6 versus 2.2 ± 0.49 × 10^5), followed by modest reduction thereafter (Fig. 6A). They were restored earlier (Fig. 6A) than MZB cells (Fig. 5B) after the 0.1 or 0.25 mg treatment. Pre-MZB cells were also decreased in the same way when HMD1-5 was administrated at 6 weeks of age (Fig. 6B). Treatment with a combination of mAbs to integrins α4 and αL eliminated pre-MZB cells in 3 days, which were restored before MZB cell restoration (Fig. 5B), implying that integrins α4 and αL also mediate lodging of pre-MZB cells (Fig. 6A). The ratio of FoB (B220+ IgMhi CD21hi CD23+) cells to total B cells was increased by HMD1-5 treatment with slight increase of T2B compartment (B220+ IgMhi CD21+ CD23hi), but there was no obvious effect on T1B compartment (B220+ IgMlo CD21+ CD23hi) (data not shown). Taken together, these results indicate that Dll1 is also required for the maintenance of pre-MZB cells in the spleen.
for a lower expression of Jagged2 on macrophages (Fig. 7C and D).

Then, we treated BWF1 mice with 0.25 mg of HMD1-5 twice a week for 2 weeks, before the appearance of serum anti-double-stranded DNA antibody (9 weeks of age) or after the onset of lupus disease (30 weeks of age). MZB cells of BWF1 mice were partially reduced when HMD1-5 was administrated at 9 weeks old (Fig. 7E). In contrast, the treatment after the disease onset resulted in no significant reduction of MZB cells, whereas B6 mice treated at the same age exhibited a marked reduction of MZB cells (Fig. 7E). A similar result was obtained with a higher dose (0.5 mg) of HMD1-5, while treatment with a mixture of anti-LFA-1 and anti-VLA-4 mAbs depleted this MZB cell population (data not shown).

**Fig. 7.** The Effect of anti-Dll1 mAb on the maintenance of MZB cells in BWF1 mice. (A) Expression of Notch receptors on splenic B (B220+T (CD3+) cells from 9-week-old BWF1 mice was analyzed by flow cytometry. Filled histograms indicate the staining with control hamster IgG. Open histograms indicate the staining with HMN1-12 (Notch1), HMN2-35 (Notch2), HMN3-133 (Notch3) or HMN4-14 (Notch4). (B) Expression of Notch2 on T1B (B220+CD1dloCD21+CD23+), T2/FOB (B220+CD1dloCD21intCD23hi), pre-MZB (B220+CD1dhiCD21hiCD23lo) or MZB (B220+CD1dhiCD21hiCD23hi) cells from 9-week-old BWF1 mice was analyzed by flow cytometry. Filled histograms indicate the staining with control hamster IgG and open histograms indicate the staining with HMN2-35. Representative ratios of mean fluorescence intensity (MFI) (HMN2-35 MFI divided by control MFI) are indicated on histograms. (C) Expression of Notch ligands on splenic macrophages (Mø: CD11b+CD11c-) and DCs (CD11b-CD11c+) from 9-week-old BWF1 mice was analyzed by flow cytometry. Filled histograms indicate the staining with control hamster IgG and open histograms indicate the staining with HMD1-5 (Dll1), HMD4-2 (Dll4), HMJ1-29 (Jagged1) or HMJ2-1 (Jagged2). Representative ratios of MFI (Notch ligand MFI divided by control MFI) are indicated on histograms. (D) Immunohistological analysis of Dll1-expressing cells in the spleen of 9-week-old BWF1 mice. Spleen sections were stained with HMD1-5 (red) and mAbs against MARCO, CD11b, CD68, F4/80 or B220 (green). Magnification: MARCO, ×100; the others, ×400. (E) B6 or BWF1 mice at the indicated ages were i.p. injected with 0.25 mg of HMD1-5 (D1) or control hamster IgG (HamIgG) twice a week for 2 weeks. The percentage of MZB cells was analyzed as in Fig. 5(B). Data are represented as the mean ± SD of three mice in each group. Similar results were obtained in two independent experiments. **P < 0.01 as compared with control IgG.
Notably, MZB cells were not decreased in aged BWF1 mice though the expression of Notch receptors and ligands showed no difference between aged (30 weeks old) and young (9 weeks old) BWF1 mice (data not shown). These results suggest that Dll1 is dispensable for the maintenance of MZB cells in BWF1 mice, especially after the onset of lupus disease.

**Discussion**

Molecular mechanisms of B cell development in the spleen have not been fully clarified. Recent studies using conditional KO mice have suggested that Notch2-Dll1 interaction is essential for the development of MZB cells (5–7, 38). In this study, we characterized the expression and function of Notch receptors and ligands, especially Dll1, by utilizing a panel of newly generated mAbs.

Expression of Notch receptors in splenocytes has been reported at mRNA levels but not at protein levels (5). In this study, we found that Notch2 was expressed highly on the surface of B cells, especially pre-MZB and MZB cells. T1B and FoB cells expressed Notch2 at 4- to 5-fold lower levels, and this difference in Notch2 expression might affect the commitment to MZB cells. Indeed, a previous report with mice having an in-frame fusion of Notch2 and β-galactosidase demonstrated a higher Notch2 expression on MZB cells than on FoB cells (39).

Although the expression of Dll1 in splenocytes has been reported at mRNA levels (7), the Dll1-expressing cells responsible for MZB cell development have not been identified. By immunohistochemical and flow cytometric analysis with anti-Dll1 mAb, we found that macrophages and erythroblasts in the red pulp expressed Dll1. Dll1 was also expressed on a subset of peripheral blood monocytes, which would become resident macrophages. These results suggest that Dll1-expressing monocytes/macrophages in the red pulp may be responsible for the development of MZB cells in the spleen.

The mechanism why MZB cells did not appear until 3 weeks of age was not clear. This was not explained by the expression of Notch2 and Dll1, which were expressed on splenic B cells and splenic macrophages, respectively, at 1 week of age. Accordingly, some MZB cells might develop in the spleen of 1-week-old mice, but they might be unable to locate in the spleen because MMM and MZM were not organized yet (our unpublished data).

To address whether Dll1 on erythroblasts plays a role in the development of MZB cells, phlebotomized mice were analyzed. MZB cells did not increase despite the increase of Dll1-expressing erythroblasts. Furthermore, erythroblasts in 1-week-old mice expressed higher Dll1 than those in 3- or 16-week-old mice. These results suggest that Dll1 on erythroblasts does not play an important role for the MZB cell development (data not shown).

A recent study using conditional KO mice has revealed an essential role of Dll1 in the MZB cell development (7). Our present study using a neutralizing anti-Dll1 mAb demonstrated that Dll1 was also required for supplying, but not for lodging, MZB cells to maintain the number of MZB cells homeostatically. Consequently, the gradual reduction of MZB cells by Dll1 blockade was correlated with the turnover rate of MZB cells, which was speculated about a month (40).

Recently formed B cells mature into FoB or MZB cells in the spleen. MZB cells develop from T1B cells via pre-MZB cells. Similar to the findings that Dll1 was necessary for the development and maintenance of MZB cells, Dll1 was important for the development and maintenance of pre-MZB cells. Consistently, Notch2 conditional KO mice also exhibited the reduction of pre-MZB cells (5). These results imply that the Notch2-Dll1 interaction is important for the development of pre-MZB cells, but not for the maturation of pre-MZB cells into MZB cells.

Interestingly, MZB cells in BWF1 mice were homeostatically maintained independently of Dll1, especially after the onset of lupus disease. It has been suggested that MZB cells play an important role in the development of lupus disease by producing auto-antibodies (24). Although mechanisms for the Dll1-independent maintenance of MZB cells in BWF1 mice remain to be explored, the Dll1 independence may be a feature of dysregulated MZB cells producing auto-antibodies. It has been recently reported that non-obese diabetic mice also have an enlarged MZB population (41) and the Dll1-independent maintenance may be responsible for this enlargement. It would be also interesting to investigate whether other autoimmune models, such as BXSB and K/BxN mice, have the Dll1-independent mechanisms.

We have previously shown that glycosylation of Notch2 extracellular domain by Fringe affects binding of Notch2 to Jagged (42). In our preliminary results, we found that Jagged1 and Jagged2 were expressed in the red pulp area of the spleen as well as Dll1 (our unpublished data). Thus, some change in the expression of Fringe in BWF1 mice might increase the affinity of Notch2 (and Notch1) to Jagged, resulting in Dll1-independent but Jagged-dependent maintenance of MZB cells. Further studies are needed to address this possibility.

Importantly, Dll1 expression was induced on inflammatory macrophages by LPS, IFN-γ and TNF-α. Since IFN-γ and TNF-α are key cytokines in various autoimmune diseases and Dll1 has been implicated in the development of T1,1 immune responses (43). Dll1 induced on inflammatory macrophages may be also involved in the pathogenesis of T1,1-mediated autoimmune disease such as rheumatoid arthritis. Further studies are underway to address this possibility.

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**Abbreviations**

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<td>BWF1</td>
<td>(NZB × NZW) F1</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>Dll1</td>
<td>Delta-like 1</td>
</tr>
<tr>
<td>Dll4</td>
<td>Delta-like 4</td>
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<tr>
<td>FoB</td>
<td>follicular B</td>
</tr>
<tr>
<td>Lps</td>
<td>leukocyte function-associated antigen</td>
</tr>
<tr>
<td>MMM</td>
<td>marginal metallophilic macrophage</td>
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<tr>
<td>MZ</td>
<td>marginal zone</td>
</tr>
<tr>
<td>MZB</td>
<td>marginal zone B</td>
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<tr>
<td>MZM</td>
<td>marginal zone macrophage</td>
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<tr>
<td>PEC</td>
<td>peritoneal exudate cell</td>
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<tr>
<td>pre-MZB</td>
<td>precursors of marginal zone B</td>
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</tbody>
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TNF
tumor necrosis factor
T1B
type-1 transitional B
T2B
type-2 transitional B

References