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Differential Activation of Anti-Erythrocyte and Anti-DNA Autoreactive B Lymphocytes by the *Yaa* Mutation[1]

Thomas Moll,* Eduardo Martinez-Soria,* Marie-Laure Santiago-Raber,* Hirofumi Amano,* Maria Pihlgren-Bosch,* Dragan Marinkovic,† and Shozo Izui[2]*

An as-yet-unidentified mutation, *Y-linked autoimmune acceleration* (*Yaa*), is responsible for the accelerated development of lupus-like autoimmune syndrome in mice. In view of a possible role for *Yaa* as a positive regulator of BCR signaling, we have explored whether the expression of the *Yaa* mutation affects the development and activation of transgenic autoreactive B cells expressing either 4C8 IgM anti-RBC or Sp6 IgM anti-DNA. In this study, we show that the expression of the *Yaa* mutation induced a lethal form of autoimmune hemolytic anemia in 4C8 transgenic C57BL/6 mice, likely as a result of activation of 4C8 anti-RBC autoreactive B cells early in life. This was further supported, although indirectly, by increased T cell-independent IgM production in spleens of nontransgenic C57BL/6 mice bearing the *Yaa* mutation. In contrast, *Yaa* failed to induce activation of Sp6 anti-DNA autoreactive B cells, consistent with a lack of increased IgM anti-DNA production in nontransgenic C57BL/6 *Yaa* mice. Our results suggest that *Yaa* can activate autoreactive B cells in a BCR-dependent manner, related to differences in the form and nature of autoantigens. ***The Journal of Immunology,*** **2005, 174: 702–709.**

It has been well established that the development of autoreactive B cells can be regulated by different mechanisms including clonal deletion, clonal anergy, and receptor editing in the bone marrow and peripheral lymphoid organs, depending on the nature of the autoantigen, its concentration in different sites, and the affinity of autoantibodies. Because the induction of self-tolerance is not complete, it has been proposed that dysregulated and excessive activation of B cells predisposes to the development of autoantibody-mediated autoimmune diseases, such as systemic lupus erythematosus (SLE)[3] and autoimmune hemolytic anemia (1). This has been supported by the finding that the production of anti-DNA autoantibodies is a common feature of genetically manipulated mice, in which B cells become abnormally hyperresponsive to antigenic stimulation (2–4).

The *Y-linked autoimmune acceleration* (*Yaa*) mutation has been shown to be responsible for the acceleration of the lupus-like autoimmune syndrome in BXSB mice and in their F_1 hybrids with NZB or NZW mice (5). *Yaa* by itself is unable to induce significant autoimmune responses in mice without an apparent SLE background (6, 7), whereas it can induce and accelerate the development of SLE in combination with autosomal susceptibility alleles present in lupus-prone mice (8). Analysis of *Yaa* and non-*Yaa* double bone marrow chimeric mice has demonstrated that anti-DNA autoantibodies were selectively produced by B cells bearing the *Yaa* gene, and that T cells from both *Yaa* and non-*Yaa* origin efficiently promoted anti-DNA autoantibody responses (9, 10). These data suggest that the *Yaa* defect is functionally expressed in B cells, but not in T cells. The expression of *Yaa* in the B cell lineage is likely responsible for a marked reduction of the marginal zone (MZ) B cell compartment early in life in spleens of mice bearing the *Yaa* mutation (11).

The molecular mechanism of the *Yaa* mutation in the accelerated development of lupus-like autoimmune syndrome has been poorly understood. The expression of the *Yaa* gene in B cells, but not in T cells, suggests that the *Yaa* defect is likely to be directly involved in the excessive activation of B cells. It can be speculated that the action of the *Yaa* mutation may be to decrease the threshold for BCR-mediated signaling or to facilitate interactions between T and B cells, thereby triggering and excessively stimulating autoreactive B cells (5). This is consistent with a previous report showing that B cells bearing the *Yaa* mutation exhibited a hyperreactive phenotype, as judged by higher proliferative responses following stimulation with LPS, anti-IgM, or CD40L (12). To better define the molecular basis of the *Yaa* defect, we determined the effect of the *Yaa* mutation on the development and activation of transgenic autoreactive B cells expressing either 4C8 IgM anti-RBC or Sp6 IgM anti-DNA. We observed that the *Yaa* mutation is unable to inhibit clonal deletion of autoreactive B cells in the bone marrow. However, it is able to activate 4C8 anti-RBC autoreactive B cells in the periphery, but not Sp6 anti-DNA B cells, suggesting a differential effect of the *Yaa* mutation on autoreactive B cells with different specificities.

Materials and Methods

Mice

C57BL/6 (B6) mice bearing the 4C8 anti-RBC IgM^a transgene or the *Yaa* mutation have been previously described (7, 13). Sp6 anti-DNA IgM^a transgenic BALB/c mice (BALB.Sp6) were obtained from Dr. A. Rolink (Pharmazentrum, Basel, Switzerland) (14). B6 mice were purchased from The Jackson Laboratory. The presence of the 4C8 or Sp6 transgene was detected by surface staining of peripheral blood B cells with biotinylated anti-IgM^a (RS-3.1). The inheritance of the 4C8 transgene in mice that died prematurely was assessed by PCR using the following primers: forward primer (5′-CTACGCATTTAGTAGTGACTGG-3′) and reverse primer

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; MZ, marginal zone; MLN, mesenteric lymph node; PeC, peritoneal cavity; Ht, hematocrit; BrMRBC, bromelain-treated mouse RBC.

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(5′-TGCAGAGACAGTGACCAGAG-3′). Their gender was determined by PCR for the Y chromosome-specific gene *Zfy* (15) using the following primers: forward primer (5′-AAGATAAGCTTACATAATCACATGGA-3′) and reverse primer (5′-CCTATGAAATCCTTTGCTGCACATGT-3′).

Flow cytometric analysis

Flow cytometry was performed using two- or three-color staining of bone marrow, spleen, mesenteric lymph node (MLN), peritoneal cavity (PeC), and peripheral blood cells, and analyzed with a FACSCalibur (BD Biosciences). The following Abs were used: anti-B220 (RA3-6B2), anti-IgM^a (RS-3.1), anti-IgM^b (MB86), anti-Sp6 Id (20.5) (14), anti-IgD^b (AF-3.33.3.2), PB493 (16), anti-CD21 (7G6), anti-CD23 (B3B4), anti-CD5 (53-7.3), and anti-CD11b (M1/70) mAb. Staining was performed in the presence of saturating concentration of 2.4G2 anti-FcγRII/III mAb.

Determination of hematocrits (Ht)

Blood samples were collected into heparinized microhematocrit tubes and centrifuged in a microfuge, as described previously (17). Percentage of packed RBC volume was directly measured after centrifugation.

Serological assays

Serum levels of total IgM were determined by ELISA as described previously (18). Serum levels of IgM^a were determined by ELISA, in which microtiter plates were coated with rabbit anti-mouse IgM Abs and developed with alkaline phosphatase-labeled rat anti-mouse IgM^a (RS-3.1) mAb. Results are expressed as micrograms per milliliter in reference to a standard curve obtained with murine IgM. Serum levels of IgM anti-DNA, anti-chromatin, and anti-DNP Abs in B6 mice were determined by ELISA using alkaline phosphatase-labeled anti-IgM (LO-MM-9) mAb, and results are expressed as titration units (units per milliliter) in reference to a standard curve established with a serum pool derived from B6 mice treated with the polyclonal B cell activator LPS. Serum IgM anti-DNA activities in Sp6 transgenic mice were quantified by ELISA using alkaline phosphatase-labeled anti-IgM^a (RS-3.1) mAb, and results are expressed as units per milliliter in reference to a standard curve established with a serum pool from LPS-injected BALB/c mice. Serum levels of IgM Abs against bromelain-treated mouse RBC (BrMRBC) were determined by a flow cytometric assay (19). Briefly, RBC from B6 mice were treated with 1 mg/ml bromelain (Sigma-Aldrich) for 30 min at 37°C. After washing three times with 1% BSA-PBS, 20 μl of a 25% BrMRBC suspension was incubated with 100 μl of serum samples diluted 1/100 in 1% BSA-PBS for 1 h at 4°C. Bound autoantibodies against BrMRBC were detected by staining with biotinylated rat anti-mouse IgM mAb (LO-MM-9) followed by streptavidin-PE, and analyzed by FACS. Results are expressed as units per milliliter in reference to a standard curve established with the median fluorescence values obtained with serial dilutions of an IgM anti-BrMRBC mAb (CP8B3D3) (20).

Cell culture

For spontaneous IgM secretion, cell suspensions were prepared from spleen, MLN, and PeC of *Yaa* and non-*Yaa* B6 male mice. Spleen cell suspensions were treated with Trizma-buffered lysis solution (150 mM NH_4Cl and 20 mM $NH_2C(CH_2OH)_3$) to eliminate RBC. Triplicates of 10^6 cells were incubated in 200 μl of DMEM containing 10% FCS at 37°C for 24 h. IgM levels in culture supernatants were determined by ELISA and are expressed in nanograms per milliliter.

Enumeration of IgM-secreting cells by ELISPOT

The number of IgM-secreting cells was assessed by ELISPOT analysis as previously described (21). MULTIscreen HA nitrocellulose-bottomed plates (Millipore) were coated with 5 μg/ml LO-MM-9 anti-IgM mAb overnight at 37°C. After washing with PBS-0.1% Tween 20 and blocking with DMEM containing 10% FCS, serial dilutions of spleen cell suspension, prepared as described for cell culture, were added to the plates and incubated for 5 h at 37°C. After washing, plates were incubated with alkaline phosphatase-conjugated LO-MM-9 overnight at 4°C, washed, and developed with 5-bromo-4-chloro-3-indolyl phosphate/NBT substrate (Sigma-Aldrich). Frequencies of IgM-secreting cells were calculated by counting wells containing between 20 and 100 spots with the use of a binocular.

Immunohistochemistry and histopathology

Spleens from 8-wk-old B6 male mice of *Yaa* or non-*Yaa* genotype were embedded in Tissue-Tek OCT compound (Miles) and snap-frozen in liquid nitrogen. Plasma cells were stained using anti-CD138 (syndecan-1) mAb (281-2; BD Pharmingen) followed by streptavidin-biotinylated HRP complex (Amersham Biosciences). Binding of HRP complex was revealed with 3-amino-9-ethylcarbazo compound. The slides were further counterstained with hematoxylin to easily distinguish follicles from red pulp. Frozen sections (10 μm) were stained for follicular dendritic cells using purified anti-CD35 (complement receptor 1) mAb (8C12; BD Pharmingen) followed by FITC-coupled mouse anti-rat IgG (Jackson ImmunoResearch) and counterstained with Texas Red-labeled goat anti-mouse IgM (Southern Biotechnology Associates) in the presence of 2.4G2 anti-FcγRII/III mAb, as described previously (22). Major organs, including spleen and liver, were obtained at autopsy, and histological sections were stained with H&E to evaluate histopathological changes.

Depletion of CD4$^+$ T cells in vivo

B6 mice were treated from birth (during the first 24 h of life) to 4 wk of age with rat anti-CD4 mAb (GK1.5), as previously described (23). The efficiency of CD4$^+$ T cell depletion was evaluated weekly by flow cytometric analysis of peripheral blood lymphocytes. As a control, mice were similarly treated with polyclonal rat IgG purified from rat serum.

Statistical analysis

Statistical analysis was performed with the Mann-Whitney *U* test. Probability values >5% were considered insignificant.

Results

Early activation of 4C8 anti-RBC autoreactive B cells in B6 mice bearing the Yaa *mutation*

To determine the effect of the *Yaa* mutation on the development and activation of autoreactive B cells, we crossed 4C8 transgenic females with B6 male mice carrying the *Yaa* mutation (B6.*Yaa)* or control male mice, and compared the development of anemia between *Yaa* and non-*Yaa* 4C8 transgenic mice. Among male offspring derived from crosses with *Yaa*-bearing progenitors, we noted early mortality (before weaning) of a substantial number of mice. At weaning, the cumulated number of female transgenic mice was approximately three times higher than that of male transgenic littermates bearing the *Yaa* mutation. When mice found dead during the first 4 wk of life were genotyped, virtually all of them were *Yaa* males carrying the 4C8 transgene. Notably, such an increased early mortality was observed neither in nontransgenic B6.*Yaa* mice nor in non-*Yaa* 4C8 transgenic mice of either sex. To confirm the early mortality of transgenic *Yaa* male mice, we genotyped mice alive at 3 wk of age, and followed their mortality rates. As shown (Fig. 1*A*), essentially all transgenic *Yaa* male mice died by 8 wk of age, whereas the mortality rates of transgenic mice lacking the *Yaa* mutation and nontransgenic *Yaa* male mice were <10% during the first 8 wk of life.

At autopsy, we consistently observed massive agglutination of RBC in spleen and, to a lesser extent, in liver from 4C8 transgenic B6.*Yaa* mice that died early in life. These histological lesions were similar to those observed in mice that died from acute anemia following an injection of 200 μg of 4C8 IgM mAb (17). In contrast, only a limited extent of agglutinated RBC was seen in spleens from non-*Yaa* transgenic mice. In parallel to the early mortality in transgenic B6.*Yaa* male mice, there was a decrease in Ht values in these mice, compared with non-*Yaa* transgenic controls ($p < 0.001$; mean Ht values ± 1 SD: 4C8$^+$.*Yaa*, 26.6 ± 9.4%; 4C8$^+$.B6, 39.8 ± 6.7%; nontransgenic controls: 43.7 ± 5.7%; Fig. 1*B*). These results indicated that the *Yaa* mutation enhanced 4C8 anti-RBC autoantibody production and caused a lethal anemia in 4C8 transgenic mice.

To determine whether the *Yaa* mutation could affect the development and/or the differentiation of 4C8 transgenic B cells, we analyzed by flow cytometry the presence of these cells in bone marrow, spleen, MLN, PeC, and peripheral blood from transgenic B6.*Yaa* and control male mice. Despite the development of a more severe anemia in 4C8$^+$ B6.*Yaa* male mice, transgenic B cells were

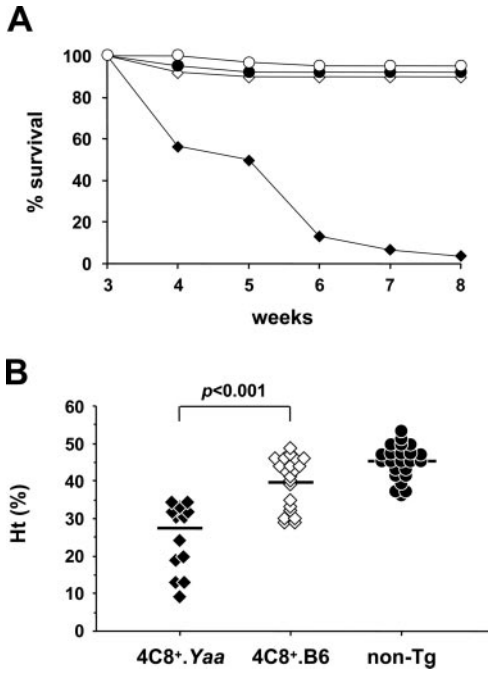

FIGURE 1. Increased mortality in 4C8 IgM anti-RBC transgenic mice carrying the *Yaa* mutation due to enhanced autoantibody production. *A*, Male 4C8 transgenic (◇, ◆) and nontransgenic littermates (○, ●) were genotyped at 3 wk of age, and their mortality in the presence (◆, ●) or absence (◇, ○) of the *Yaa* mutation was assessed. *B*, Ht of 4- to 6-wk-old 4C8$^+$.*Yaa*, 4C8$^+$ non-*Yaa*, and nontransgenic (non-Tg) control male mice.

hardly detectable in any of the lymphoid tissues analyzed in these mice, as in the case of non-*Yaa* transgenic mice (Fig. 2), in agreement with the previous findings in 4C8 transgenic mice (13, 24, 25). Notably, 4C8 transgenic mice displayed only very limited amounts of serum IgM, as determined for total IgM and transgenic IgM^a, even in the presence of the *Yaa* mutation (Table I). These low concentrations of serum IgM were owed to a very small number of B cells and a rapid adsorption of 4C8 anti-RBC mAb to circulating RBC, as previously observed in mice injected with 4C8 mAb (17). These results indicated that the *Yaa* mutation neither inhibited clonal deletion of 4C8 anti-RBC autoreactive B cells in the bone marrow nor promoted their clonal expansion in the periphery.

Table I. *Serum levels of total IgM and IgM(a) in 4C8 and Sp6 transgenic male mice with or without the* Yaa *mutation*

Mice	Transgene	*Yaa*	IgM[a]	IgM(a)[a]
B6	4C8	+	4.1 ± 2.5	3.2 ± 1.8
B6	4C8	−	1.9 ± 1.3	1.8 ± 1.2
B6	−	+	210 ± 111[b]	ND[c]
B6	−	−	90 ± 34[b]	ND[c]
(BALB × B6)F$_1$	Sp6	+	194 ± 69	213 ± 62
(BALB × B6)F$_1$	Sp6	−	224 ± 51	232 ± 45
(BALB × B6)F$_1$	−	+	438 ± 142[b]	206 ± 97[b]
(BALB × B6)F$_1$	−	−	213 ± 96[b]	97 ± 32[b]

[a] Serum concentrations of total IgM and IgM(a) (a allotype) were determined at 4 wk of age for 4C8 transgenic mice and at 6 wk of age for Sp6 transgenic mice. Results are expressed in micrograms per milliliter (means ± 1 SD of 8–12 mice in each group).
[b] $p < 0.001$.
[c] Not detectable.

Increased spontaneous IgM secretion in young B6 mice bearing the Yaa *mutation*

The early mortality due to the development of severe anemia in young 4C8 transgenic mice bearing the *Yaa* mutation suggested that *Yaa* could induce the activation of B cells very early in life. To test this possibility, we compared serum IgM levels between nontransgenic B6.*Yaa* and non-*Yaa* male mice at 2, 3, 4, and 8 wk of age. At 2 wk of age, serum levels of IgM in *Yaa* and non-*Yaa* males were almost comparable (Fig. 3*A*). However, at 3 wk of age, B6.*Yaa* male mice displayed significantly increased levels of IgM

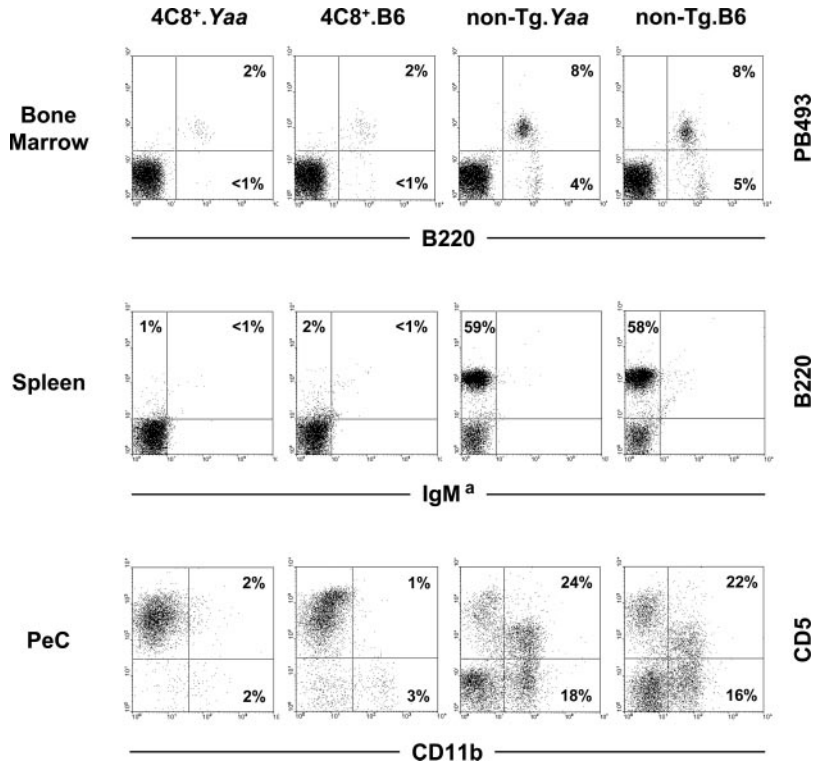

FIGURE 2. Development of 4C8 transgenic B cells in different lymphoid tissues of 4- to 6-wk-old 4C8$^+$.*Yaa*, 4C8$^+$ non-*Yaa*, and nontransgenic (non-Tg) *Yaa* and non-*Yaa* male mice. Representative results obtained from four to six mice in each group are shown. Numbers indicate percentages based on total gated mononuclear cells. In the bone marrow, 4C8 transgenic mice both with and without the *Yaa* mutation showed a predominant developmental block at the stage of immature (PB493$^+$B220^{int}) B cells and consequently lacked mature (PB493$^-$B220^{high}) B cells. In the periphery, as illustrated by spleen-cell staining, 4C8$^+$ mice hardly bore any B220$^+$ B cells, neither of transgenic (IgM^a) nor of endogenous (IgM^b) origin, independent of the presence or absence of the *Yaa* mutation. Staining of mononuclear PeC cells in non-Tg control mice with mAb against CD5 and CD11b (Mac-1) separated B1a (CD11b$^+$CD5$^+$) and B1b (CD11b$^+$CD5$^-$) cells from T cells (CD11b$^-$CD5$^+$) and conventional B2 cells (double-negative). All B cell subsets were hardly detectable in the PeC of 4C8 transgenic mice with or without the *Yaa* mutation.

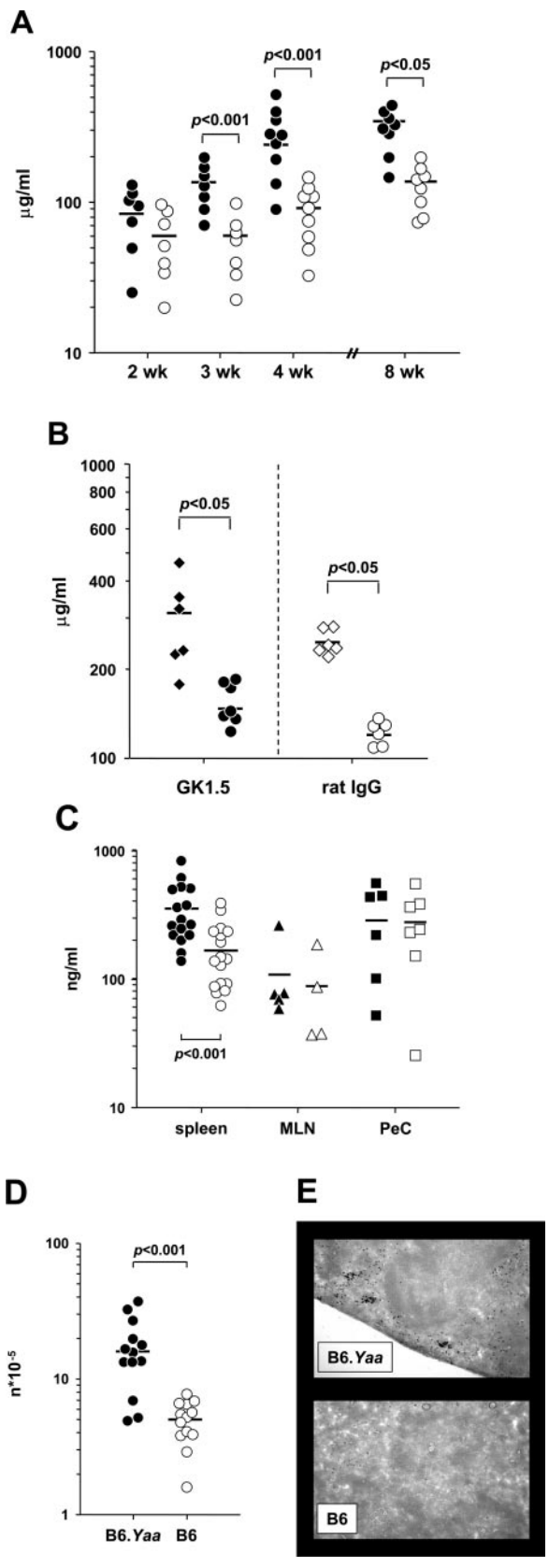

FIGURE 3. Increase of spontaneous IgM secretion by spleen cells early in life in B6 mice carrying the *Yaa* mutation, but without contribution of $CD4^+$ T cells. *A*, Total serum IgM levels in 2- to 8-wk-old B6.*Yaa* (●) and B6 control male mice (○) were determined by ELISA. *B*, $CD4^+$ T cells in B6.*Yaa* (◆, ◇) and B6 male mice (●, ○) were depleted by treatment with GK1.5 mAb from birth (◆, ●) or mice received polyclonal rat IgG as control (◇, ○). Total serum IgM levels were determined by ELISA at 4 wk of age. *C*, Spleen, MLN, or PeC cells prepared from 8-wk-old B6.*Yaa* (●, ▲, ■) or B6 control male mice (○, △, □) were cultured for 24 h and spontaneously secreted IgM was quantified by ELISA. A statistically significant difference was found only for spleen cells. *D*, The frequency of IgM-secreting cells among total spleen cells prepared from adult B6.*Yaa* or B6 control male mice was determined by ELISPOT assay. In average, B6.*Yaa* male mice contain 17 IgM-secreting cells per 10^5 total spleen cells, whereas B6 male mice contain 5 IgM-secreting cells per 10^5 spleen cells. *E*, Frozen sections of spleens from adult B6.*Yaa* or B6 control male mice were stained with hematoxylin and anti-CD138 mAb for plasma cells (×40).

(mean ± 1 SD, 131 ± 43 μg/ml), compared with wild-type male mice (58 ± 24 μg/ml; $p < 0.001$), and the differences remained significant until 8 wk of age (4 wk, $p < 0.001$; 8 wk, $p < 0.05$). In contrast, serum levels of total IgG, measured at 8 wk of age, were not different between *Yaa* and non-*Yaa* B6 male mice (*Yaa*, 11.2 ± 3.3 mg/ml; non-*Yaa*, 10.1 ± 4.9 mg/ml). Notably, when B6.*Yaa* male mice were depleted of $CD4^+$ T cells by treatment with GK1.5 anti-CD4 mAb from birth, they still displayed increased serum levels of IgM, compared with similarly treated non-*Yaa* B6 mice (means ± 1 SD at 4 wk of age: depleted B6.*Yaa*, 310 ± 131 μg/ml; depleted B6, 156 ± 25 μg/ml; $p < 0.05$; control B6.*Yaa*, 250 ± 24 μg/ml; control B6, 122 ± 10 μg/ml; $p < 0.05$; Fig. 3*B*).

To determine the site of increased IgM secretion in B6 mice bearing the *Yaa* mutation, the spontaneous secretion of IgM was measured by culturing mononuclear cells from spleen, MLN, and PeC of 8-wk-old B6 male mice with or without the *Yaa* mutation. Levels of IgM secreted by *Yaa* spleen cells during a 24-h culture were markedly increased, compared with those by non-*Yaa* spleen cells (means ± 1 SD: B6.*Yaa*, 350 ± 187 ng/ml; B6, 169 ± 92 ng/ml; $p < 0.001$; Fig. 3*C*). In contrast, no increased secretion of IgM was observed by MLN and PeC cells from B6.*Yaa* male mice (MLN: B6.*Yaa*, 109 ± 86 ng/ml; B6, 87 ± 70 ng/ml; PeC: B6.*Yaa*, 295 ± 201 ng/ml; B6, 276 ± 170 ng/ml). Enhanced IgM secretion by *Yaa* spleen cells was further confirmed by a 3- to 5-fold increase in the frequency of IgM-secreting cells, as determined by ELISPOT assay (Fig. 3*D*). Concordantly, immunohistochemical analysis of the spleen showed an increased accumulation in B6.*Yaa* mice of $CD138^+$ plasma cells, located in the red pulp (Fig. 3*E*).

Because $CD11b^+$ B1 cells have been considered to be the major source of serum IgM (26), we determined whether an enhanced IgM secretion in B6.*Yaa* male mice was related to a possible increase in B1 cells in the periphery. However, the flow cytometry analysis did not show any differences in the total number of B1 cells, including both B1 subsets ($CD5^+$ B1a and $CD5^-$ B1b), in spleen and PeC between 8-wk-old B6.*Yaa* and control male mice (data not shown). It should be stressed that the number of mature follicular B cells was comparable between B6.*Yaa* and non-*Yaa* male mice, even though the size of the MZ B cell compartment in B6.*Yaa* male mice was substantially reduced, as described previously (11). Furthermore, we analyzed whether the *Yaa* mutation promoted an accelerated development of mature B cells in the spleen, which may predispose to an early activation of B cells. However, flow cytometric analysis of splenic B cells for different immature and mature B cell subsets, as well as immunohistochemical analysis of the development of follicular dendritic cells did not reveal significant differences between B6.*Yaa* and non-*Yaa* male mice at 3 and 5 wk of age, respectively (data not shown).

To determine the specificity of IgM, the production of which was enhanced by the presence of the *Yaa* mutation, we tested the reactivity of serum IgM from 6-wk-old B6.*Yaa* male mice to different Ags, including DNA, chromatin, DNP, and BrMRBC (Fig.

4). In parallel to increases in total IgM, sera from B6.*Yaa* male mice exhibited significantly increased titers of IgM anti-chromatin, anti-DNP, and anti-BrMRBC, compared with those from non-*Yaa* male mice (means ± 1 SD: anti-chromatin: B6.*Yaa*, 14.0 ± 10.5 U/ml vs B6, 4.4 ± 2.7 U/ml; anti-DNP: 11.9 ± 10.6 vs 1.5 ± 1.1; anti-BrMRBC: 3.8 ± 1.1 vs 2.6 ± 0.2; for all: $p < 0.001$). In contrast, IgM anti-DNA activities were hardly elevated in B6.*Yaa* male mice (B6.*Yaa*, 5.4 ± 4.0, vs B6, 4.4 ± 1.3).

Lack of activation of Sp6 anti-DNA autoreactive B cells in B6 mice bearing the Yaa *mutation*

The analysis of the specificity of serum IgM in B6.*Yaa* and control male mice suggested that enhanced IgM production occurring in B6.*Yaa* mice did not simply reflect an increased polyclonal activation of B cells, but was possibly related to the specificity of the BCR. To test this possibility, we determined the effect of the *Yaa* mutation on the activation of B cells expressing a transgenic Sp6 IgM^a anti-DNA Ab in (BALB.Sp6 × B6)F_1 mice. Serum levels of Sp6 IgM^a anti-DNA activities were comparable between transgenic *Yaa* and non-*Yaa* F_1 male mice at 6 wk of age (means ± 1 SD: *Yaa*: 92.0 ± 44.3 U/ml; non-*Yaa*: 110.2 ± 74.2 U/ml), whereas such activities were hardly detectable in sera from nontransgenic F_1 mice (2.5 ± 0.7 U/ml; Fig. 5*A*). Notably, Sp6 anti-DNA Abs did not show any significant binding to chromatin (data not shown). These results were further confirmed by in vitro analysis for spontaneous secretion of transgenic Sp6 IgM^a Abs by *Yaa* and non-*Yaa* spleen cells, as assessed by ELISA and ELISPOT assays (Fig. 5*B*). Moreover, no differences in serum levels of total IgM and transgenic IgM^a were found in Sp6 transgenic mice with or without the *Yaa* mutation, whereas the potential of the *Yaa* mutation to induce increased levels of serum IgM was confirmed also in nontransgenic F_1 controls (Table I).

Flow cytometric analysis on bone marrow and spleen cells showed that the size of the immature and mature B cell compartments in 8-wk-old Sp6 transgenic males bearing the *Yaa* mutation was not different from that of transgenic non-*Yaa* males, and that the majority of B cells in spleen expressed the transgenic Sp6 Id (Fig. 6). However, it should be stressed that mature $PB493^{-}B220^{high}$, long-lived recirculating B cells were significantly reduced in bone marrow from Sp6 transgenic mice, independently of the *Yaa* genotype (means of three mice ± 1 SD: *Yaa*, 3.9 ± 0.6%; non-*Yaa*, 4.7 ± 0.6%), as compared with nontransgenic mice (*Yaa*, 9.5 ± 0.6%; non-*Yaa*, 8.1 ± 0.9%). This was consistent with the finding that the percentage of B cells in peripheral blood was significantly reduced in Sp6 transgenic *Yaa* (means of eight mice: 32.8 ± 4.3%) and non-*Yaa* male mice (means of nine mice: 28.1 ± 5.1%), compared with nontransgenic littermates (means of nine mice: *Yaa*, 58.3 ± 7.5%; non-*Yaa*, 57.8 ± 8.9%; $p < 0.001$) (Fig. 6). Despite the reduction of the recirculating B cells, the numbers of mature follicular and MZ B cells in the spleen of Sp6 transgenic mice were not diminished compared with those of nontransgenic littermates, as we have previously shown (11).

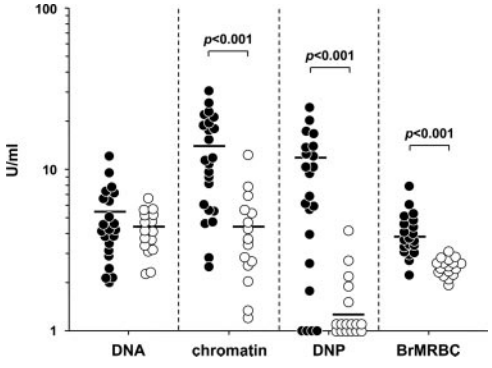

FIGURE 4. Autoreactive specificities of IgM autoantibodies in B6.*Yaa* mice. Sera from 6-wk-old B6.*Yaa* (●) or B6 control male mice (○) were analyzed by ELISA (anti-DNA, anti-chromatin, anti-DNP) or FACS (anti-BrMRBC). Serum levels of IgM anti-chromatin, anti-DNP, and anti-BrMRBC, but not anti-DNA, were significantly increased in B6.*Yaa* over B6 mice.

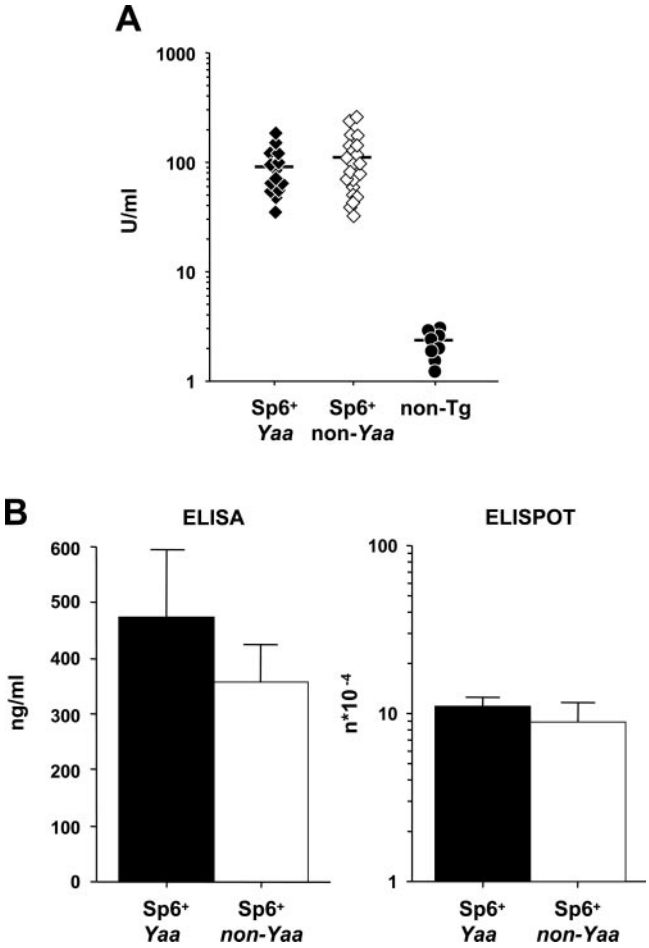

FIGURE 5. No enhancement of the secretion of Sp6 transgenic IgM^a anti-DNA autoantibodies by the *Yaa* mutation in Sp6 transgenic mice. *A*, Sera from 6-wk-old (BALB.Sp6 × B6.*Yaa*)F_1 or (BALB.Sp6 × B6)F_1 male mice, as well as from nontransgenic (non-Tg) controls (with or without the *Yaa* mutation) were analyzed by ELISA for levels of IgM^a anti-DNA. No statistically significant difference was found between the two transgenic strains. *B*, Transgenic IgM^a secreted by spleen cells during 24 h of culture and plasma cell frequencies were determined by ELISA and ELISPOT, respectively, in (BALB.Sp6 × B6)F_1 male mice carrying or not the *Yaa* mutation. Results from a representative experiment are shown (three mice per group), and no statistically significant differences were found.

Discussion

In the present study, we have demonstrated that the expression of the *Yaa* mutation induces a lethal form of autoimmune hemolytic anemia in 4C8 IgM transgenic mice, likely as a result of an increased activation of anti-RBC autoreactive B cells early in life. This is consistent with the demonstration of high serum IgM levels and an increased IgM secretion in spleen from nontransgenic B6 mice bearing the *Yaa* mutation. In contrast, the *Yaa* mutation fails

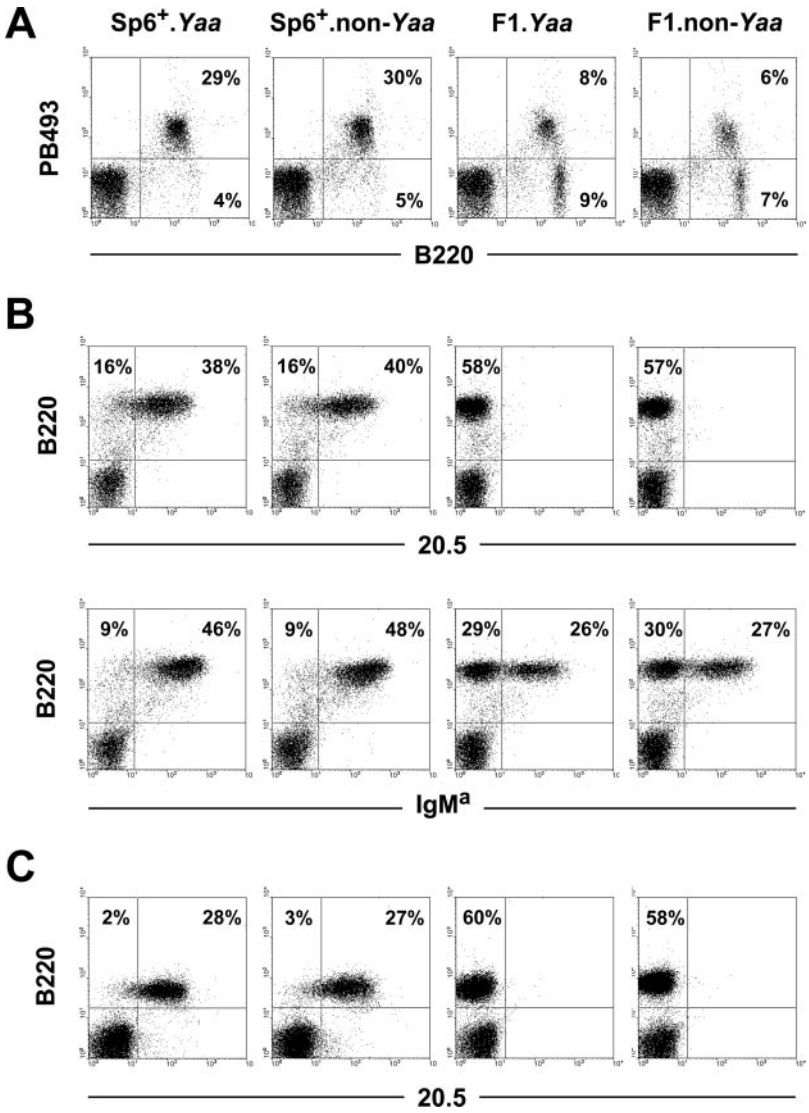

FIGURE 6. Development of Sp6 transgenic B cells in bone marrow (*A*), spleen (*B*), and peripheral blood (*C*) of 8-wk-old (BALB/c × B6)F_1 mice. Representative results obtained from three mice for bone marrow and spleen cells, and from eight to nine mice for PBMC are shown. Numbers indicate percentages based on total gated mononuclear cells. Even though immature ($PB493^+B220^{int}$) B cells accumulated in the bone marrow of Sp6 transgenic mice, independently of the presence of the *Yaa* mutation, percentages of total B cells in the spleen were comparable with those of nontransgenic controls. In contrast, the percentages of B cells in peripheral blood were significantly reduced in Sp6 transgenic mice with or without the *Yaa* mutation, consistent with the finding that recirculating ($PB493^-B220^{high}$) B cells were reduced in bone marrow of these mice. Notably, the vast majority of peripheral B cells from $Sp6^+$ mice expressed the transgenic Sp6 Id.

to induce activation of Sp6 IgM anti-DNA autoreactive B cells. The differential effect of the *Yaa* mutation on these two types of autoreactive B cells may be related to differences in the form and nature of the autoantigens involved in stimulation of the respective B cells.

It is striking to see that essentially all of the 4C8 transgenic mice carrying the *Yaa* mutation spontaneously develop a lethal anemia early in their lives (even before 4 wk of age). The development of severe anemia in these mice was documented by decreases in Ht values and histological findings (markedly agglutinated RBC in red pulp of spleen). The pathogenesis of anemia in the 4C8 transgenic *Yaa* mice is in agreement with our previous finding that the injection of 4C8 IgM mAb induces anemia as a result of massive agglutination of RBC in spleen and liver, but is not due to complement-mediated intravascular hemolysis (17).

It is clear that the development of lethal anemia in the context of the *Yaa* mutation is not due to failure of clonal deletion of 4C8 anti-RBC autoreactive B cells in the bone marrow. However, we did not find any sign of expansion of the 4C8 transgenic B cells in any of the peripheral lymphoid tissues analyzed in *Yaa* mice. This suggests that the development of lethal anemia early in life in $4C8^+$ *Yaa* mice is likely to be a result of a transient activation of mature 4C8 transgenic B cells. This idea is supported, although indirectly, by the demonstration that nontransgenic B6.*Yaa* male mice had increased serum levels of IgM from 3 wk of age, and displayed enhanced levels of spontaneous IgM secretion, as well as increased numbers of IgM-secreting cells in spleen. This is in agreement with the earlier observation that spleen cells from BXSB.*Yaa* male mice exhibit an increased IgM Ab production early in their lives, compared with those from BXSB females (27).

In the 4C8 transgenic model, it has been repeatedly shown that B1 cells are the major subset to become activated and secrete 4C8 anti-RBC autoantibodies (25, 28, 29). Because B1 cells largely contribute to the production of serum IgM without T cell help, an excessive activation of B1 cells could be partly responsible for an increased IgM production in B6.*Yaa* mice. This is consistent with the finding that B6.*Yaa* mice had higher serum levels of IgM anti-BrMRBC Abs, the production of which is largely dependent on the activation of B1 cells (30). However, we did not find any measurable increases in the number of B1 cells in PeC, spleen, and lymph nodes of 4C8 transgenic *Yaa* mice developing severe anemia. In addition, the analysis of nontransgenic *Yaa* mice failed to show an expansion of B1 cells in PeC and an increased IgM production by PeC B cells. Thus, our data rather argue against the idea that B1 cells are the target of *Yaa*.

We have shown that spleen is the major site for increased IgM secretion in B6.*Yaa* mice, and that this process is largely independent of the presence of $CD4^+$ T cells, because their depletion

failed to down-modulate hypersecretion of IgM in these mice. Because MZ B cells are the major subset to secrete IgM without T cell help in spleen, *Yaa* may promote the activation of this particular B cell subset in a T cell-independent manner. However, our previous analysis showed a markedly reduced number of MZ B cells in B6.*Yaa* mice (11). MZ B cells are known to be very rapidly activated upon stimulation, migrating into the red pulp and differentiating into plasma cells (31, 32). Because we noted an accumulation of plasma cells in the red pulp of *Yaa* spleens, one can speculate that the reduction of MZ B cells in *Yaa* mice may be a consequence of excessive and continuous activation of MZ B cells by environmental T-independent Ags, and their migration into the red pulp and differentiation into plasma cells. In this regard, it is worth mentioning that we have recently observed an increased level of IgM secretion in spleen, a diminished MZ B cell compartment, but normal numbers of B1 cells in B6 mice congenic for the *New Zealand autoimmunity 2* (*Nba2*) locus (33), which is a major locus contributing to lupus susceptibility in NZB mice (34). In agreement, our preliminary studies have shown that the introduction of *Nba2* induced an accelerated development of severe anemia in the 4C8 transgenic mice. Furthermore, we have previously detected an increase, instead of a reduction, of the MZ B cell compartment in Sp6 anti-DNA transgenic *Yaa* mice (11). This could be due to the lack of spontaneous activation of these autoreactive B cells present in the MZ, and the subsequent failure of migration into the red pulp, even in the presence of the *Yaa* mutation. Consistently, our preliminary study has shown that anti-hen egg lysozyme IgM transgenic mice bearing the *Yaa* mutation, in which B cells lack antigenic stimulation, have an increased MZ B cell compartment, as observed in Sp6 transgenic *Yaa* mice.

In contrast to 4C8 anti-RBC autoreactive B cells, it is striking to observe that the *Yaa* mutation failed to activate Sp6 anti-DNA autoreactive B cells, as judged by the lack of any increases in Sp6 IgM^a anti-DNA Abs in sera and splenocyte culture supernatants. Differential effect of *Yaa* cannot be explained by differences in the functional states of 4C8 and Sp6 B cells, because 4C8 B cells present in the periphery are expected to be more anergic than the corresponding Sp6 B cells, because of a more pronounced deletion of the former cells in the bone marrow. Notably, Sp6 transgenic mice spontaneously produce substantial amounts of Sp6 anti-DNA Abs. Because the recirculating pool of mature B cells was markedly diminished in Sp6 transgenic mice, one cannot exclude the possibility that *Yaa* differentially activates distinct B cell subpopulations. Alternatively, it may be that the action of the *Yaa* mutation is more dependent on BCR specificity, and does not simply reflect a polyclonal activation of B cells. In fact, when the specificity of serum IgM in nontransgenic B6.*Yaa* mice was analyzed, we observed no increase in IgM anti-DNA activities, whereas IgM anti-chromatin and anti-BrMRBC activities were substantially elevated. As discussed above, *Yaa* may promote more efficiently the activation of MZ B cells, which are readily stimulated by particulate forms of bloodborne Ags in a T cell-independent manner (35, 36). Thus, one can speculate that certain forms of autoantigens, such as membrane-bound, high-density epitopes, could more efficiently trigger the activation of *Yaa*-bearing B cells without T cell help, as may be the case for 4C8 anti-RBC transgenic B cells. This is in agreement with the observation in young B6.*Yaa* mice of spontaneous production of IgM anti-BrMRBC autoantibodies, because the protease treatment possibly discloses repetitive autoantigenic epitopes on the RBC surface. B cells specific for chromatin autoantigens may also be more efficiently activated through contact with apoptotic bodies that likely display suitable membrane-bound autoantigenic determinants. In contrast, this may not be the case for Sp6 anti-DNA B cells, which are unable to recognize chromatin.

In the present study, we have shown that the *Yaa* mutation is able to activate 4C8 anti-RBC autoreactive B cells, but not Sp6 anti-DNA autoreactive B cells. This suggests that *Yaa* can activate autoreactive B cells without T cell help, but dependent on BCR specificity, and thus likely related to the form and nature of autoantigens. However, it should be stressed that the *Yaa* mutation is known to promote the production of various autoantibodies of IgG class, including anti-DNA, in a $CD4^+$ T cell-dependent manner (37). The level of activation induced in naturally occurring DNA-specific B cells bearing the *Yaa* mutation upon contact with DNA autoantigens may still be too weak to induce their differentiation into IgM producers. However, the presence of *Yaa* in these B cells may be sufficient to promote subsequent interaction with autoreactive Th cells. Thus, *Yaa* could reduce the threshold for B cell activation following BCR engagement, which may result not only in triggering a direct activation of B cells upon contact with appropriate autoantigens but also in potentiating a weak interaction with autoreactive Th cells. This idea is consistent with our previous observation that *Yaa* is able to potentiate IgG Ab responses against foreign Ags only in mice that are genetically (*H2*-linked) low-responding, but not high-responding (38). In addition, if *Yaa* is indeed involved in the activation of MZ B cells, one can speculate that *Yaa*-bearing MZ B cells directly activated by particular autoantigens may become efficient APCs, thereby promoting subsequent T cell-dependent autoimmune responses. In this regard, it is worth noting that a recent study has shown that a fraction of activated MZ B cells can migrate into B cell follicles, and participate in the germinal center formation in response to T-dependent Ags (39). The understanding of the mechanism responsible for the hyperreactive phenotype of *Yaa* B cells is of paramount importance for the elucidation of the molecular abnormality caused by the *Yaa* mutation. Ultimately, advances in our understanding of the nature of the *Yaa* defect should give important insights into the development of lupus-like systemic autoimmune disease.

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