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The *Yaa* Mutation Promoting Murine Lupus Causes Defective Development of Marginal Zone B Cells[1]

Hirofumi Amano,[2]* Eri Amano,[2]* Thomas Moll,* Dragan Marinkovic,† Nabila Ibnou-Zekri,* Eduardo Martinez-Soría,* Isabelle Semac,* Thomas Wirth,† Lars Nitschke,‡ and Shozo Izui[3]*

The accelerated development of systemic lupus erythematosus (SLE) in BXSB male mice is associated with the presence of an as yet unidentified mutant gene, *Yaa* (Y-linked autoimmune acceleration). In view of a possible role of marginal zone (MZ) B cells in murine SLE, we have explored whether the expression of the *Yaa* mutation affects the differentiation of MZ and follicular B cells, thereby implicating the acceleration of the disease. In this study, we show that both BXSB and C57BL/6 *Yaa* mice, including two different substrains of BXSB *Yaa* males that are protected from SLE, displayed an impaired development of MZ B cells early in life. Studies in bone marrow chimeras revealed that the loss of MZ B cells resulted from a defect intrinsic to B cells expressing the *Yaa* mutation. The lack of selective expansion of MZ B cells in diseased BXSB *Yaa* males strongly argues against a major role of MZ B cells in the generation of pathogenic autoantibodies in the BXSB model of SLE. Furthermore, a comparative analysis with mice deficient in CD22 or expressing an IgM anti-trinitrophenyl/DNA transgene suggests that the hyperreactive phenotype of *Yaa* B cells, as judged by a markedly increased spontaneous IgM secretion, is likely to contribute to the enhanced maturation toward follicular B cells and the block in the MZ B cell generation. *The Journal of Immunology,* **2003, 170: 2293–2301.**

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The BXSB strain of mice spontaneously develops an autoimmune syndrome with features of systemic lupus erythematosus (SLE)[4] that affects males much earlier than females (1). This accelerated development of SLE in male BXSB mice is due to the presence of an as yet unidentified mutant gene located on the Y chromosome, designated *Yaa* (Y-linked autoimmune acceleration) (2). The *Yaa* gene by itself is unable to induce significant autoimmune responses in mice without an apparent SLE background, while it can induce and accelerate the development of SLE in combination with autosomal susceptibility alleles present in lupus-prone mice (3, 4). The selective production of anti-DNA autoantibodies by B cells bearing the *Yaa* gene in *Yaa* and non-*Yaa* double bone marrow chimeric mice revealed that the *Yaa* defect is expressed in B cells (5, 6). Based on these results, it can be speculated that the *Yaa* defect may decrease the threshold for B cell Ag receptor (BCR)-mediated signaling, thereby triggering and excessively stimulating autoreactive B cells (7).

Newly generated B cells in the bone marrow emigrate to the spleen, in which two types of transitional mature B cell precursors (T1 and T2) exist (8). In the spleen, immature transitional B cells can further differentiate into follicular or marginal zone (MZ) B cells, which differ in their localization and cell surface markers. Follicular, recirculating B cells are $IgM^{int}IgD^{high}CD21^{int}CD23^{high}CD1^{low}CD9^{low}$, and resident MZ B cells located at the junction of white and red pulps are $IgM^{high}IgD^{low}CD21^{high}CD23^{neg/low}CD1^{high}CD9^{high}$ (9–11). Although follicular B cells respond to T-dependent Ags, MZ B cells have recently been proposed to play a critical role in host defense against T-independent blood-borne pathogens (12, 13). In addition, several recent studies have claimed a potential role for MZ B cells in the spontaneous development of autoantibodies in systemic autoimmunity. $CD1^{high}$ B cells have been reported to produce large amounts of IgM anti-DNA Abs in lupus-prone (NZB × $NZW)F_1$ mice (14), which apparently have an increased number of MZ B cells (15). In addition, it has been shown that mice overexpressing B cell-activating factor of the TNF family (BAFF) spontaneously develop an SLE-like syndrome in association with a marked increase in T2 and MZ B cells (16, 17). Because BAFF is apparently a potent survival factor for immature T2 B cells, it can be speculated that autoreactive T2 B cells could escape deletion and differentiate into autoreactive MZ B cells in these mice. This is consistent with the findings that autoreactive B cells are accumulated in the MZ (18, 19). However, it still remains to be established that MZ B cells indeed secrete pathogenic autoantibodies implicated in SLE.

Molecular mechanisms regulating the differentiation into MZ vs follicular B cells have not been fully defined. However, studies on several different genetically manipulated mice have suggested that the impaired development of MZ B cells could be related to defects in chemotactic migration to the MZ (20–22) and to hypersensitive BCR signaling, favoring an accelerated maturation toward follicular B cells (23–26). The latter hypothesis has further been supported by the demonstration that the specificity and surface density of BCR are the critical factors determining the lineage commitment to different B cell subsets (27, 28). Because the *Yaa* mutation may act by enhancing BCR signaling, it could bring along a reduction of MZ B cell development. Alternatively, if the accelerated development of SLE by the *Yaa* gene is associated with an increased production of pathogenic autoantibodies by MZ

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[4] Abbreviations used in this paper: SLE, systemic lupus erythematosus; BAFF, B cell-activating factor of the TNF family; BCR, B cell Ag receptor; int, intermediate; MZ, marginal zone; neg, negative; NIP, (4-hydroxy-3-iodo-5-nitrophenyl)acetyl; TI-2, thymus-independent type 2; TNP, trinitrophenyl; *Yaa*, Y-linked autoimmune acceleration.

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B cells, the number of MZ B cells may rather be increased in mice bearing the *Yaa* mutation. We show in this study that the development of MZ B cells is markedly diminished in the presence of the *Yaa* mutation and not expanded during the course of SLE in BXSB mice, and discuss the possible mechanisms by which the *Yaa* mutation leads to the MZ B cell defect and autoimmunity.

Materials and Methods

Mice

BXSB mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BXSB (H-2^b) male, but not female mice develop an accelerated SLE, which results in part from the action of the *Yaa* gene (1), and do not express MHC class II I-E molecules because of a defect in the *Ea* gene encoding the I-E α-chain (29). BXSB mice lacking the *Yaa* gene or bearing the H-2^d haplotype (BXSB.H-2^d), or an *Ea* transgene (BXSB.Eα), and C57BL/6 (B6) mice bearing the *Yaa* mutation have been previously described (4, 29–31). The B6.C20 strain carrying the IgH^a allotype, instead of the IgH^b allotype, on the B6 background was kindly provided by M. Bosma (Philadelphia, PA). $CD22^{-/-}$ mice with a pure B6 background were developed as described previously (32). BXSB mice expressing an Sp6 anti-trinitrophenyl (TNP)/DNA IgM transgene (33) were created by backcross procedures at the eighth generation. The analysis of transgenic IgM^a and endogenous IgM^b expression in Sp6 transgenic mice showed that the majority (~90%) of splenic B cells expressed the transgene.

Preparation of bone marrow chimeras

Three- to 4-mo-old BXSB.Eα *Yaa* or non-*Yaa* male recipients (I-E^+) were irradiated at 850 rad and reconstituted with 5×10^6 bone marrow cells from 3- to 4-mo-old BXSB *Yaa* or non-*Yaa* male donors (I-E^-), as described previously (5). Two months later, chimerism in recipients was controlled by the absence of I-E-positive circulating B cells by flow cytomeric analysis. In some experiments, a mixture of donor bone marrow cells from *Yaa* B6 male mice bearing the IgH^b allotype and non-*Yaa* B6.C20 male mice bearing the IgH^a allotype was injected into irradiated B6 male mice. As control, a mixture of bone marrow cells from IgH^a and IgH^b non-*Yaa* male mice was injected into irradiated B6 male mice. Chimerism in recipients was controlled by the presence of IgM^a and IgM^b allotype-positive circulating B cells 2 mo after the reconstitution.

Flow cytometric analysis

Flow cytometry was performed using two- or three-color staining of spleen cells, and analyzed with a FACSCalibur (BD Biosciences, Mountain View, CA). The following Abs were used: anti-CD21 (7G6), anti-CD23 (B3B4), anti-CD22 (CY34), anti-CD19 (1D3), anti-B220 (RA3-6B2), anti-IgM^a (RS-3.1), anti-IgM^b (MB86), anti-IgD (AF6-122), anti-CD1d (1B1), anti-CD9 (KMC8), anti-I-A (Y-3P), anti-I-E (Y-17), anti-LFA-1 α-chain (CD11a; H35.89.9), and anti-β_1 integrin (CD29; Ha2/5) mAb, and polyclonal goat anti-human IgG (BD PharMingen, San Diego, CA). Human BAFF-human IgG Fc (BAFF-Fc) fusion protein was a kind gift of J. Tschopp (Lausanne, Switzerland). Staining was performed in the presence of saturating concentration of 2.4G2 anti-FcγRII/III mAb.

Immunohistochemistry

Spleens from 2-mo-old BXSB mice of both *Yaa* and non-*Yaa* genotypes were embedded in Tissue-Tek OCT compound and snap frozen in liquid nitrogen. Frozen sections (4 μm) were stained with PE-labeled anti-IgM (1B4B1; Southern Biotechnology, Birmingham, AL), FITC-labeled anti-IgD (11-26c.2a; PharMingen), and FITC-labeled MOMA-1 (Serotec, Oxford, U.K.) mAb in the presence of 2.4G2 anti-FcγRII/III mAb, as described previously (26).

Immunizations and ELISA

Two-month-old B6 *Yaa* and non-*Yaa* male mice were immunized i.v. with 50 μg of thymus-independent type 2 (TI-2) Ag, NIP-Ficoll (Biosearch Technologies, San Francisco, CA), and bled on days 0 and 7. Serum levels of IgM and IgG3 anti-(4-hydroxy-3-iodo-5-nitrophenyl)acetyl (NIP) Abs were determined by ELISA, using alkaline phosphatase-labeled rat anti-mouse IgM (LO-MM-9) and anti-mouse IgG3 (H139.61.1) mAb. Results are expressed as U/ml in reference to a standard curve established by using a pooled sera from B6 mice immunized with NIP-Ficoll. In addition, IgM concentrations in culture supernatants were determined by IgM-specific ELISA, as described previously (34).

Purification of splenic B cells and cell culture

B cells were purified from spleen by adherence of macrophages for 1 h at 37°C on plastic plates and subsequent treatment with IgM anti-Thy-1.2 (AT-83) mAb in the presence of rabbit complement (Cedarlane, Ontario, Canada). The purity of B cells, as documented by flow cytometric analysis, was superior to 95%. For spontaneous IgM secretion, 5×10^5 splenic B cells purified from *Yaa* and non-*Yaa* B6 male mice were incubated in 0.2 ml of DMEM containing 10% FCS at 37°C for 24 h. For proliferative responses of splenic B cells, 2×10^5 spleen cells were incubated with 1, 5, or 25 μg/ml of B7-6 anti-IgM mAb in 0.2 ml of DMEM-10% FCS at 37°C, and cultures were pulsed with [^{3}H]thymidine for the final 6 h of 3 days' culture.

Western blot analysis

Total lysates of purified splenic B cells from 2-mo-old BXSB and B6 mice of the *Yaa* and non-*Yaa* genotypes (three mice for each group) were separated on a 10% minigel and transferred to nitrocellulose (Hybond-C; Amersham Pharmacia Biotech, Dübendorf, Switzerland) with a semidry blotting apparatus (Bio-Rad, Glattbrugg, Switzerland). After 2 h of blocking at room temperature in TTBS (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.05% Tween 20) containing 5% low-fat, dry milk powder (TTBS-MP), the filters were incubated with polyclonal rabbit anti-Lyn (amino-terminal) Abs (Santa Cruz Biotechnology, Heidelberg, Germany) in TTBS-MP overnight at 4°C. Thoroughly washed filters were incubated with alkaline-phosphatase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) for 1 h at room temperature. Chemiluminescence development was conducted with the Immun-Star Pack reagents (Bio-Rad), and the filters were exposed to X-OMAT Kodak films.

RT-PCR

Five micrograms of total RNA were prepared from purified splenic B cells of 2-mo-old BXSB and B6 mice of both *Yaa* and non-*Yaa* genotype (three mice for each group) by RNeasy Mini kit (Qiagen AG, Basel, Switzerland). The first strand of cDNA (20 μl) was synthesized with an oligo(dT) primer and total RNA. For amplification with *Taq* DNA polymerase (Roche, Basel, Switzerland), the following primers for *Aiolos* and *SHP-1* genes were used: Aiolos forward primer (5′-GGCATCTTTTACTCAGAAAGG) and reverse primer (5′-TGGCTTGGTCCATCATCCG), and SHP-1 forward primer (5′-CCTGGACATTTCTTGTGCG) and reverse primer (5′-GTTCTCATCTGGACCTAGC). PCR products obtained following amplification of 5 μl of cDNA diluted 1/50, 1/250, and 1/1250 were visualized after electrophoresis through 2% agarose gels by staining with ethidium bromide. The PCR products obtained with optimal cDNA concentration, which gave an exponential phase of amplification, were quantified by densitometric analysis. A reference control gene (GAPDH) was also amplified with forward primer (5′-TGAAGGTCGGTGTGAACGGATTTGG) and reverse primer (5′-ACGACATACTCAGCACCAGCATCAC) to standardize amounts of RNA and to allow calculation of relative amounts of gene expression. To ensure the absence of sample contamination, a reaction mixture with no added cDNA was run in parallel with each PCR.

Measurement of intracellular Ca^{2+} mobilization

A total of 10^7 spleen cells from B6 *Yaa* and non-*Yaa* male mice was loaded with 4.5 μM Indo-1 (Molecular Probes, Eugene, OR) and 0.04% pluronic F-127 in RPMI (pH 7.4) with 1% FCS for 45 min at 37°C. After Indo-1 loading, cells were stained on ice with FITC-labeled anti-B220 mAb. Cells were washed, and IgM on the B cell surface was cross-linked at 37°C with 10, 30, or 90 μg/ml of B7-6 anti-IgM mAb. Increases of intracellular Ca^{2+} in splenic $B220^+$ B cells were recorded in real time for 6 min with a FACSVantage (BD Biosciences). The anti-B220 staining had no effect on Ca^{2+} flux, as was checked by comparison with unstained B cells.

Statistical analysis

Statistical analysis was performed with the Wilcoxon two-sample test. Values of $p > 5\%$ were considered insignificant.

Results

Reduction of MZ B cells in BXSB and B6 mice bearing the Yaa *mutation*

To investigate whether the *Yaa* mutation affected the size of the MZ B cell compartment, flow cytometric analysis of spleen cells from lupus-prone BXSB mice at 2 mo of age was performed. Analysis of cell surface expression of CD21 and CD23 on B220-positive population defines immature T1 B cells ($CD21^{neg}CD23^{neg}$),

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follicular B cells (CD21^{int}CD23^{high}), and MZ B cells (CD21^{high} CD23$^{neg/low}$). When compared with BXSB males or females lacking the *Yaa* mutation, MZ B cells were substantially (~4-fold) diminished in BXSB *Yaa* male mice (Fig. 1, *A* and *C*). This phenotypic defect was further confirmed with the use of other markers, including CD1d and CD9 (Fig. 1*B*). Histologically, spleens from BXSB *Yaa* male mice showed normal anatomical structures. The resident metallophilic, MOMA-1$^+$ MZ macrophages were correctly localized. However, in agreement with the flow cytometric analysis, a characteristic rim of IgM$^+$IgD$^-$ MZ B cells at the periphery of the follicles and separated by MOMA-1$^+$ macrophages was poorly visible in the spleens of BXSB *Yaa* males, as compared with non-*Yaa* BXSB males (Fig. 2).

The observed reduction of MZ B cells in BXSB *Yaa* male mice could be secondary to the accelerated development of autoimmune responses occurring in these mice, rather than a direct effect of the *Yaa* gene defect on MZ B cell generation. To exclude this possibility, we determined the size of the MZ B cell compartment in two different substrains of BXSB male mice (BXSB.H-2^d and BXSB.Eα). These two substrains of BXSB male mice carry the *Yaa* mutation, but fail to develop SLE during the first year of life, because of the presence of the H-2^d haplotype (30) or the transgene encoding an I-E α-chain (29), respectively. Despite the absence of significant autoantibody production, the development of the CD21^{high}CD23$^{neg/low}$ MZ B cell compartment in these two BXSB substrains was markedly limited, and indistinguishable from that of conventional BXSB *Yaa* male mice (Fig. 1, *A* and *C*). The association of the MZ B cell defect with the *Yaa* mutation was further confirmed by analysis in 2-mo-old B6 *Yaa* male mice (Fig. 1, *A* and *C*), which lack significant autoantibody production. More significantly, 6- to 8-mo-old conventional BXSB *Yaa* male mice developing severe SLE did not shown any sign of selective expansion of MZ B cells (Fig. 1, *A* and *C*), arguing against a major role of MZ B cells in the production of pathogenic lupus autoantibodies in the BXSB model of SLE. Although we noted a slight reduction of MZ B cells in 8-mo-old BXSB non-*Yaa* males, differences between *Yaa* and non-*Yaa* males were still highly significant (data not shown).

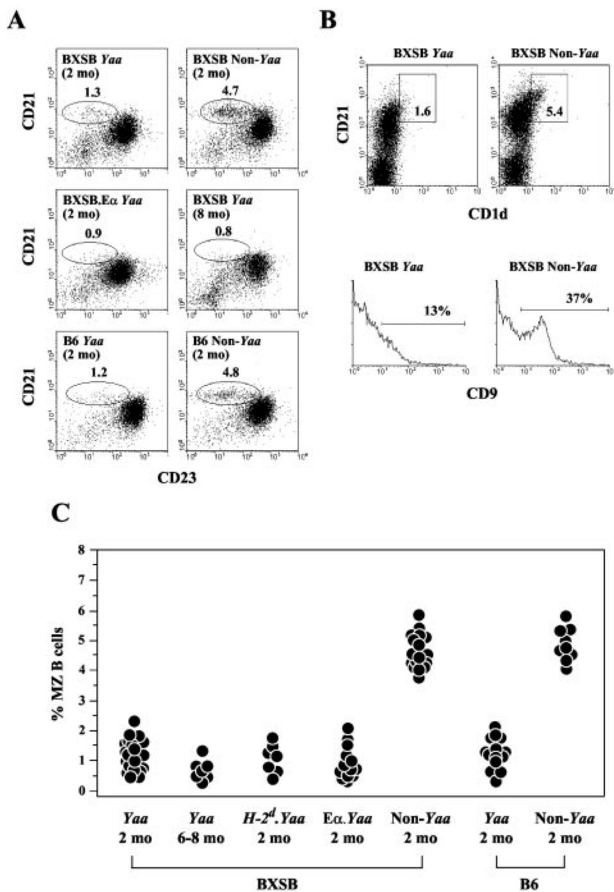

FIGURE 1. Reduction of MZ B cells in BXSB and B6 mice bearing the *Yaa* mutation. *A*, Spleen cells from BXSB, BXSB.Eα, and B6 male mice of the *Yaa* or non-*Yaa* genotype at 2 and 8 mo of age were stained with a combination of anti-B220, anti-CD21, and anti-CD23 mAb, and gated for B220$^+$ cells. Mean percentages of MZ B cells (CD21^{high}CD23$^{neg/low}$) in total spleen are indicated. Representative results from 8–20 mice in each group are shown. *B*, Spleen cells from 2-mo-old BXSB mice were stained with anti-CD21 and anti-CD1d mAb or with a combination of anti-IgM, anti-IgD, and anti-CD9 mAb. Percentages of MZ B cells (CD21^{high} CD1d^{high}) are indicated. Histograms show CD9 staining of the IgM^{high} IgD^{low} population of B cells enriched in MZ B cells. Percentages of CD9^{high} cells in the IgM^{high}IgD^{low} B cell population are indicated. Representative results from five to seven mice are shown. *C*, Percentages of MZ B cells (CD21^{high}CD23$^{neg/low}$) in spleen from BXSB, BXSB.H-2^d, BXSB.Eα, and B6 male mice of the *Yaa* or non-*Yaa* genotype at 2 and 6–8 mo of age. Means ($\pm$SD) of each group are: 2-mo-old BXSB *Yaa*, 1.3 $\pm$ 0.8%; 6- to 8-mo-old BXSB *Yaa*, 0.8 $\pm$ 0.4%; 2-mo-old BXSB.H-2^d *Yaa*, 1.0 $\pm$ 0.5%; 2-mo-old BXSB.Eα *Yaa*, 0.9 $\pm$ 0.7%; 2-mo-old BXSB non-*Yaa*, 4.7 $\pm$ 0.7%; 2-mo-old B6 *Yaa*, 1.2 $\pm$ 0.4%; 2-mo-old B6 non-*Yaa*, 4.8 $\pm$ 1.1%. The results obtained with BXSB female mice were essentially identical with those of non-*Yaa* BXSB male mice (means of 5 mice $\pm$ SD: 4.8 $\pm$ 1.0%).

Reduction of MZ B cells due to an intrinsic defect of B cells expressing the Yaa *mutation*

To determine whether the impaired MZ B cell development in mice bearing the *Yaa* mutation resulted from a defect in B cells themselves or in the stromal microenvironment that supports the

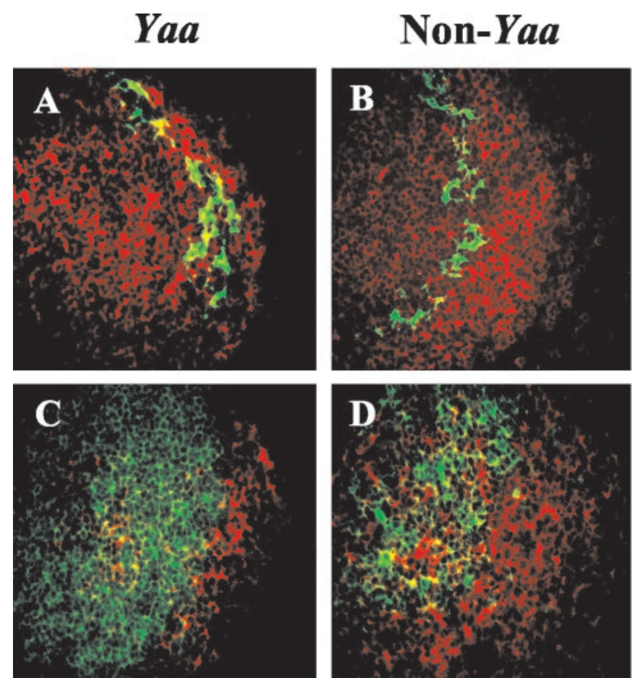

FIGURE 2. Reduction of the MZ B cell compartment in BXSB *Yaa* male mice. Spleen sections from 2-mo-old BXSB *Yaa* and non-*Yaa* male mice were stained with PE-labeled anti-IgM (red) and FITC-labeled anti-MOMA-1 (green) (*A*, *Yaa* and *B*, Non-*Yaa*) or PE-labeled anti-IgM (red) and FITC-labeled anti-IgD (green) mAb (*C*, *Yaa* and *D*, Non-*Yaa*). Representative results obtained from four mice in each group are shown.

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development of MZ B cells, we performed reciprocal bone marrow cell reconstitution experiments between BXSB *Yaa* and non-*Yaa* male mice. The transfer of bone marrow cells from BXSB non-*Yaa* males into irradiated BXSB *Yaa* males efficiently reconstituted MZ B cells (means of 7 mice ± SD: 4.4 ± 0.5%), as documented by flow cytometric analysis (Fig. 3*A*). The extent of the MZ B cell development observed in these mice was comparable to that observed in non-*Yaa* mice reconstituted with non-*Yaa* bone marrow cells (means of 4 mice ± SD: 4.6 ± 1.1%). In contrast, MZ B cells were poorly developed in irradiated BXSB non-*Yaa* males reconstituted with bone marrow cells from BXSB *Yaa* males (means of 5 mice ± SD: 1.4 ± 0.3%).

To confirm that the impaired development of MZ B cells in *Yaa* mice resulted from a defect intrinsic to B cells bearing the *Yaa* mutation, the development of MZ B cells was assessed in irradiated B6 male mice reconstituted with a mixture of bone marrow cells from *Yaa* B6 (IgH^b) and non-*Yaa* B6.C20 (IgH^a) male mice. As control, irradiated B6 male mice were reconstituted with bone marrow cells from both non-*Yaa* B6 and B6.C20 male mice. The analysis of surface IgM allotypes revealed a selective accumulation of IgM^a-positive B cells of non-*Yaa* origin in the MZ, but not in the follicular B cell compartment of *Yaa*/non-*Yaa* mixed chimeras, which contrasted with the comparable localization of B cells bearing either IgM allotype in control chimeras (Fig. 3*B*). Notably, the size of the MZ B cell compartment was somehow lower in *Yaa*/non-*Yaa* mixed chimeras (means of 4 mice ± SD: 3.3 ± 1.5%) than that in control chimeras (means of 3 mice ± SD: 4.7 ± 0.9%), most likely due to the defective development of MZ B cells of *Yaa* origin.

Reduction of T2 B cells in BXSB and B6 mice bearing the Yaa *mutation*

It has been considered that T2 B cells are the immediate precursors differentiating into either MZ or follicular B cells (17, 24). Therefore, the distribution of immature T1 ($CD21^{neg}CD23^{neg}IgM^{high}IgD^{neg}$) and T2 ($CD21^{high}CD23^{high}IgM^{high}IgD^{high}$) B cells (8) was examined in 2-mo-old BXSB and B6 male mice in relation to the *Yaa* mutation. The proportion of newly formed T1 B cells appeared almost comparable in both *Yaa* and non-*Yaa* males of either strain, while the size of the T2 B cell compartment was substantially diminished in both BXSB and B6 *Yaa* males (Fig. 4*A*), suggesting an accelerated maturation of T2 B cells toward follicular B cells in *Yaa* male mice. Notably, a more pronounced decrease of T2 B cells than T1 B cells was similarly observed in B6 mice deficient in the negative BCR regulator CD22 (data not shown), which are also defective in the generation of MZ B cells (26).

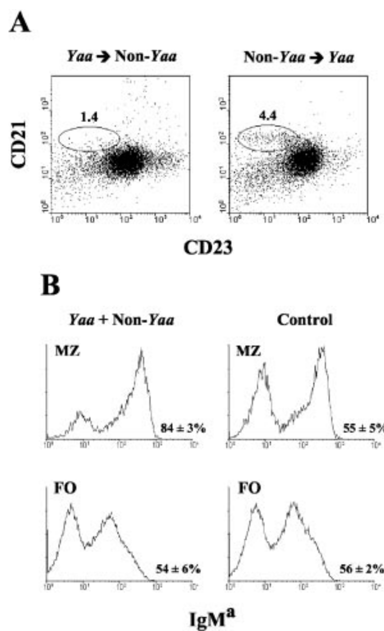

FIGURE 3. Impaired development of MZ B cells of *Yaa* origin in bone marrow radiation chimeras. *A*, Irradiated *Yaa* or non-*Yaa* BXSB male mice were reconstituted with bone marrow cells from BXSB *Yaa* or non-*Yaa* males. Two months after reconstitution, spleen cells were stained with a combination of anti-B220, anti-CD21, and anti-CD23 mAb, and gated for $B220^+$ cells. Mean percentages of MZ B cells ($CD21^{high}CD23^{neg/low}$) in spleen are indicated. Representative results from five to seven mice in each group (*Yaa*→Non-*Yaa* and Non-*Yaa*→*Yaa*) are shown. *B*, Irradiated B6 mice were reconstituted with a mixture of bone marrow cells from *Yaa* B6 (IgH^b) and non-*Yaa* B6.C20 (IgH^a) male mice (*Yaa* + Non-*Yaa*) or from non-*Yaa* B6 and B6.C20 male mice (control chimera). Two months after reconstitution, spleen cells were stained with a combination of anti-CD21, anti-CD23, and anti-IgM mAb. Histograms show non-*Yaa*-derived IgM^a staining of MZ ($CD21^{high}CD23^{neg/low}$) and follicular (FO; $CD21^{int}CD23^{high}$) B cells in both mixed chimeras, and mean percentages (±SD) of IgM^a-positive B cells in each compartment from three to four mice of each group are indicated. Note a selective accumulation of IgM^a-positive B cells of non-*Yaa* origin in the MZ of *Yaa*/non-*Yaa* mixed chimeras. This was confirmed by IgM^b staining of *Yaa* B cells (data not shown).

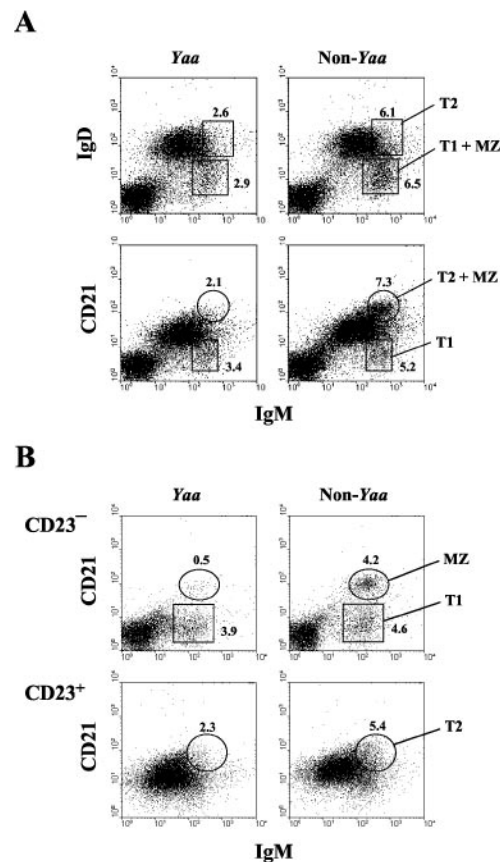

FIGURE 4. Reduction of T2 B cells in 2-mo-old BXSB *Yaa* mice. *A*, Spleen cells were stained with a combination of anti-IgM, anti-IgD, and anti-CD21 mAb, in which percentages of T1 ($CD21^{neg}IgM^{high}$) and T2 ($IgM^{high}IgD^{high}$) B cells are indicated. *B*, Spleen cells were stained with a combination of anti-IgM, anti-CD21, and anti-CD23 mAb. Within the $CD23^+$ and $CD23^-$ cell gates, percentages of MZ ($IgM^{high}CD21^{high}CD23^-$), T1 ($IgM^{high}CD21^{neg}CD23^-$), and T2 ($IgM^{high}CD21^{high}CD23^+$) B cells are indicated. Essentially identical results were obtained with B6 *Yaa* male and $CD22^{-/-}$ mice (data not shown). Representative results from three to five mice are shown.

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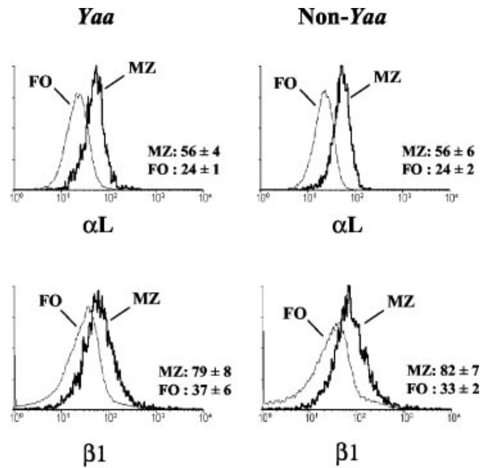

FIGURE 5. Lack of differences in expression levels of α_L- and β_1-containing integrins on MZ and follicular B cells between *Yaa* and non-*Yaa* B6 male mice. Spleen cells from 2-mo-old *Yaa* and non-*Yaa* B6 males were stained with a combination of anti-CD21, anti-CD23, and anti-α_L or anti-β_1 mAb. Histograms show α_L or β_1 staining of MZ (CD21^{high}CD23$^{neg/low}$) and follicular (FO; CD21^{int}CD23^{high}) B cells from *Yaa* and non-*Yaa* mice. Mean fluorescence intensities (±SD) of α_L and β_1 from five B6 mice of the *Yaa* or non-*Yaa* genotype are indicated.

It has been shown that BAFF secreted by dendritic cells, monocytes/macrophages, and T cells (16, 35, 36) is apparently a potent survival factor for T2 B cells, as overexpression of BAFF led to a remarkable expansion of the T2 B cell population, accompanied by an increase in MZ B cells (17). Thus, the reduction of T2 and MZ B cells in *Yaa* male mice could be related to a lower expression of BAFF-binding receptors. However, this possibility was excluded, because the flow cytometric analysis using BAFF-Fc did not show any significant differences in the binding of BAFF-Fc on T2, MZ, and follicular B cells from *Yaa* and non-*Yaa* BXSB and B6 males (data not shown).

A more recent study has demonstrated that an up-regulated expression of integrins, LFA-1 ($\alpha_L\beta_2$) and $\alpha_4\beta_1$, on MZ B cells and the interaction with their respective ligands, ICAM-1 and VCAM-1, expressed on resident stromal cells in the MZ is critical for the localization and retention of MZ B cells (37). Staining with anti-LFA-1 α-chain (α_L) and anti-β_1 integrin mAb confirmed higher levels of these two integrins in MZ B cells than in follicular B cells in non-*Yaa* B6 male mice (Fig. 5). However, we did not find any significant differences in the level of surface staining by anti-α_L and anti-β_1 mAb on MZ and follicular B cells between *Yaa* and non-*Yaa* mice. In addition, flow cytometric analysis failed to show the appearance of the MZ-phenotype B cells in the peripheral blood of *Yaa* mice, as opposed to mice treated with anti-α_L and anti-α_4 mAb, causing the displacement of MZ B cells into the blood (37).

Decreased levels of CD21 expression on follicular B cells from BXSB and B6 mice bearing the Yaa *mutation*

It has recently been reported that the absence of MZ B cells in Aiolos$^{-/-}$ mice is accompanied by the enhanced maturation of follicular B cells characterized by a lower level of CD21 expression (24). This was interpreted as a result of hypersensitive BCR signaling in Aiolos-deficient B cells, favoring the maturation of T2 B cells toward follicular B cells. In fact, CD22-deficient follicular B cells, which are hyperresponsive to BCR triggering, also exhibited a down-regulation of CD21 expression (Fig. 6). Because the *Yaa* defect may lead to an excessive activation of B cells, possibly through lowering the threshold for BCR-mediated signaling, we assessed whether the CD21 expression was similarly altered in follicular B cells from BXSB or B6 male mice carrying the *Yaa* mutation. The intensity of CD21 staining on follicular CD21^{int}CD23^{high} B cells was significantly reduced in *Yaa* males, as compared with non-*Yaa* males in both strains of mice ($p < 0.005$; Fig. 6). Although the differences were relatively small, follicular B cells from *Yaa* mice consistently showed lower levels of CD21 in several independent analyses. Notably, we did not find any measurable differences on splenic B cells between *Yaa* and non-*Yaa* BXSB and B6 male mice in their expression levels of different BCR coreceptors, CD22, CD19, and FcγRIIB, and MHC class II I-A, as determined by flow cytometric analysis. This was also the case for Lyn, SHP-1, and Aiolos, all of which are known to be implicated in BCR signaling and the MZ B cell development, as assessed by Western blot or semiquantitative RT-PCR analysis (data not shown).

Increased spontaneous secretion of IgM in Yaa *B6 male mice, but comparable responses to TI-2 Ag between* Yaa *and non-*Yaa *B6 male mice*

CD21 down-regulation in follicular B cells bearing the *Yaa* mutation suggested that these B cells resemble lymphocytes that have been constitutively activated, as the expression of CD21 is known to be reduced following BCR-mediated activation of B cells (38, 39). If this is the case, one can expect an increased spontaneous secretion of IgM Abs by splenic B cells bearing the *Yaa* mutation. In fact, when 2-mo-old *Yaa* and non-*Yaa* B6 male mice were compared, the amount of IgM spontaneously secreted during a 24-h culture by splenic B cells bearing the *Yaa* mutation was ~10-fold higher than that of non-*Yaa* counterparts (Fig. 7*A*). However, *Yaa*

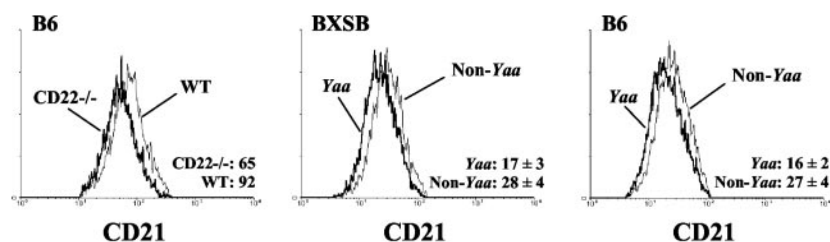

FIGURE 6. Decreased expression of CD21 in follicular B cells from 2-mo-old CD22$^{-/-}$ and *Yaa* mice. Spleen cells were stained with a combination of anti-B220, anti-CD21, and anti-CD23 mAb. Within the B220^{+} cell population, CD21^{int}CD23^{high} follicular B cells were gated, with maximal exclusion of T2 (CD21^{high}CD23^{high}) cells. Histograms show CD21 staining of follicular B cells, in which the presence of contaminating T2 cells was too limited to influence the value of peak fluorescence intensity of CD21. Mean fluorescence intensities (±SD) of CD21 from five BXSB and B6 mice of the *Yaa* or non-*Yaa* genotype and from two CD22$^{-/-}$ and wild-type B6 mice are indicated. Differences in the fluorescence intensity of CD21 between *Yaa* and CD22$^{-/-}$ mice are due to the fact that these analyses were conducted in the laboratory of S. Izui and L. Nitschke, respectively, by using different batches of mAb.

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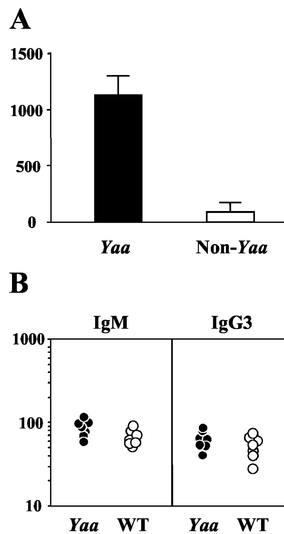

FIGURE 7. Increased secretion of IgM by splenic B cells from B6 male mice bearing the *Yaa* mutation, but comparable Ab responses to TI-2 Ag, NIP-Ficoll. *A*, Spontaneous secretion of IgM in vitro after a 24-h incubation of splenic B cells from 2-mo-old B6 male mice of the *Yaa* or non-*Yaa* genotype was determined by ELISA. IgM concentrations in supernatants from three different mice (means ± SD) are expressed as ng/ml of IgM. *B*, Serum levels of IgM and IgG3 anti-NIP Abs were determined by ELISA 7 days after an i.v. injection of 50 μg of NIP-Ficoll into 2-mo-old B6 mice. Results are expressed as units per milliliter of anti-NIP Abs. Serum levels of IgM and IgG3 anti-NIP Abs before the immunization were <10 and <1 U/ml, respectively, in *Yaa* and non-*Yaa* B6 males.

B cells did not show any significant differences in Ca^{2+} influx and proliferative responses following BCR cross-linking with B7-6 anti-IgM mAb at any dose tested (data not shown).

It has been shown that MZ B cells play a major role in humoral responses against TI-2 Ags (20). To test whether the reduction of MZ B cells in B6 *Yaa* mice affected their immune responses to TI-2 Ags, 2-mo-old B6 mice were immunized i.v. with NIP-Ficoll. NIP-specific IgM and IgG3 concentrations in B6 *Yaa* males during the response to the TI-2 Ag were comparable to those of non-*Yaa* counterparts (Fig. 7*B*).

Increased development of MZ B cells in BXSB Yaa *male mice expressing an Sp6 IgM anti-TNP/DNA transgene*

To further define the possible mechanism responsible for the *Yaa*-linked MZ B cell defect, we determined the effect of the *Yaa* mutation on the development of MZ B cells expressing a transgene encoding an Sp6 IgM anti-TNP/DNA Ab in BXSB mice. Studies in several different Ig transgenic mice have shown that the proportion of MZ B cells varies depending on the specificity of BCR, and that partially autoreactive B cells tend to be more accumulated in the MZ B cell compartment (19, 40). In agreement with results obtained in mice expressing a VH81X H chain transgene conferring self-reactivity (40), the flow cytometric analysis revealed that the proportion of MZ B cells was significantly enlarged in 2-mo-old non-*Yaa* BXSB Sp6 transgenic male mice (means of 5 mice ± SD: 9.4 ± 2.1%), as compared with nontransgenic littermates (means of 4 mice ± SD: 4.6 ± 1.3%) (Fig. 8*A*). In contrast to a compromised development of MZ B cells in *Yaa* BXSB nontransgenic males (means of 4 mice ± SD: 1.1 ± 1.5%), the size of the MZ B cell compartment in Sp6 transgenic BXSB *Yaa* males was markedly increased (means of 5 mice ± SD: 7.5 ± 2.4%), reaching a level almost comparable to that of non-*Yaa* BXSB transgenic mice. These results suggest that MZ B precursors expressing the

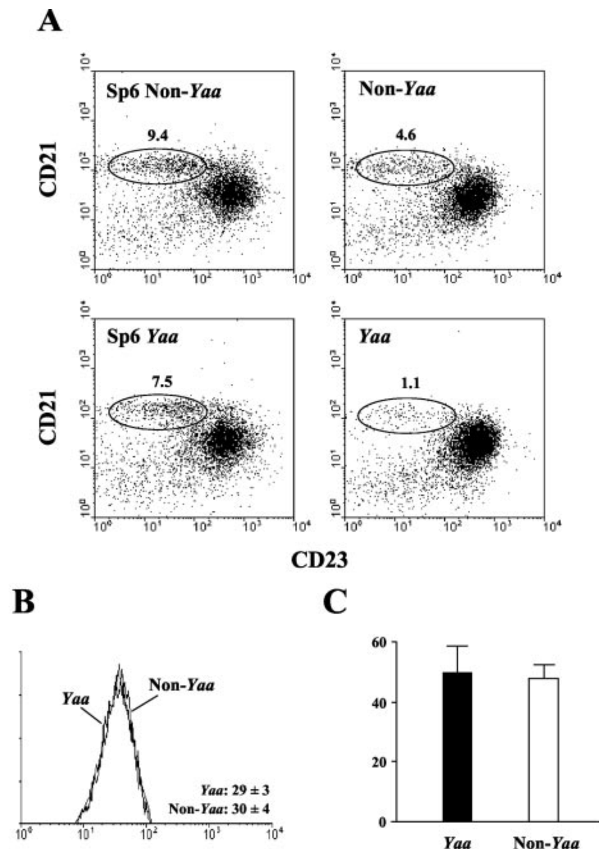

FIGURE 8. Increased MZ B cell development, and lack of CD21 down-modulation on follicular B cells and of increased spontaneous IgM secretion by splenic B cells in BXSB *Yaa* male mice expressing an Sp6 IgM anti-TNP/DNA transgene. *A*, Spleen cells from 2-mo-old *Yaa* and non-*Yaa* BXSB Sp6 transgenic males and their nontransgenic littermates were stained with a combination of anti-B220, anti-CD21, and anti-CD23 mAb, and gated for $B220^{+}$ cells. Mean percentages of MZ B cells ($CD21^{high}CD23^{neg/low}$) in spleen are indicated. Representative results from four to five mice in each group are shown. *B*, Histograms show comparable CD21 staining of follicular ($CD21^{int}CD23^{high}$) B cells between *Yaa* and non-*Yaa* Sp6 transgenic male mice. Mean fluorescence intensity (±SD) of CD21 from five Sp6 transgenic male mice in each group is given. *C*, Spontaneous secretion of IgM in vitro after a 24-h incubation of splenic B cells from 2-mo-old BXSB Sp6 transgenic male mice of the *Yaa* or non-*Yaa* genotype was determined by ELISA. IgM concentrations in supernatants from three different mice (means ± SD) are expressed as nanograms per milliliter of IgM.

Yaa mutation have the potential to migrate toward the MZ almost as efficiently as those of non-*Yaa* origin. Notably, the levels of CD21 expression on follicular B cells and of spontaneous IgM secretion by splenic B cells did not significantly differ between *Yaa* and non-*Yaa* Sp6 transgenic male mice (Fig. 8, *B* and *C*).

Discussion

In the present study, we have demonstrated that lupus-prone BXSB and nonautoimmune B6 mice bearing the *Yaa* mutation display a defect in the development of MZ B cells, as documented by flow cytometric or histological analysis. This defect was similarly observed in two BXSB *Yaa* substrains (BXSB.H-2^{d} and BXSB.Eα), which are protected from SLE (29, 30), indicating that the defect in the MZ B cell generation is directly linked to the *Yaa* mutation, and not secondary to the development of lupus-like autoimmune responses. Studies in bone marrow chimeras revealed that the selective loss of MZ B cells in *Yaa* mice results from a defect intrinsic to B cells expressing the *Yaa* mutation. In addition, the lack

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of preferential expansion of MZ B cells in aged BXSB *Yaa* males developing severe SLE strongly argues against a major role of MZ B cells in the generation of pathogenic autoantibodies in the BXSB model of SLE.

Several recent studies have proposed a possible role for MZ B cells in the spontaneous production of lupus autoantibodies in mice. First, the size of the MZ B cell compartment has been reported to be enlarged in lupus-prone (NZB × NZW)F_1 mice (15), and it was claimed to be linked to the *Nba2* locus (41), which provides the major contribution to lupus susceptibility in this model of SLE (42). Second, $CD1d^{high}$ B cells, a phenotype of MZ B cells, have been shown to produce more IgM autoantibodies than follicular B cells in (NZB × NZW)F_1 mice (14). Third, mice overexpressing BAFF developed a lupus-like autoimmune syndrome in parallel to an expansion of MZ B cells (16). Finally, low affinity autoreactive B cells could be positively selected into the MZ (18, 19, 40). However, as shown in the present study, a specific reduction, rather than an expansion, of MZ B cells in aged BXSB *Yaa* males developing a lethal form of SLE suggests that the MZ B cells are not critically involved in the *Yaa*-mediated accelerated development of SLE in these mice. However, our results cannot totally exclude the possibility that low numbers of MZ B cells in BXSB *Yaa* male mice may in part be due to an increased activation of autoreactive B cells in the MZ and their exit from this compartment. In fact, it has been reported that MZ B cells undergo rapid migration to lymphoid follicles after exposure to blood-borne bacterial products (43). Clearly, the experiment in lupus-prone mice genetically deficient in the MZ B cell compartment would provide a more definite conclusion to this issue.

It has been shown that MZ B cells are capable of differentiating into plasma cells very rapidly upon stimulation with polyclonal B cell activators (10). It may be possible that MZ B cells bearing low affinity self-reactive BCR produce so-called natural autoantibodies of the IgM isotype upon contact with blood-borne pathogens. These natural IgM autoantibodies could play an important role in self tolerance, possibly by promoting the elimination of pathogenic self Ags or maintaining central tolerance through opsonization of self Ags with complement (44), as shown in mice deficient in secretory IgM, which are predisposed to the production of IgG autoantibodies (45, 46). If this is the case, the loss of MZ B cells occurring in mice bearing the *Yaa* mutation could rather promote autoimmune responses, because of a limited production of beneficial natural IgM autoantibodies. This could partially explain the increased production of IgG autoantibodies in $CD22^{-/-}$ or $Lyn^{-/-}$ mice having decreased MZ B cell compartments (47–49). Furthermore, it is worth mentioning that, in contrast to recent findings based on linkage analysis in (B6 × NZB)F_2 mice (41), the development of MZ B cells was substantially reduced in B6 mice congenic for the *Nba2* locus (unpublished data), which produce antinuclear autoantibodies characteristic of SLE (42).

The mechanism by which the *Yaa* mutation affects the MZ B cell development remains speculative, because the molecular defect caused by the *Yaa* mutation has not yet been defined. Studies in $Aiolos^{-/-}$ mice, which are deficient in MZ B cells, revealed that their B cells were more readily activated in response to BCR triggering, and that the follicular B cells show hyperreactive phenotypes (24). Therefore, it has been proposed that the strength of the signal elicited via the BCR regulates the lineage commitment of mature B cells into follicular vs MZ B cells, in which relatively strong signals favor follicular B cell generation, while weaker signals induce differentiation into MZ B cells. This hypothesis is consistent with the increased development of MZ B cells in Btk-deficient mice, in which BCR signal strength is decreased (23, 24), and the impaired MZ B cell generation in $CD22^{-/-}$ mice, in which BCR signaling is enhanced (26). Significantly, the present study revealed that B cells bearing the *Yaa* mutation share the phenotype of hyperreactive B cells, as shown by a decreased expression level of CD21 and a markedly increased spontaneous production of IgM Abs by splenic B cells in young B6 *Yaa* mice, which do not show any sign of autoantibody production. In addition, it can be speculated that a pronounced decrease of T2 B cells in the *Yaa* mice, as is the case in $CD22^{-/-}$ and $Aiolos^{-/-}$ mice (24), may be a result of the accelerated and preferential differentiation of *Yaa*-bearing T2 cells into follicular B cells. Thus, an attractive hypothesis is that the *Yaa* mutation may act as a positive BCR regulator, thereby modulating the maturation of MZ B cells. However, unlike B cells deficient in Aiolos or CD22 (24), *Yaa* B cells did not show an enhanced Ca^{2+} release upon BCR stimulation by anti-IgM mAb, although we cannot exclude the possibility that the effect of the *Yaa* mutation on BCR signaling may be too subtle to be detected under this experimental condition.

An alternative possibility is that a signal derived from surrounding cells may play an additional role in the activation, differentiation, or survival of mature B cells in spleen, in which the *Yaa* mutation is implicated, thereby affecting the development of MZ B cells. It has been reported that the development of MZ B cells is markedly reduced in CD19-deficient mice (23, 50), despite the fact that CD19 functions as a positive BCR regulator through the formation of the BCR coreceptor complex with CD21 (51). However, because of the presence of CD19 in excess of CD21 on the cell surface, it has been speculated that CD19 may act as a receptor for unidentified ligands (51), thereby regulating the generation or survival of MZ B cells in a BCR-independent manner. In addition, we have recently observed that mice deficient in CD40 or CD40 ligand have an increased proportion of MZ B cells (unpublished data). Although the expression levels of CD19 and CD40 are not altered in splenic B cells from *Yaa* mice, it might be worth investigating whether the *Yaa* mutation can modulate the CD19 or CD40 signaling pathway.

It is also possible that the *Yaa* mutation leads to defects in the motility and responsiveness to chemokines critical for the migration of MZ B precursors to the appropriate site. This hypothesis has been used to explain the loss of MZ B cells in mice lacking Pyk-2 tyrosine kinase, Lsc (the murine homologue of human p115 Rho GTP exchange factor), or DOCK-2 (a hemopoietic cell-specific CDM family protein) (20–22). In addition, a more recent study has demonstrated a critical role of integrins, LFA-1 and $\alpha_4\beta_1$, in the localization and retention of MZ B cells (37). However, the expression levels of these integrins on MZ and follicular B cells in *Yaa* mice were not different from those in non-*Yaa* mice. Furthermore, we observed that BXSB *Yaa* male mice expressing the Sp6 anti-TNP/DNA transgene developed a MZ B cell compartment almost comparable to that of non-*Yaa* counterparts. Therefore, we consider a migration defect to the MZ or a localization and retention defect within the MZ as a less likely explanation for the loss of MZ B cells in *Yaa* mice. In addition, the experiment with the Sp6 transgenic mice further supports the dependence on BCR specificity and signaling in maturation and survival of MZ B cells. The increase of MZ B cell compartment in the Sp6 transgenic mice is consistent with the finding that low affinity self-reactive B cells tend to be accumulated in the MZ (19, 40). It has been speculated that self Ags may very weakly trigger such autoreactive B cells, thereby favoring the differentiation into MZ B cells (23, 24, 40). In this regard, it is of interest to note that the follicular B cells bearing the Sp6 transgene and *Yaa* mutation no longer showed hyperreactive phenotypes, consistent with the normal development of the MZ B cell compartment in the Sp6 transgenic *Yaa* mice. One possible explanation for this is that these

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autoreactive B cells could become partially anergic, thus counteracting the action of the *Yaa* mutation. This possibility warrants further investigation by using different Ig transgenic mice bearing the *Yaa* mutation.

It is significant that follicular B cells in *Yaa* mice exhibit hyperreactive phenotypes, as judged by an increased spontaneous secretion of IgM in vitro. This was further supported by serological analysis, showing increased levels of serum IgM in 5- to 6-wk-old young B6 and BXSB *Yaa* males, as compared with non-*Yaa* males (unpublished data). This is in agreement with the earlier observation that spleen cells from BXSB *Yaa* males exhibit an increased polyclonal IgM Ab production early in their life, as compared with those from BXSB female mice (52). This would also explain why Ab responses against TI-2 Ags were not diminished in the *Yaa* mice (31), in contrast to lower Ab responses against TI-2 Ags in several, but not all, mice deficient in the MZ B cell development (13, 20, 26, 53). It has been reported that mice overexpressing CD19 or deficient in CD22 or Lyn, in which B cells become abnormally hyperresponsive to antigenic stimulation, spontaneously produced increased levels of autoantibodies (48, 49, 54, 55). Therefore, it is reasonable to assume that the hyperreactive phenotype of *Yaa* B cells is implicated in the *Yaa*-mediated acceleration of autoantibody production in lupus-prone mice.

In conclusion, we have shown in the present study that the *Yaa* mutation causes an impaired development of MZ B cells, and that the lack of selective expansion of MZ B cells in diseased BXSB *Yaa* male mice argues against a critical role of MZ B cells in the generation of pathogenic autoantibodies in this SLE model. It can be hypothesized that in the presence of the *Yaa* mutation, B cells may be more readily activated by putative natural in vivo ligand(s), thereby contributing not only to the enhanced maturation into follicular B cells and the block of MZ B cell generation, but also to the accelerated development of SLE. The understanding of the mechanism responsible for the *Yaa*-associated MZ B cell defect and the hyperreactive phenotype of *Yaa* B cells is of paramount importance for the elucidation of the molecular abnormality caused by the *Yaa* mutation, and hence the development of lupus-like systemic autoimmune disease.

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