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Notch2 Haploinsufficiency Results in Diminished B1 B Cells and a Severe Reduction in Marginal Zone B Cells

Colleen M. Witt,* Woong-Jai Won,* Vincent Hurez,† and Christopher A. Klug‡

Recent studies have implicated a role for Notch in the generation of marginal zone (MZ) B cells. To further investigate the role of Notch2 in the B cell lineage, we have analyzed the effects of reduced Notch2 signaling in mice expressing one functional allele of Notch2 (Notch2<sup>+/−</sup>). Notch2<sup>+/−</sup> mice have reduced B1 B cells of the peritoneal cavity and show a severe reduction in MZ B cells of the spleen. The reduction in MZ B cells was not due to the disruption of splenic architecture, disregulated terminal differentiation, nor to increased apoptosis within the MZ B cell compartment. Rather, our data suggest that Notch2 haploinsufficiency leads to impaired development of MZ B cells, possibly by impacting the formation of immediate MZ B precursors. These results provide evidence that Notch2 plays a determining role in the development and/or the maintenance of B1 B and MZ B cells. The Journal of Immunology, 2003, 171: 2783–2788.

The generation of mature B cell subsets occurs through a series of differentiative steps that includes the expression of a functional surface Ig receptor and successful passage through selective checkpoints (1, 2). Once mature, each subset exhibits unique functional properties, which in their totality provide an optimal defense against a diverse array of foreign pathogens. Although much insight has been gained into the functional properties of mature B cells, the mechanisms that drive the generation and selection of immature B cells into the various mature B cell subsets remain unclear.

Mammalian Notch proteins (Notch1–4) are highly conserved type I transmembrane receptors that regulate cell fate decisions in numerous developmental contexts (3, 4). Until recently, most of what is known of Notch function in hemopoiesis has come from studies of Notch1 that have demonstrated its critical role in T cell development (5–8). However, recent studies have shown that the conditional inactivation of RBP-Jκ, a primary mediator of Notch signaling, leads to the loss of marginal zone (MZ) B cells (9), suggesting a role for Notch signaling beyond the T cell lineage. Also, our recent studies have shown that expression of an activated form of Notch2 in hemopoietic progenitors blocks the development of conventional B2 cells and promotes the selective development of B1 B cells, a mature B cell subset that shares many phenotypic and functional properties with MZ B cells. These studies, taken with our findings that Notch2 protein is preferentially expressed in B1 B and MZ B cells, implicate a role for Notch2 in the generation of mature B cell subsets.

Previously, an inactivating mutation within the Notch2 locus was generated by the targeted in-frame insertion of the Escherichia coli lac-Z gene into the ankyrin repeat region of Notch2 (10). This mutation, which completely abolishes the signaling function of Notch2, results in embryonic lethality at approximately embryonic day 10.5. Mice that are heterozygous for this mutation (Notch2<sup>+/−</sup>) are viable and provide a means to assess the effects of reduced Notch2 signaling on the development of mature B cell populations. To this end, Notch2<sup>+/−</sup> and age- and sex-matched wild-type littermates were analyzed using a combination of flow cytometric and immunohistological approaches. Our results show that haploinsufficiency of Notch2 leads to a reduction in both the frequency and absolute number of B1 B cells in the peritoneal cavity. In addition, Notch2<sup>+/−</sup> mice have a severe reduction in the frequency and absolute number of MZ B cells. The reduction in MZ B cells is not due to disrupted splenic architecture, disregulated terminal differentiation, nor increased apoptosis within the MZ B or transitional immature B cell populations, but rather due to impaired development of MZ B cells. This study provides evidence that Notch2 plays a direct and determining role in the generation of both the MZ and B1 B cell subsets.

Materials and Methods

Mice

Notch2<sup>+/−</sup> mice, previously described (10), were backcrossed for >10 generations onto the C57BL-Ly-5.2 background. All mice used in these experiments were either 4- or 12-wk-old females, as indicated. Control mice used for analyses of Notch2<sup>+/−</sup> mice were age-matched wild-type female littermates.

Flow cytometry and Abs

For analyses of the B1 B cell population, peritoneal cavity cells were isolated by lavage, and single cell suspensions of harvested spleens were prepared according to standard protocols. FACS analysis was done using the following Abs: FITC-conjugated anti-B220 and PE-conjugated anti-CD5 (BD Pharmingen, San Diego, CA).Splenic populations were analyzed using FITC-conjugated CD23 and PE-conjugated CD21 (BD Pharmingen) and anti-mouse IgM coupled to Cy5 (Jackson ImmunoResearch, West Grove, PA). Cells were resuspended in propidium iodide, and viable...
cells were electronically gated and analyzed on FACSCalibur (BD Biosciences, San Jose, CA).

**Notch2** mouse expression

Notch2<sup>−/−</sup> mice express an in-frame fusion of Notch2 and the *E. coli* β-galactosidase (β-gal) protein driven by the endogenous Notch2 promoter, permitting the analysis of Notch2 protein expression by assaying β-gal activity within cells. Single cell suspensions from the spleens of Notch2<sup>−/−</sup> and wild-type littermates, serving as negative controls, were stained with Abs (described below), washed, and loaded with fluorescein di-β-D-galactopyranoside (FDG) using Fluorescent laccase Flow Cytometry kit (Molecular Probes, Eugene, OR), as described in the manufacturer's protocol. Cells were resuspended in propidium iodide, and viable cells were electronically gated and analyzed on MoFlo (Cytomation, Fort Collins, CO). Notch2 protein expression was determined by assessing β-gal activity within specific subpopulations, indicated by the production of fluorescein upon reaction of FDG with *E. coli* β-gal. Abs included biotinylated anti-CD23 and PE-conjugated CD21 (BD PharMingen), streptavidin-conjugated Texas Red (Southern Biotechnology Associates, Birmingham, AL), and anti-mouse IgM coupled to Cy5 (Jackson ImmunoResearch).

**Detection of apoptosis by TUNEL**

Splenocytes were stained with the above-described Abs and fixed in 2% paraformaldehyde for 60 min at room temperature, washed, and resuspended in 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Permeabilized cells were washed, resuspended in 50 μl of TUNEL reaction mixture (In Situ Cell Death Detection Kit; Roche, Basel, Switzerland), and incubated in the dark for 60 min at 37°C under humidified conditions. For negative controls, cells were incubated in TUNEL reaction mixture without addition of terminal transferase. For positive controls, fixed and permeabilized cells were treated with DNase I (3 U/ml in 50 mM Tris-HCl, pH 7.5, 1 mg/ml BSA) for 10 min at room temperature before incubation with TUNEL reaction mixture. Subpopulations were analyzed by flow cytometry for the detection of FITC-labeled cells.

**Immunofluorescence analysis of splenic sections**

Cryosections from Notch2<sup>−/−</sup> and wild-type littermates were prepared at 4-μm thickness, fixed in acetone, and blocked with 10% horse serum. Sections were stained with MOMA-1 (rat IgG2a; a gift from G. Kraal, Vrije Universiteit Medical Center, The Netherlands) and developed with biotinylated goat anti-rat IgG, followed by incubation with streptavidin-4-aminomethylcoumarin-3-acetic acid (Vector Laboratories, Burlingame, CA). Sections were stained with ERTR-9 (also provided by G. Kraal), washed, and incubated with a mixture of PE-conjugated anti-rat IgM (BD PharMingen) for development of ERTR-9 and with Alexa 488-conjugated anti-mouse IgM (MB66). Sections were washed and mounted with Fluormount G (Southern Biotechnology Associates) and viewed with a Leica/Leitz DMRB fluorescence microscope equipped with appropriate filter cubes (Chromatechnology, Battleboro, VT). Images were acquired with a C5810 series digital color camera (Hamamatsu Photonic System, Bridgewater, NJ).

**Results**

**Notch2<sup>−/−</sup> mice have a reduction in peritoneal B1 B cells**

To investigate the effects of Notch2 haploinsufficiency on B1 B cell development, we performed flow cytometric analyses of this population in Notch2<sup>−/−</sup> mice, which had been backcrossed for greater than 10 generations onto wild-type C57BL/6 mice. Age- and sex-matched wild-type littermates served as controls. Although FACS analyses at 4 wk of age showed only a slight decrease in the relative frequencies and absolute numbers of B220<sup>low</sup>CD5<sup>intermediate</sup> cells (B1a) in the peritoneal cavity of Notch2<sup>−/−</sup> mice (data not shown and Table I), analyses at 12 wk of age showed that the relative frequencies were ~50% of those of control littermates (23.4 ± 5.7% for Notch2<sup>−/−</sup> vs 42.4 ± 6.5% for controls; *p* < 0.001; Fig. 1, A and B). Because the overall cellularity of the peritoneal cavity was similar in Notch2<sup>−/−</sup> mice, these frequencies represented a ~2-fold reduction in the absolute numbers of B1a B cells (Table I). Similarly, there was a reduction in the frequencies of B220<sup>low</sup>CD5<sup>low</sup> B1b B cells (12.4 ± 4.3 for Notch2<sup>−/−</sup> vs 19.0 ± 3.1% for controls; *p* < 0.001), which resulted in ~1.6-fold decrease in the absolute numbers of B1b B cells (Table I). Similar results were obtained when peritoneal cavity cells were analyzed using anti-IgM and anti-Mac-1 staining (data not shown). Analyses of the B2 B cell population of the peritoneal cavity showed increased frequencies in Notch2<sup>−/−</sup> mice as compared with controls, although values did not reach statistical significance (Fig. 1B and Table I). The observation that Notch2 haploinsufficiency leads to a reduction in B1 B cells is consistent with our recent studies, which showed that expression of activated Notch2 in hemopoietic progenitors leads to the selective development of these cells. Collectively, these studies provide the first evidence that the Notch2 family member plays a role in the development and/or the maintenance of the B1 B cell subset.

**Notch2<sup>−/−</sup> mice have a severe reduction in MZ B cells**

Follicular (FO) B cell and MZ B cell subsets in the spleen reside in distinct anatomical locations and display unique phenotypic and functional properties (11). Flow cytometric analysis was used to assess the effects of Notch2 haploinsufficiency on these cells from Notch2<sup>−/−</sup> and control littermates. Analyses were performed at 4 wk of age, early in the development of mature splenic B cell subsets, and at 12 wk of age when splenic subsets are well established. Analyses at 12 wk of age showed that while the overall frequencies of splenic B cells in Notch2<sup>−/−</sup> mice were similar to their wild-type littermates (Fig. 2A), there was a dramatic reduction in the frequencies of MZ B cells in Notch2<sup>−/−</sup> mice, with an average frequency of 1.7 ± 0.7% in Notch2<sup>−/−</sup> mice vs 9.2 ± 1.2% in wild-type littermates (*p* < 0.001; *n* = 14 for each genotype; Fig. 2A, B). Coincident with the severe reduction in this subset was an increase in the relative frequencies of FO B cells, although this increase did not reach statistical significance. Similar results were obtained from analyses of younger mice, with the exception that the disparity in frequencies of MZ B cells was less severe in mice analyzed at 4 wk of age (5.6 ± 1.1% in wild type vs 1.1 ± 0.6% in Notch2<sup>−/−</sup>; *n* = 14 for each genotype; Fig. 2B). The absolute numbers of MZ B cells in young mice were 4-fold reduced as compared with controls in Notch2<sup>−/−</sup> mice.
compared with the more severe 8-fold reduction observed in mice with fully developed MZ B cell compartments (Table I).

One explanation for the loss of MZ B cells in Notch2−/− mice is that Notch2 may be necessary for the maintenance of this B cell subset. In this case, Notch2 haploinsufficiency could lead to their disregulated terminal differentiation into plasma cells. However, analyses of spleens (Fig. 2C) and bone marrow showed no significant increase in the frequencies or absolute numbers of IgM−Syn-1high or IgA−Syn-1high natural plasma cells in unimmunized Notch2−/− mice as compared with their wild-type littermates (data not shown). Taken with the observation that Ig serum levels were not elevated in Notch2−/− mice (data not shown), these results suggest that the severe loss of MZ B cells in Notch2−/− mice was not due to disregulated terminal differentiation of MZ B cells.

**Splenic architecture is grossly normal in Notch2−/− mice**

Another factor that could contribute to the reduction of MZ B cells in Notch2−/− mice might be the disruption of splenic architecture, which may be necessary for MZ B cell recruitment and/or retention. To address this possibility, splenic cryosections from Notch2−/− and control littermates were stained with Abs against markers that delineate the follicles and the MZ. Analysis by fluorescence microscopy revealed no gross abnormalities in Notch2−/− splenic architecture, as follicles appeared intact with MZ macrophages (ERTR-9+ cells) present forming the MZ outer boundary (Fig. 3A). MOMA-1+ metallophilic macrophages were also present, forming the characteristic inner ring of the MZ that surrounds FO B cells (IgM+ cells) (Fig. 3B). However, it should be noted that while MOMA-1+ metallophilic macrophages were present in the MZ of Notch2+/− spleens, less intensely stained MOMA-1+ cells were also observed dispersed throughout the red pulp (Fig. 3C). Similar results were also observed in analyses of splenic cryosections from RAG-1−/− mice (J. Kearney and F. Martin, personal communication). Analysis at higher magnification of MOMA-1+ cells localized to the red pulp showed them to be large and of myeloid-like morphology (Fig. 3D). Because the MOMA-1-reactive Ag expressed on metallophilic macrophage is unknown, it is not clear whether the distribution of MOMA-1+ cells represents the abnormal localization of metallophilic macrophages or the aberrant expression of the MOMA-1 Ag on resident cells of the red pulp. Regardless, the MZ appeared to be overall intact, making it unlikely that the loss of MZ B cells in Notch2−/− mice was due to disrupted splenic architecture.

**MZ B cells are developmentally impaired in Notch2−/− mice**

Upon emigration from the bone marrow, newly formed B cells enter the spleen and progress through transitional stages (TR1 and TR2), where it is believed the MZ vs FO B cell fates are determined (12). The frequencies of Notch2−/− TR1 B cells, which represent the earliest arrivals into the spleen, were similar to those of wild-type littermates (Fig. 4A), indicating that there was no gross impairment in the emigration of immature B cells into the spleen. Importantly, while the overall frequencies of TR2 B cells
were also similar in Notch2+/− and wild-type littermates, a small population of CD23^IgM^highCD21^high cells that fall within the TR2 population, which are believed to be the immediate precursors of MZ B cells (MZP) (13–15), was markedly reduced (Fig. 4A). Furthermore, flow cytometric analysis of Notch2 protein expression showed that while Notch2 protein levels were low in TR2 cells when gated as a whole population, Notch2 was highly expressed in the CD23^IgM^highCD21^high subpopulation (MZP) (Fig. 4B). These results suggest that Notch2 may be functioning in developing MZP and that Notch2 haploinsufficiency leads to defects in their formation and/or their survival. To address the latter possibility, splenic subpopulations were analyzed using a TUNEL assay to detect the presence of apoptotic cells. As shown in Fig. 4C, we detected no increase in apoptosis in the CD23^IgM^highCD21^high precursor population or in the TR2 population as a whole (Fig. 4C). Nor was any increase in apoptosis detected in TR1 stage B cells or in the MZ B cell compartment itself (Fig. 4C). These results, taken with all of the above, suggest that Notch2 haploinsufficiency leads to impaired formation of MZ B cells, possibly by impacting the generation of MZP during the transitional stages of immature B cell development.

Discussion

In this study, we have shown that mice expressing only one functional allele of Notch2 have ~2-fold reduction in the frequency and absolute number of B1 B cells. The observation that B1 B cell numbers are similar to those of controls at early time points and diminish with the age of the mice suggests that the maintenance and/or self-renewing capacity of these cells are affected by haploinsufficiency of Notch2. This does not exclude the possibility that the development of B1 B cells is also affected by reduction in Notch2 dosage. Although the majority of B1 B cells in the adult mouse are derived during fetal B lymphopoiesis, small numbers continue to be generated from B cell progenitors in the bone marrow during adult life (16). Although it is not clear to what degree
The reduction in B1 B cells in Notch2+/− mice was also associated with a severe reduction in MZ B cells of the spleen, a mature B cell subset that shares several phenotypic and functional properties with B1 B cells (11, 17). The observation that Notch2 haploinsufficiency affects MZ B cells is consistent with recent studies that showed that the conditional inactivation of RBP-Jκ, a primary effector protein for the Notch signaling pathway, also resulted in the loss of the MZ B cell compartment (9). The severe reduction in MZ B cells in Notch2+/− mice indicates that other Notch family members do not effectively compensate for reduced Notch2 function in MZ B cell development. These results suggest that Notch2 signaling through RBP-Jκ is necessary for the formation of the MZ B cell subset.

Of particular interest is the observation that the B1 B cell population was not affected by the conditional inactivation of RBP-Jκ, which is in contrast to the reduction in these cells observed in Notch2+/− mice. Several studies have documented alternative Notch signaling pathways that do not depend on the actions of RBP-Jκ (18–22). Therefore, the differential phenotypes obtained from the inactivation of RBP-Jκ and Notch2 haploinsufficiency may reflect the use of an RBP-Jκ-independent Notch2 signaling pathway in the development and/or the maintenance of B1 cells.

The fact that the reduction in B1 B cells is in association with reduced MZ B cells suggests not only a role for Notch2 in the development of these subsets, but also supports the notion that these subsets share at least some common elements in their developmental pathways. Although the mechanisms that drive the selection of immature B cells into the various mature B cell subsets are still being defined, there is growing evidence that MZ B and B1 B cells are positively selected into their respective compartments (1, 23). Support for this model comes from recent studies that showed that newly formed immature B cells, expressing a transgenic B cell receptor enriched in the MZ B cell compartment, were positively selected into the MZ B cell pool, and that their enrichment was achieved by their preferential proliferation and survival during selective checkpoints (24).

According to this model, a potential role for Notch2 may be to facilitate positive selection through enhanced proliferation and/or survival of immature B cells with BCR specificities suited to either the MZ B or the B1 B cell pools. Such a role is consistent with the known function of Notch in cellular proliferation (25–27) and with numerous other studies that have demonstrated Notch-mediated protection from apoptosis (28–31). In addition, NF-κB activity is often involved in the promotion of cell survival (32–36), and the loss of certain NF-κB family members results in impaired MZ B cell development (37–41). Thus, recent evidence that the Notch signaling pathway targets NF-κB activity (42–44) may reflect an important connection between Notch, NF-κB activity, and MZ B cell development. Alternatively, it has been proposed that the strength of signaling received through the BCR in immature B cells determines their selection into either the FO B, MZ B, or B1 B cell populations (15). This view raises the possibility that Notch2 signaling modulates the strength of BCR signaling in such a way as to drive the selection of immature B cells into either the MZ B or B1 B cell pools. Finally, cell fate determined by strength of signal or by BCR specificity coupled to positive selection are not mutually exclusive models, and evidence suggests that aspects of both models are likely to be used in the generation of mature B cell subsets. Therefore, it remains possible that Notch2 functions at more than one level, by both instructive and permissive means, to promote the development of the MZ B and B1 B cell subsets.

FIGURE 4. Notch2+/− MZP are diminished in Notch2+/− mice. Representative plot of splenic cells from Notch2+/− and same-sex wild-type littermates analyzed at 12 wk of age stained with anti-IgM, anti-CD23, and anti-CD21. Top panel, Frequencies of IgM<sup>+</sup>CD21<sup>+</sup>TR2 B cells among CD23<sup>+</sup> gated cells are shown in rectangular gate. CD23<sup>+</sup>IgM<sup>+</sup>CD21<sup>+</sup>MZP are shown in circular gate. Bottom panel, Frequencies of IgM<sup>+</sup>CD21<sup>+</sup>TR1 B cells among CD23<sup>+</sup> gated cells are shown. B, Notch2 protein expression in CD23<sup>+</sup>IgM<sup>+</sup>CD21<sup>+</sup> TR2 B cells (top) and in CD23<sup>+</sup>IgM<sup>+</sup>CD21<sup>+</sup>MZP subpopulation (bottom). Notch2+/− mice express an in-frame fusion of Notch2 and E. coli β-gal from the endogenous Notch2 promoter. Splenocytes from Notch2+/− and wild-type littermates, serving as negative controls, were stained as described in A and loaded by hypotonic shock with FDG and analyzed by FACS. Notch2 protein-expression cells are indicated by the production of FITC upon reactivation of FDG β-gal. Histogram overlays of negative control (filled) and Notch2+/− (open) cells gated on the TR2 population as a whole (top), and of the MZP subpopulation within the TR2 population (bottom), are shown. C, Representative results using TUNEL assay to identify frequencies of apoptotic cells by flow cytometry. Histograms are overlays of negative control (dashed), FITC<sup>+</sup> cells (solid) that identify apoptotic cells within each gated population. Stained cells treated with DNase I served as positive controls (data not shown) and were >95% FITC<sup>+</sup>. Results are representative of three independent experiments of n = 2 for each genotype.
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