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Analysis of Marginal Zone B Cell Development in the Mouse with Limited B Cell Diversity: Role of the Antigen Receptor Signals in the Recruitment of B Cells to the Marginal Zone*

Naoki Kanayama,* Marilia Cascalho,† and Hitoshi Ohmori2*†

The quasimonoclonal (QM) mouse provides an intelligible model to analyze the B cell selection as the competition between two major 4-hydroxy-3-nitrophénylacetate-specific B cell populations whose BCR are comprised of the knock-in $V_{H}^{17.2.25}$ (VHT)-encoded H chain and the $\lambda_1$ or $\lambda_2$ L chain. In this study, we show the QM system is useful to examine how BCR signals guide a subset of B cells to the marginal zone (MZ). Compared with the control C57BL/6 mice, the QM mice had $\sim$2.7-fold increased number of B cells exhibiting the MZ B cell phenotype and a larger MZ area in the spleen. Interestingly, $V_{H}^{T/A2}$ B cells significantly predominated over $V_{H}^{T/A1}$ B cells in MZ-$V_{H}^{T/A1}$: $V_{H}^{T/A2} \approx 3:7$ and transitional 2-B cell subsets, while these two populations were comparable in immature, transitional 1, and mature counterparts. Thus, the biased use of $A2$ in the MZ B cells may be the result of selection in the periphery. The enlargement of MZ B cell compartment and the preferred recruitment of the $V_{H}^{T/A2}$ B cells were further augmented by doubling the $V_{H}^{T}$ gene, but dampened by the dysfunction of Bruton’s tyrosine kinase, suggesting a positive role of BCR signaling in this selection. Comparison of Ag specificity between $V_{H}^{T/A1}$ and $V_{H}^{T/A2}$ IgM mAbs revealed a polyreactive nature of the $V_{H}^{T/A2}$ BCR, including the reactivity with ssDNA. Taken together, it is suggested that polyreactivity (including self-reactivity) of BCR is crucial in driving B cells to differentiate into the MZ phenotype. The Journal of Immunology, 2005, 174: 1438-1445.

Marginal zone (MZ) B cells belong to a subset of mature B cells that localize in the vicinity of the marginal sinus surrounding lymphoid follicles in the spleen (1, 2). MZ B cells exhibit characteristic surface markers (IgM$^{\text{high}}$/IgD$^{\text{low}}$ CD21$^{\text{high}}$CD23$^{\text{low}}$) that are different from those of conventional follicular (FO) B cells (IgM$^{\text{low}}$/IgD$^{\text{high}}$CD21$^{\text{intermediate}}$CD23$^{\text{low}}$), and show partially activated properties and very long life spans (3-6). MZ B cells are considered to play a vital role in the first line of defense against blood-borne bacterial infection by rapidly responding to Ags, often in a T cell-independent (TI) fashion (7-9).

Signals mediated by the BCR may be critical in the commitment of B cells to MZ B cell compartment (reviewed in Refs. 2, 10, and 11). Although the origin of MZ B cells remains controversial, newly formed (NF) B cells are most likely the immediate precursors (12-15), while an origin from FO B cells is also suggested (16, 17). Accumulating evidence indicates that MZ B cells may be positively selected by BCR signals that might be distinct from those required for generating FO B cells. For instance, IgH transgenic (Tg)-B cells expressing M167 or VH81X 1d, either of which is related to auto-reactivity, can be enriched into the MZ (13, 18). Stimulation with autologous Ags or endogenous microfloras might play a role in the positive selection of MZ B cells (2). In addition, gene knockouts of positive regulators for BCR signaling, including CD19, impaired the development of MZ B cells without markedly affecting the generation of FO B cells (10, 13, 14). In contrast, the dysfunction of Bruton’s tyrosine kinase (Btk) that is an essential component of the BCR signalosome led to defects in the development of FO B cells with the MZ B cell compartment being intact (12, 14). Although an optimal strength of BCR signals tuned by positive and negative regulators may be favorable for the generation of MZ B cells (11-13, 19), a model remains to be established that can accommodate contradictory data from knockout lines for BCR signaling.

If the development of MZ B cells can be assessed in Ig Tg mice whose B cell repertoire is restricted and expresses BCR with a defined Ag specificity, the selection process toward MZ B cells will be more profoundly understood in terms of the intensity of BCR signals. For this purpose, we analyzed the MZ B cell compartment in the quasimonoclonal (QM) mouse whose B cell repertoire is restricted (20). The QM mouse is a strain bearing the site-directed $V_{H}$ gene, in which one of the $J_{H}$ loci is replaced by the 17.2.25 $V_{H}^{DI_{H}}$ segment ($V_{H}^{T}$) encoding 4-hydroxy-3-nitrophénylacetate (NP)-specific mAb and the other $J_{H}$ and both $J_{C}C_{L}$ loci are disrupted. Thus, the $V_{H}^{T}$ H chains are expressed on $\sim$80% of peripheral B cells, about one-half of which use $A1$ as the L chain ($V_{H}^{T/A1}$) and the other half use $A2$ ($V_{H}^{T/A2}$) (21). Recently, we have reported that the QM mice provide a useful experimental system to analyze the B cell clonal selection and the recruitment of the selected cells to affinity maturation pathway as the result of the competition between two major B cell populations, $V_{H}^{T/A1}$ and $V_{H}^{T/A2}$ (21). QM mice may also be useful to examine the development of MZ B cells, because we found that the MZ B cells were dominated by $V_{H}^{T/A2}$ B cells, while there was no such bias in the

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2 B cells were further augmented by doubling the VHT gene, but dampened by the dysfunction of Bruton’s tyrosine kinase, suggesting a positive role of BCR signaling in this selection. Comparison of Ag specificity between $V_{H}^{T/A1}$ and $V_{H}^{T/A2}$ IgM mAbs revealed a polyreactive nature of the $V_{H}^{T/A2}$ BCR, including the reactivity with ssDNA. Taken together, it is suggested that polyreactivity (including self-reactivity) of BCR is crucial in driving B cells to differentiate into the MZ phenotype. The Journal of Immunology, 2005, 174: 1438-1445.

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Materials and Methods

Mice

C57BL/6 and MRL/Mp lpr/lpr mice were purchased from Charles River Laboratories Japan. X-linked immunodeficient (Xid) (CBA/N) mice were purchased from Japan SLC. QC mice (VHT/Tg, 3k β2m, 3, λ), VHT/JH (VHT/VHT, Jβ2m), and B6 mice (VHT/Tg, Tc) were generated by the backcross of QC mice onto C57BL/6 mice at least for nine generations (22). QC mice homozygous for the VHT Tg gene (VHT/VTg/Tg) and B6 mice (VHT/Tg, Tc) were generated by QC mice (VHT/VTg, Tc). The heterozygous (VHT/VTg) mice were used usually, unless otherwise stated. The QC (VHT/VTg, Tc) mice were crossed with Xid mice to generate the QM mouse strain (QM Xid) carrying a point mutation in the exon 1 of btk (VHT/VHT, Jβ2m, 3k β2m, λ, λ', Xid/Xid) (23). Genotypes were determined with PCR using specific primers to targeted loci and a mutant btk locus: AAGAAGCTATATGACGGC for germline Cβ2m, CTTGGCTTCTACGTGTCGC for IgK, and ATCTGCCGAAATGGTCTGGATCTGTCGTG for germline Igλ as sense primers; CAACATATCCCTCACAGTATG for antisense of 3k β2m intron; CTCGTGGCTTACTCGGATCTGGTGTC for Cβ2m and CTTGGATGACGTGTCGTC for ATAC for Jβ2m; GTGGAAGATGTTGGACGTGACGG for germline Igλ, and TACGGATGTTGACGTG for a mutant btk locus. Mice at 8–15 wk of age were used throughout present experiments. All mice were treated in accordance with the guidelines approved by the Committee of Laboratory Animal Care, Okayama University.

Flow cytometric analysis

Single cell suspensions were prepared from the spleen, lymph nodes (axillary, brachial, inguinal, and popliteal), and bone marrow. Cells were stained with anti-mouse Abs in PBS containing 0.2% BSA, 0.1% sodium azide, and, if necessary, 50 µg/ml normal rat IgG as a blocking reagent (Vector Laboratories). FITC or CyChrome-conjugated second Abs were labeled with HRP. Absorbance at 405 nm was measured by using a microplate reader MPR A4i (ToxoB).

Binding to ssDNA was measured, as reported previously (32). Calf thymus DNA (Sigma-Aldrich) was dissolved in SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 8) and shared by passing through 26-gauge needle. DNA was heat denatured and chilled on ice immediately before use. Microplates were coated by 50 µg/ml ssDNA and blocked with 1% BSA. Bound Abs were detected, as described above. Data for specific binding to ssDNA were represented after subtraction of background binding to BSA on uncoated microplates. mAbs in use were bound to BSA in a negligible level.

Results

Expansion of MZ B cells in QM mice

QM mice that predominantly express the knob-in VHT-encoded H chains possess two major B cell populations, VHT1/Tg1 and VHT2/Tg2, each of which constitutes 45–50% of lymph node (LN) B cells (20, 21). We have reported that these two B cell populations shared comparable affinity for the hapten NP, but the VHT2/Tg2 showed 50–100-fold higher affinity than the VHT1/Tg1 for an NP analog, pNP (21). Thus, it is possible that autologous or environmental Ags differentially stimulate VHT1/Tg1 and VHT2/Tg2 B cells, and affect the subsequent commitment of these two B cell populations to different B cell compartments. If there is a bias in the proportion of these two B cells in some compartments, the QM mice will provide a useful system for analyzing how BCR specificity regulates the fate of B cells to differentiate into specialized subsets. This prompted us to assess B cell subsets in peripheral lymphoid tissues of QM mice.

By means of flow cytometry, splenic B cells of QM mice were separated into MZ and FO B cells in terms of the surface expression of CD21 and CD23 (3) (Fig. 1A). CD21high/CD23low/- MZ and CD21high/CD23high FO B cells in QM mice exhibited distinct surface phenotypes: large B220highIgM+IgDlowCD19highCD43low/- HSAhighCD86high cells and small B220highIgM+IgDlowCD19low/- HSAhighCD86high cells, respectively (Fig. 1B). Interestingly, the proportion of CD21high/CD23low/- B220 MZ B cells in the QM mouse spleen was significantly higher (4.6-fold) than in the control C57BL/6 mouse spleen (Fig. 1A, top row, and C). Reciprocally, the proportion of CD21high/CD23high B220+ FO B cells was decreased in QM mice. CD21high/CD23low/- B cells were almost absent in the LN that did not possess the MZ structure (Fig. 1A, top row). Importantly, MZ B cells of QM mice were significantly increased not only in the proportion, but also in the absolute number (2.7-fold) compared with those of the control mice, while total B220 cells and FO B cells were decreased in QM mice (Fig. 1C). When splenic B cells were separated in terms of CD21 and IgM levels, the increase of CD21highIgM+ B220 MZ-type B cells in QM mice was similarly confirmed (Fig. 1A, bottom row). NF B cells that were defined as CD21-CD23+ or CD21- IgM+ were unchanged in the proportion, but decreased in the cell number (Fig. 1, A and C).

To examine the anatomical distribution of the expanded B cells with the MZ B cell phenotype in the spleen, histochemical analysis of frozen section was performed. Both in QM and C57BL/6 mice,
the MZ could be observed as the outer layer of the B cell-rich area that is separated from the follicle by a layer of MOMA-1/M2 metalophilic macrophages (Fig. 2). The MZ area of QM mice was larger than that of the control mice (Fig. 2, A and B). Additionally, cells in the enlarged MZ layer were stained as IgM/IgD, while those in the follicle as IgM/IgD (Fig. 2D), indicating that the expanded MZ B cells, which were characterized as IgM high/IgD low with flow cytometric analysis (Fig. 1B), were localized in the MZ.

Predominance of V_{H1} T/12 B cells in the MZ

We have previously shown that each V_{H1} T/1 and V_{H1} T/2 B cell population has a comparable size in the LN of QM mice (21). As MZ B cells of QM mice were increased in comparison with those of the control mice, we examined whether or not the ratio of V_{H1} T/1 to V_{H1} T/2 was maintained in this specialized microenvironment. CD21^{high}/CD23^{low/mid} MZ B cells expressed the V_{H1} T/1d with higher frequency than CD21^{int}/CD23^{high} FO B cells (MZ B, 88.0 ± 1.7%; FO B, 73.0 ± 6.6%; p < 0.01) (Fig. 3). Very interestingly, ~70% of the MZ B cells were found to bear \lambda 2-chains, while \lambda 1-chains were expressed on only 10–20% of the MZ B cells (\lambda 1, 17.9 ± 5.7%; \lambda 2, 70.0 ± 2.8%; p < 0.001) (Fig. 3). A similar repertoire shift was observed when the splenic MZ B cells were assessed as CD21^{high}/IgM^{high} B cells (data not shown). In contrast, the biased usage of \lambda 2-chains was not observed in the FO B cells of the spleen (\lambda 1, 42.3 ± 5.7%; \lambda 2, 43.4 ± 4.1%) (Fig. 3) as well as in the recirculating equivalents in the LN (21). When B cells prepared from the spleen were stimulated with LPS or anti-CD40 mAb, \lambda 2 B cells responded more rapidly than \lambda 1 B cells (data not shown), thus suggesting that the \lambda 2 B cell pool more abundantly contains the MZ B cells that have been reported to be...
more responsive to LPS or anti-CD40 than the FO counterparts (3, 4). Collectively, these results show that the V_{H}T/A2 B cells were preferentially recruited to the MZ in QM mice. Thus, to investigate why the V_{H}T/A2 B cells were committed to the MZ more efficiently in comparison with the V_{H}T/A1 counterparts will lead to the elucidation of the role of BCR in MZ B cell development.

The bias to V_{H}T/A2 in the MZ is determined in the periphery

NF B cells have been reported to be precursor candidates of MZ B cells (12, 13). To examine whether the preferential use of \( \lambda_2 \)-chains in the MZ B cells was due to the biased generation of \( \lambda_2 \)-bearing B cells in the bone marrow or to the selection done in the periphery, we examined the \( \lambda_1 \)-chain usage of immature B cells in the bone marrow and transitional B cells in the spleen. In contrast to the bias to \( \lambda_2 \)-chains in the MZ B cells, immature B cells expressed \( \lambda_1 \)-chains with rather higher frequency than \( \lambda_2 \)-chains (Fig. 4). This \( \lambda_1 \) bias in the immature stage also has been reported in wild-type or several other IgH Tg mice (33, 34). Transitional B cells in the spleen were separated by expression levels of CD23, CD21, and IgM (12). Similarly to immature B cells in the bone marrow, the \( \lambda_2 \) bias was not found in CD23-lowIgM-high transitional 1 (T1) B cells (Fig. 5), and in CD23-highIgM-high mature follicular B cells (Figs. 3 and 5). Interestingly, \( \lambda_2^+ \) B cells significantly accumulated in CD23-lowIgM-high transitional 2 (T2) B cell compartment (Fig. 5). T2 cells can be subdivided by CD1d expression (35), and CD1d- T2 cells have been presumed as precursors of MZ B cells (MZP) (11, 19). CD23-IgM-highCD1d- MZP cells were also predominantly occupied by \( \lambda_2^+ \) B cells (Fig. 5). Therefore, these results suggest that the biased \( \lambda_1 \)-chain usage of the MZ B cells was determined in the periphery, probably at the T2 stage in the spleen.

BCR signals are responsible for the repertoire bias of MZ B cells

To examine how the intensity of BCR signals regulates the differentiation to MZ B cells, we analyzed MZ B cell development in QM mice whose BCR signals were either strengthened or weakened by genetic manipulations. It has been shown that B cell development is affected by the expression level of BCR, which varies with the hemizygous or homozygous allelic status of Ig transgenes (36–41). In the QM mice that were homozygous for the V_{H}T gene (QM (V_{H}T/V_{H}T)), BCR signals are considered to be augmented owing to an increased density of BCR. In contrast, the QM mice bearing the Xid mutation (QM Xid) were generated by crossing QM (V_{H}T/V_{H}T) mice with CBA/N mice to observe MZ B cell development under conditions in which BCR signals were attenuated by the defect in Btk.

Splenic B cells of QM (V_{H}T/V_{H}T) mice were compared with those of the original QM (V_{H}T/JH) mice by flow cytometry (Fig. 6). The MZ B cells of QM (V_{H}T/V_{H}T) mice were significantly increased both in the proportion (1.3-fold) and cell number (1.8-fold), while the number of FO B cells was not meaningfully changed (Fig. 6, A and B). Interestingly, the \( \lambda_2/\lambda_1 \) ratio of MZ B cells was found to be further increased in QM (V_{H}T/V_{H}T) (from 4.3 to 6.9) (Fig. 6C). Next, splenic B cells of QM Xid mice were compared with those of the control littermate QM (V_{H}T/V_{H}T) mice (Fig. 7). The absolute numbers of total splenic B220+ and FO B cells in QM Xid mice were markedly decreased (4.3- and 3.4-fold, respectively) compared with those in the control littermates (Fig. 7B), consistent with the previous report describing an impaired B cell development in Btk-deficient mice (42). Notably, the MZ B cells of QM Xid mice were reduced by 0.53 and 0.14 times in the proportion and the absolute number, respectively (Fig. 7, A and B). The proportion of MZ B cells appeared to be still higher in QM Xid mice than wild-type C57BL/6 mice (C57BL/6, 7.7 ±
0.5%; QM Xid, 26.9 ± 8.3%; p < 0.01). However, the number of MZ B cells was severely reduced in QM Xid mice (C57BL/6, 2.1 ± 0.4 × 10⁶; QM Xid, 8.7 ± 3.6 × 10⁵; p < 0.01) (Figs. 1 and 7). More importantly, the bias toward the \( \lambda \)2-chain in MZ B cells was significantly canceled in QM Xid mice (Fig. 7C). Taken together, these results suggest that signals delivered by the \( V_\gamma/T/\lambda_2 \) BCR have a positive role in the selection and/or survival of the \( V_\gamma/T/\lambda_2 \) B cells to be committed to MZ B cells.

### B cells with an autoreactivity are enriched in MZ B cell compartment

To address the possibility as to whether the difference between \( V_\gamma/T/\lambda_1 \) and \( V_\gamma/T/\lambda_2 \) BCRs in Ag specificity including autoreactivity is involved in determining the recruitment of the latter B cells to the MZ, the specificities of the corresponding IgM mAbs toward various haptenes and natural compounds were examined.

\( V_\gamma/T/\lambda_1 \) and \( V_\gamma/T/\lambda_2 \) IgM mAbs showed comparable affinity to the NP hapten, as reported previously (Fig. 8) (21). In contrast, the \( V_\gamma/T/\lambda_2 \) IgM mAb was more efficiently bound to other nitrophenyl moieties, including TNP and 3-nitrotyrosine, which has been known as an inflammation-associating marker (43, 44), than the \( V_\gamma/T/\lambda_1 \) IgM mAb. More importantly, the \( V_\gamma/T/\lambda_2 \) mAb reacted with a self Ag, ssDNA, but the \( V_\gamma/T/\lambda_1 \) mAb did not. In addition, only the \( V_\gamma/T/\lambda_2 \) mAb was polyreactive to several protein Ags, \(-\)galactosidase, insulin, thyroglobulin, and cytochrome c. Neither mAb showed detectable reactivity to dsDNA or phosphorylcholine (data not shown). Thus, it is suggested that a polyreactivity of the \( V_\gamma/T/\lambda_2 \) BCR, including the reactivity to ssDNA, is one of the factors directing the recruitment of the \( V_\gamma/T/\lambda_2 \) B cells to the MZ B cell compartment.
FIGURE 8. Ag specificities of V_{H}/T1A and V_{H}/T2A IgM mAbs. The Ag specificity was assessed by measuring the binding of serially diluted mAbs to microplates coated with NP-BSA (NP), TNP-BSA (TNP), tyrosine-nitrated BSA (3-nitrotyrosine), ssDNA, β-galactosidase, insulin, thyroglobulin, or cytochrome c. V_{H}/T1A (●) and V_{H}/T2A (○) IgM mAbs. As the controls of binding toward ssDNA, sera collected from C57BL/6 mice (n = 4) (●) and MRL/lpr/lpr mice (n = 5) (▲) were used with 1/500-fold dilution. Data are presented as the mean of triplicate assays. Bar indicates the SD.

Discussion

In QM mice whose ~80% of B cells express V_{H}/T1A or V_{H}/T2A BCR, we show that MZ and T2 B cells were predominantly occupied by the V_{H}/T2A B cells, whereas immature, T1, and FO B cell compartments were comparably populated by V_{H}/T1A and V_{H}/T2A B cells. The present results suggest that this bias is formed by the selection of V_{H}/T1A B cells at the T2 B cell stage due to the fact that the V_{H}/T2A BCR, but not V_{H}/T1A BCR, showed a polyreactivity including an autoreactivity to a self Ag ssDNA. Although the QM system may be nonphysiological and have limitations in analyzing MZ B cell development due to its limited B cell diversity, this characteristic feature, in contrast, may enable us to analyze the B cell differentiation as the competition/selection between the two major B cell populations. As reported previously, we showed that QM mice were advantageous in analyzing the selection and affinity maturation of B cells during an Ab response (21).

Analysis of the V_{H}/T2A bias of the MZ B cells in QM mice suggests that the Ag specificity of the V_{H}/T2A BCR has a critical role in the positive selection of the MZ B cells. The comparison between Ag specificities of V_{H}/T1A and V_{H}/T2A BCRs revealed that these two receptors showed similar reactivity to NP, but the latter bound more strongly to ssDNA, nitrophenyl haptens including 3-nitrotyrosine (Fig. 8) and pNP (21), and several protein Ags, suggesting a polyreactive nature of the V_{H}/T2A BCR. In addition, 3-nitrotyrosine on autologous proteins have been detected in inflammation sites (43). Thus, the polyreactivity, which potentially involves an autoreactivity, of V_{H}/T2A BCR may be responsible for the population bias of MZ B cells. These findings are consistent with the previous reports that weak reactivity with self Ags may be the population bias of MZ B cells. These findings are consistent with the previous reports that weak reactivity with self Ags may be responsible for the differentiation to MZ B cells (13, 18). For instance, it has been reported that IgH Tg-B cells expressing the phosphocholine-binding Id M167 or the Id VH81X that reacts with unidentified self Ags, but not Tg B cells specific for xeno- neic hen egg lysozyme, were enriched into the MZ (13). Similarly, an increase of MZ B cells has been observed in the Tg mouse line that coexpresses a human natural Ab against human Fcγ and a soluble human IgG (45), suggesting that soluble self Ags can be involved in the positive selection of MZ B cells.

The V_{H}/T2A B cells in the MZ are considered to be immune-competent, because V_{H}/T2A B cells more readily responded to in vitro stimulation by LPS or anti-CD40 than the V_{H}/T1A counterparts (our unpublished data). The robust activation of B cells has been reported to occur by immunizing QM mice with a T cell-independent (TI)-Ag NP-Ficoll, indicating that the MZ B cells of QM mice can participate in TI Ag response (46). The MZ B cells bearing M167- or VH81X-autoreactive BCR have been previously shown to respond to TI Ags in vitro and in vivo (8, 13). In contrast, in 3H9 anti-DNA IgH knockin mice, anti-DNA B cells have been shown to escape deletion by coexpressing λ and κ L chains, and preferentially colonize in the MZ (47), suggesting that the MZ is a site in which autoreactive B cells are sequestered and rendered nontoxic. The V_{H}/T2A B cells were distributed not only in the MZ, but also in the splenic follicle and the LN (Figs. 1 and 3), and the recirculating V_{H}/T2A B cells have been shown to participate in T cell-dependent response to pNP-CGG (21). Therefore, the QM system indicates that an immunocompetent B cell clone bearing an autoreactive BCR is preferentially recruited into the MZ, but not necessarily excluded from the follicle.

The intensity of BCR signals may be critical for determining the fate of B cells to be recruited into MZ B cells. The preferred recruitment of V_{H}/T2A B cells was further augmented by rendering the V_{H}/T gene homozygous, but dampened by the impairment of Btk, suggesting a positive role of BCR signaling in this selection (Figs. 6 and 7). Likewise, the gene dose-dependent increase of MZ B cells has been reported in anti-RBC or anti-p-azophenylarsonate IgH Tg mouse line (41, 48). Thus, when the V_{H}/T gene was made homozygous, it is possible that the intensity of the augmented signals delivered by the homoyzogous BCR was within the optimal range for the development of MZ B cells, but did not exceed a threshold level required for negative selection. In contrast, augmented BCR signals in Aiolos knockout mice have been shown to lead to the absence of MZ B cells, suggesting that strong BCR signals favor the formation of FO over MZ B cells (19). In the QM system, Ag specificity might be more effective on BCR signals required for MZ B cell development than manipulations of BCR signaling examined in the present study, because the manipulations specifically affected the recruitment of V_{H}/T2A B cells to the MZ (Figs. 6 and 7).

In CBA/N mice with a Xid phenotype, there was a marked decrease in the number of FO B cells, while the number of MZ B cells was affected to a lesser extent (12, 14, 19). Thus, the proportion of MZ B cells apparently increased in these mice. In QM mice, however, the recruitment of V_{H}/T2A B cells to the MZ was found to depend on Btk profoundly, because introduction of Xid mutation led to the significant decrease of the MZ B cells and the compromise of the B cell repertoire (Fig. 7). The enrichment of IgH Tg B cells with 81X or M167 Id into the MZ also has been reported to require functional Btk (13). Recently, it has been shown that BCR signals via Btk activate integrin α_{β}_{1}-mediated
adhesion of B cells to VCAM-1 (49), which may be involved in the B cell recruitment to the MZ (50). The observations in the QM mouse system in which major B cells are clonally defined in their Ag specificity suggest that Btk has an essential role in determining optimal intensity of BCR signals for B cell recruitment to the MZ.

In QM mice, MZ B cells were significantly increased in the absolute number (Fig. 1). Is the increase the result from accumulation of newly generated B cells from the bone marrow, or proliferation of a small subset of bone marrow- or fetal-derived cells? It has been proposed that the suppressed generation and diversification of B cells may lead to the expansion of long-lived B cell, including B-1 and MZ B cells, which contribute to maintain the production of natural Abs in a homeostatic manner (2, 5, 6). In addition, mice lacking serum IgM have been reported to show a MZ expansion, which was reversed by the administration of polyclonal IgM, but not of a mAb (51). However, although the B cell diversity in the spleen and LN of QM mice is contracted to two major populations (Fig. 3) (21), a variety of non-VH-encoded Abs is produced from small populations of B cells whose VH-T segments have been secondarily replaced with endogenous VH segments (52), and the MZ expansion in QM mice was not affected by adoptive transfer of the control C57BL/6 mouse sera that contained greater variety of Abs (our unpublished data). In the monoclonal BT mice whose B cells express only VH-T and tRNAs (52), and the MZ expansion in QM mice was not affected by stimuli having a crucial role in repertoire selection of precursor B cells at the T2 stage. But, this should be further examined in other mouse systems.

The results shown in Fig. 5 indicate that the repertoire bias observed in the MZ also occurred at the T2 and MZP stages in QM mice. MZP cells, a subpopulation of T2 cells, have been presumed as immediate precursors of MZ B cells, because MZP cells disappeared in parallel with MZ B cells in Atioslos- or Notch2-deficient mice (19, 54, 55). The relationship between BCR and Notch signals required for the selection of MZP cells is still unclear. The present results suggest that BCR signals probably given by Ag stimuli have a crucial role in the repertoire selection of precursor B cells for MZ B cells at the T2 stage. But, this should be further examined in other mouse systems.

Although there is the biased distribution of VH-T/A2 B cells to the MZ, this population is present both in the MZ and follicle (Fig. 3). In the Xid background, both MZ and FO B cells expressing the VH-T/A2 BCR were markedly reduced, while FO B cells expressing the VH-T/A1 BCR were affected to a lesser extent (Fig. 7), implying that these two VH-T/A2 B cell populations belonging to different compartments are maintained in a parallel manner. Recent reports have shown that VCAM-1+ and/or ICAM-1+ stromal cells and MZ macrophages have a critical role in the retention of B cells in the MZ B cell niche (50, 56). Because this niche is limited in the capacity to retain MZ B cells, an excess of VH-T/A2 B cells may reside in the follicle and acquire FO B cell phenotypes. Another possibility is that a part of FO B cells can be precursors of MZ B cells (16, 17). By analyzing the molecular basis controlling the recruitment of VH-T/A2 B cells into these two microenvironments, the QM system will give a clue to gain insight into the development mechanism of MZ B cells.

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