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Evidence for Genes in Addition to *Tlr7* in the *Yaa* Translocation Linked with Acceleration of Systemic Lupus Erythematosus[1]

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The accelerated development of systemic lupus erythematosus (SLE) in male BXSB mice is associated with the genetic abnormality in its Y chromosome, designated *Yaa* (*Y-linked autoimmune acceleration*). Recently, the *Yaa* mutation was identified to be a translocation from the telomeric end of the X chromosome (containing the gene encoding TLR7) onto the Y chromosome. In the present study, we determined whether the *Tlr7* gene duplication is indeed responsible for the *Yaa*-mediated acceleration of SLE. Analysis of C57BL/6 mice congenic for the *Nba2* (*NZB autoimmunity 2*) locus (B6.*Nba2*) bearing the *Yaa* mutation revealed that introduction of the *Tlr7* null mutation on the X chromosome significantly reduced serum levels of IgG autoantibodies against DNA and ribonucleoproteins, as well as the incidence of lupus nephritis. However, the protection was not complete, because these mice still developed high titers of anti-chromatin autoantibodies and retroviral gp70-anti-gp70 immune complexes, and severe lupus nephritis, which was not the case in male B6.*Nba2* mice lacking the *Yaa* mutation. Moreover, we found that the *Tlr7* gene duplication contributed to the development of monocytosis, but not to the reduction of marginal zone B cells, which both are cellular abnormalities causally linked to the *Yaa* mutation. Our results indicate that the *Yaa*-mediated acceleration of SLE as well as various *Yaa*-linked cellular traits cannot be explained by the *Tlr7* gene duplication alone, and suggest additional contributions from other duplicated genes in the translocated X chromosome. ***The Journal of Immunology,*** **2008, 181: 1556–1562.**

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). Genetic analysis of the F_1 hybrids of male BXSB mice with other lupus-prone mice demonstrated that the accelerated development of SLE in male BXSB mice is linked to the Y chromosome of the BXSB strain. Thus, the genetic abnormality present in BXSB Y chromosome has been called *Yaa* (*Y-linked autoimmune acceleration*; Ref. 2). Studies of *Yaa* and non-*Yaa* double-bone marrow-chimeric mice have demonstrated that anti-DNA autoantibodies are selectively produced by B cells bearing the *Yaa* mutation, and that T cells from both *Yaa* and non-*Yaa* origin efficiently promote anti-DNA autoantibody responses (3, 4). These data indicate that the *Yaa* defect is functionally expressed in B cells, but not in T cells. The expression of the *Yaa* defect in B cells has been further supported by a loss of marginal zone (MZ) B cells in spleen, which results from a defect intrinsic to *Yaa*-bearing B cells, independently of the development of SLE (5). More recently, the *Yaa* mutation was shown to be a consequence of a translocation from the telomeric end of the X chromosome and onto the Y chromosome (6, 7). Based on the presence of the gene encoding TLR7 in this translocated segment of the X chromosome, the possible role of TLR7 in the activation of autoreactive B cells (8, 9) and the development of SLE (10, 11), the *Tlr7* gene duplication has been proposed to be the etiological basis for the *Yaa*-mediated enhancement of disease (6, 7, 12).

To determine whether the *Tlr7* gene duplication is indeed responsible for the *Yaa*-mediated acceleration of SLE, in the present study, we generated C57BL/6 (B6) mice congenic for the *Nba2 (NZB autoimmunity 2*) locus bearing the *Tlr7* null mutation on the X chromosome and carrying *Yaa* (i.e., expressing a single *Yaa* copy of the *Tlr7* gene, namely, B6.*Nba2 Tlr7*$^{-}$*/Yaa*). Male B6.*Nba2* mice bearing the *Yaa* mutation (B6.*Nba2 Tlr7*$^{+}$*/Yaa*), with both X chromosome *Tlr7* and *Yaa Tlr7* genes, develop a lethal form of SLE (13). We compared the development of lupus-like autoimmune syndrome (autoantibody production and glomerulonephritis) in B6.*Nba2*-congenic male mice of the *Tlr7*$^{-}$*/Yaa*, *Tlr7*$^{+}$*/Yaa* and non-*Yaa* (*Tlr7*$^{+}$*/y*) genotypes. We herein report that B6.*Nba2 Tlr7*$^{-}$*/Yaa* males developed less severe lupus nephritis than B6.*Nba2 Tlr7*$^{+}$*/Yaa* males, but more severe renal disease compared with non-*Yaa* B6.*Nba2* males. Our data demonstrate that the *Yaa*-mediated acceleration of SLE is not caused only by the *Tlr7* gene duplication, implying the involvement of additional gene(s) present in the translocated X chromosome in the *Yaa* defect.

Materials and Methods

Mice

The generation of B6.*Nba2 Tlr7*$^{+}$*/Yaa*-congenic mice homozygous for the NZB-derived *Nba2* lupus susceptibility locus (flanked by markers *D1Mit47* and *D1Mit461* in chromosome 1) and B6 *Tlr7*$^{+}$*/Yaa*-consomic mice have

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[4] Abbreviations used in this paper: SLE, systemic lupus erythematosus; MZ, marginal zone; B6, C57BL/6; RNP, ribonucleoproteins; gp70 IC, gp70-anti-gp70 immune complexes.

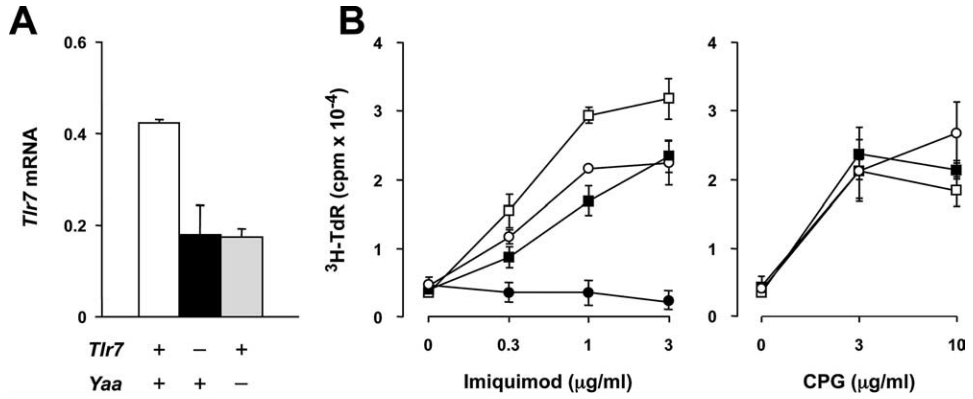

FIGURE 1. Expression levels of *Tlr7* in splenic B cells from 2-mo-old male *Tlr7*$^+$*/Yaa*, *Tlr7*$^-$*/Yaa*, and *Tlr7*$^+$*/y* B6 mice. *A*, Quantitative real-time RT-PCR analysis of *Tlr7* mRNA abundance in purified B cells from B6 *Tlr7*$^+$*/Yaa* (□), *Tlr7*$^-$*/Yaa* (■), and *Tlr7*$^+$*/y* (▨) mice. Levels of *Tlr7* mRNA (means of three mice ± SD) were quantified relative to a standard curve generated with serial dilutions of a reference cDNA preparation and normalized using TATA-binding protein mRNA. Note a comparable *Tlr7* expression in B cells from *Tlr7*$^-$*/Yaa* and *Tlr7*$^+$*/y* B6 mice, which contrasted to increased expression of *Tlr7* mRNA in B cells from male *Tlr7*$^+$*/Yaa* mice ($p < 0.001$). *B*, Proliferative responses of purified B cells from B6 *Tlr7*$^+$*/Yaa* (□), *Tlr7*$^-$*/Yaa* (■) and *Tlr7*$^+$/y (○) mice after stimulation with different concentrations of imiquimod (TLR7 agonist) or CpG (TLR9 agonist). Note significantly enhanced proliferative responses by *Tlr7*$^+$*/Yaa* B cells stimulated with 1 and 3 μg/ml of imiquimod, as compared with *Tlr7*$^-$*/Yaa* and *Tlr7*$^+$*/y* B cells ($p < 0.05$). The specificity of imiquimod as a TLR7 agonist was controlled by the lack of proliferative responses of *Tlr7*$^{-/-}$B cells (●). Results (means of three mice ± SD) are expressed as cpm of [^{3}H]TdR incorporation.

been described previously (13, 14). *Tlr7*$^{-/-}$ mice, generated by gene targeting in 129/Ola-derived ES cells (15), were backcrossed for seven generations on a B6 background, using marker-assisted selection, as described previously (16). The *Tlr7* genotype was determined by PCR analysis using wild-type-specific primer (5′-ACGTGATTGTGGCGGTCAGAGG ATAAC-3′), mutant-specific primer (5′-CCAGATACATCGCCTACC TACTAGACC-3′), and common primer (5′-ATCGCCTTCTATCGCCT TCTTGACGAG-3′). *Tlr7*$^-$*/Yaa* B6 and B6.*Nba2* mice were established by selective crossing of *Tlr7*$^{-/-}$ B6 females with B6.*Yaa* or male B6.*Nba2.Yaa* mice. Animal studies described in the present study have been approved by the Ethical Committee for Animal Experimentation of the Faculty of Medicine, University of Geneva. Blood samples were collected by orbital sinus puncture and sera were stored at −20°C until use.

Purification of splenic B cells and cell culture

B cells were purified from spleen by treatment with IgM anti-Thy-1.2 (AT-83) mAb in the presence of rabbit complement (Cedarlane). The purity of B cells, as documented by flow cytometric analysis, was superior to 90%. For proliferative responses of splenic B cells, 2×10^5 purified B cells were incubated with different concentrations of a TLR7 agonist imiquimod R837 (Invivogen) or a TLR9 agonist CpG oligonucleotide (type B CpG 1018; a gift of Dr. Eyal Raz, University of San Diego, San Diego, CA) in 0.2 ml of DMEM, 10% FCS at 37°C, and cultures were pulsed with [^{3}H]TdR for the final 16 h of a 3-day culture.

Quantitative RT-PCR

RNA from splenic B cells was purified with TRIzol reagents (Invitrogen). The abundance of *Tlr7* mRNA was quantified by real-time RT-PCR with cDNA prepared from RNA. *Tlr7* cDNA was amplified using a forward primer (5′-GTACCAAGAGGCTGCAGATTAGAC-3′) and reverse primer (5′-AGCCTCAAGGCTCAGAAGATG-3′). PCR was performed using the iCycler iQ Real-Time PCR Detection System (Bio-Rad) and iQ SYBR green Supermix (Bio-Rad). Results were quantified relative to a standard curve generated with serial dilutions of a reference cDNA preparation from spleen and normalized using TATA-binding protein mRNA.

Flow cytometric analysis

Flow cytometry was performed using two- or three-color staining of PBMC and spleen cells and were analyzed with a FACSCalibur (BD Biosciences). The following Abs were used: anti-CD21 (7G6), anti-CD23 (B3B4), anti-B220 (RA3-6B2), anti-CD69 (H1.2F3), anti-CD11b (M1/70), and anti-F4/80 mAb. Staining was performed in the presence of saturating concentration of 2.4G2 anti-FcγRII/III mAb. The mean percentage of CD11b$^+$ monocytes (±SD) among PBMC in 8-mo-old male B6 mice ($n = 12$) was 8.3 ± 2.2. Mice displaying percentages of monocytes >3 SD above the mean of B6 males (>14.9%) were considered as positive for monocytosis.

Serological assays

Serum levels of IgG autoantibodies against chromatin, dsDNA, and ribonuclear protein (RNP) were determined by ELISA. Chromatin, prepared from chick erythrocytes, and calf thymus RNP complexes (ImmunoVision) were directly coated to ELISA plates, whereas dsDNAs were coated to ELISA plates precoated with poly-L-lysine (Sigma-Aldrich). Then, the plates were incubated with 1/100 diluted serum samples, and the assay was developed with alkaline phosphatase-labeled goat anti-mouse IgG. Results are expressed in units per milliliter in reference to a standard curve derived from a serum pool of MRL-*Fas*^{lpr} mice. Serum levels of gp70-anti-gp70 immune complexes (gp70 IC) were quantified by ELISA combined with the treatment of sera with 10% polyethylene glycol (average molecular weight, 6000), which precipitates only Ab-bound gp70, but not free gp70, as described (17). Results are expressed as micrograms per milliliter of gp70 by referring to a standard curve obtained from a serum pool of NZB mice.

Histopathology

Kidney samples were collected when mice were moribund or at the end of the experiment at 14 mo of age. Histological sections were stained with periodic acid-Schiff reagent. The extent of glomerulonephritis was graded on a 0 to 4 scale based on the intensity and extent of histopathological changes, as described previously (14). Glomerulonephritis with grade 3 or 4 was considered a significant contributor to clinical disease and/or death.

Statistical analysis

Unpaired comparisons for *Tlr7* mRNA expression, B cell proliferative responses, and percentages of MZ B cells and CD69$^+$ B cells were analyzed by Student's *t* test. Analysis for serological parameters and the intensity of glomerular lesions were performed with the Mann-Whitney *U* test. The Kaplan-Meier log-rank test was used for the statistical analysis for mortality rates. Probability values >5% were considered insignificant.

Results

Expression of Tlr7 *in male B6 mice of the* Tlr7$^+$/Yaa, Tlr7$^-$/Yaa, *or* Tlr7$^+$/y *genotype*

Similar to previous studies (6, 7), real-time quantitative RT-PCR analysis confirmed a 2-fold increased abundance of *Tlr7* mRNA in B cells from male B6 *Tlr7*$^+$*/Yaa* mice, as compared with B cells from male B6 *Tlr7*$^+$*/y* non-*Yaa* mice (Fig. 1*A*). B cells from male B6 *Tlr7*$^-$*/Yaa* mice bearing the *Tlr7* null mutation on the X chromosome expressed *Tlr7* mRNA at levels comparable with those of male B6 *Tlr7*$^+$/y non-*Yaa* mice, consistent with having the same copy number of the *Tlr7* gene. An enhanced expression of *Tlr7* in

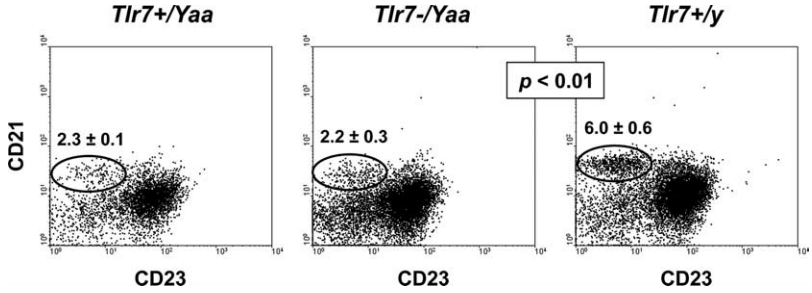

FIGURE 2. Comparable reduction of MZ B cells in male 2-mo-old *Tlr7*$^{+}$*/Yaa* and *Tlr7*$^{-}$*/Yaa* B6 mice. Spleen cells from *Tlr7*$^{+}$*/Yaa*, *Tlr7*$^{-}$*/Yaa* and *Tlr7*$^{+}$*/y* B6 mice were stained with a combination of anti-B220, anti-CD21, and anti-CD23 mAb. Representative staining profiles for CD21 and CD23 on B220^{+} B cells are shown. Percentages of CD21^{high}CD23$^{-/low}$ MZ B cells (mean of five mice ± SD) are indicated. Differences between *Yaa* and non-*Yaa* males were significant ($p < 0.01$).

B6 *Tlr7*$^{+}$*/Yaa* mice was reflected by increased proliferative responses of B cells in vitro after stimulation with a TLR7 agonist, imiquimod (Fig. 1*B*). However, such an augmented response was no longer observed with B cells from male B6 *Tlr7*$^{-}$*/Yaa* mice. Notably, proliferation of B cells in response to a TLR9 agonist, CpG oligonucleotides, was comparable among three different genotypes of male B6 mice (Fig. 1*B*).

No contribution of Tlr7 *gene duplication to loss of MZ B cells in male B6* Tlr7^{+}/Yaa *mice*

One of the unique cell abnormalities associated with the *Yaa* mutation is a loss of the CD21^{high}CD23$^{-/low}$ MZ B cell subset, which is independent of the development of accelerated autoimmune responses (5). To define whether the *Tlr7* gene duplication is responsible for this *Yaa*-linked B cell abnormality, we compared the size of the MZ B cell compartment in three different genotypes (*Tlr7*$^{-}$*/Yaa*, *Tlr7*$^{+}$*/Yaa* and *Tlr7*$^{+}$*/y*) of 2-mo-old male B6 mice. Our results, however, showed that the extent of the MZ B cell loss was essentially identical between male B6 *Tlr7*$^{-}$*/Yaa* and *Tlr7*$^{+}$*/Yaa* mice (Fig. 2). This indicated that the loss of MZ B cells occurring in B6 *Tlr7*$^{+}$*/Yaa* mice was unrelated to the *Tlr7* gene duplication, but rather to the duplication of one or several other genes present in the translocated part of the X chromosome.

Contribution of Tlr7 *gene duplication to enhanced production of autoantibodies against dsDNA and RNP, but not against chromatin and retroviral gp70 in male B6.*Nba2 Tlr7^{+}/Yaa *mice*

Using the B6.*Nba2* model of SLE (13), we determined whether the *Tlr7* gene duplication could account for enhanced production of different lupus autoantibodies in mice bearing the *Yaa* mutation. For this purpose, we established a colony of B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice bearing the *Tlr7* null mutation on the X chromosome by intercrossing *Tlr7*$^{-/-}$ B6 females and B6.*Nba2.Yaa* males. As shown in Fig. 3*A*, the *Tlr7* mutant gene in B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice is flanked by a ~6-Mb segment from the 129 X chromosome. In addition, analysis of chromosome 1 in B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice confirmed the absence of a distal 129-derived interval in the *Nba2*

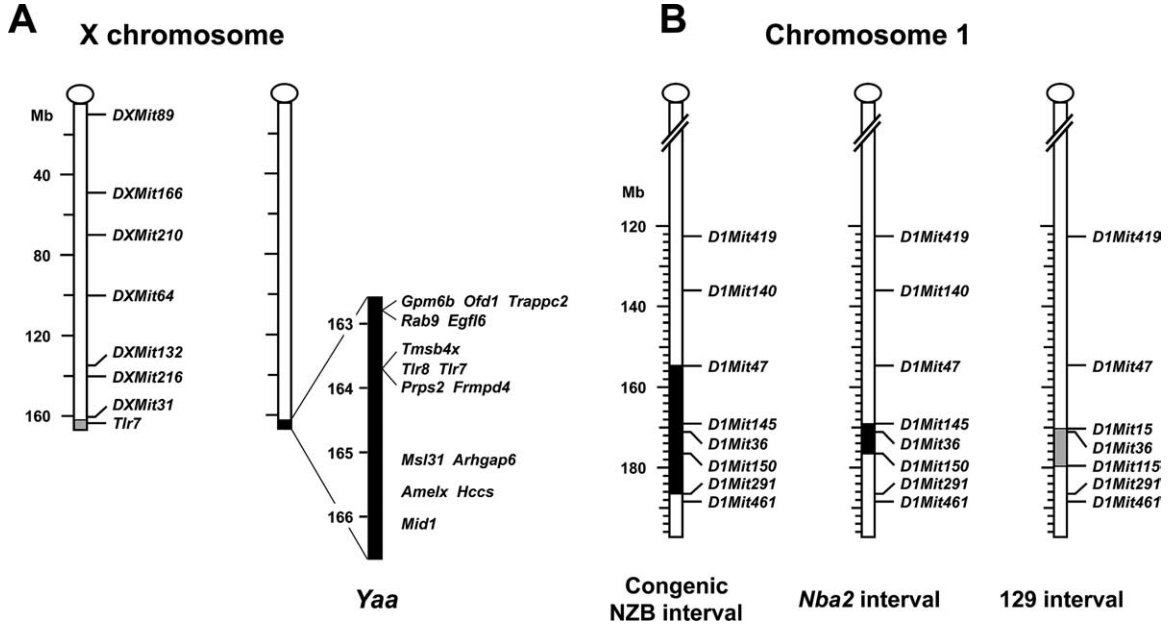

FIGURE 3. Genetic map of the X chromosome and the telomeric region of the chromosome 1 in B6.*Nba2 Tlr7*$^{-}$*/Yaa* male mice. (*A*) The segment of ~6 Mb telomeric end of the X chromosome derived from *Tlr7*$^{-/-}$ 129 mice (gray bar) was introduced into B6.*Nba2 Tlr7*$^{+}$*/Yaa* mice (*left*). *DXMit31* and *Tlr7* are located at 160.41 and 163.74 Mb, respectively, from the centromere, and the size of the X chromosome is 166.65 Mb, based on the Ensembl Genome Browser database (www.ensembl.org/Mus_musculus/index.html). The segment of the X chromosome translocated onto the Y chromosome in *Yaa* mice (■) (6, 7) and all the known genes present in this segment are indicated (*right*). *Gpm6b*, glycoprotein m6b; *Ofd1*, oral-facial-digital syndrome 1 gene homologue; *Trappc2*, trafficking protein particle complex 2; *Rab9*, RAB9, member RAS oncogene family; *Egfl6*, EGF-like-domain multiple 6; *Tmsb4x*, thymosin β4; *Prps2*, phosphoribosylpyrophosphate synthetase 2; *Frmpd4*, FERM and PDZ domain containing 4; *Msl31*, male-specific lethal-3 homolog 1; *Arhgap6*, Rho GTPase-activating protein 6; *Amelx*, amelogenin X chromosome; *Hccs*, holocytochrome c synthetase; *Mid1*, midline 1. *B*, The NZB chromosomal region (■) present in the B6.*Nba2* congenic strain, the *Nba2* interval (■; Ref. 36) and the 129 interval (▨) implicated in antinuclear autoantibody production and lupus nephritis (18) are shown. Note the absence of the 129-derived susceptibility interval in B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice. The positions of the microsatellite markers used to establish the genetic map are indicated.

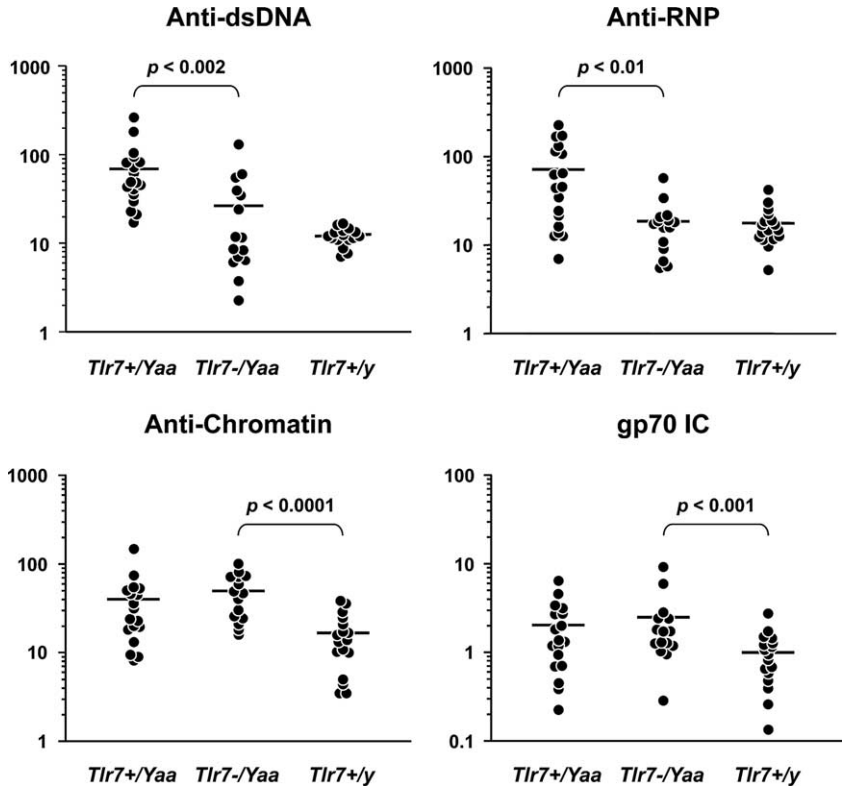

FIGURE 4. Serum levels of IgG anti-dsDNA, anti-chromatin and anti-RNP autoantibodies, and gp70 IC in male 5-mo-old *Tlr7*$^{+}$*/Yaa*, *Tlr7*$^{-}$*/Yaa*, and *Tlr7*$^{+}$*/y* B6.*Nba2* mice. Each symbol represents an individual animal (18 *Tlr7*$^{+}$*/Yaa*, 15 *Tlr7*$^{-}$*/Yaa*, and 17 *Tlr7*$^{+}$*/y* B6.*Nba2* mice). Results are expressed as units per milliliter for anti-dsDNA, anti-chromatin and anti-RNP autoantibodies, and as micrograms per milliliter for gp70 IC. The mean values are indicated by the horizontal line.

region (Fig. 3*B*), which has been implicated in susceptibility to anti-nuclear autoantibody production and lupus nephritis (18).

The spontaneous production of IgG anti-nuclear autoantibodies (anti-dsDNA, anti-chromatin, and anti-RNP) and of retroviral gp70 IC in B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice were compared with those from B6.*Nba2 Tlr7*$^{+}$*/Yaa* and *Tlr7*$^{+}$/y mice expressing two and one copies of the *Tlr7* gene, respectively. At 5 mo of age, serum levels of anti-dsDNA (mean ± SD, 28 ± 35 U/ml) and anti-RNP (19 ± 13 U/ml) autoantibodies in B6.*Nba2 Tlr7*$^{-}$*/Yaa* males were lower than those in B6.*Nba2 Tlr7*$^{+}$*/Yaa* males (anti-dsDNA, 70 ± 52 U/ml, $p < 0.002$; anti-RNP, 72 ± 67 U/ml, $p < 0.01$; Fig. 4). The levels of anti-dsDNA and anti-RNP autoantibodies in B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice were not significantly different from those in B6.*Nba2 Tlr7*$^{+}$*/y* non-*Yaa* males (anti-dsDNA, 12 ± 3 U/ml; anti-RNP, 18 ± 9 U/ml). In contrast, serum titers of autoantibodies against chromatin (47 ± 25 U/ml) and of gp70 IC (2.5 ± 1.8 μg/ml) in B6.*Nba2 Tlr7*$^{-}$*/Yaa* males were comparable with those in B6.*Nba2 Tlr7*$^{+}$*/Yaa* males (anti-chromatin, 39 ± 34 U/ml; gp70 IC, 2.1 ± 1.7 μg/ml) but higher than those in B6.*Nba2 Tlr7*$^{+}$*/y* non-*Yaa* males (anti-chromatin, 19 ± 8 U/ml, $p < 0.0001$; gp70 IC, 1.0 ± 0.6 μg/ml, $p < 0.001$; Fig. 4). These data indicated that the *Tlr7* gene duplication was responsible for enhanced autoimmune responses against dsDNA and RNP, but not against chromatin and retroviral gp70, occurring in B6.*Nba2 Tlr7*$^{+}$*/Yaa* mice.

Because we have previously shown that the percentage of B cells positive for the early activation marker CD69 was higher in 2 mo-old B6.*Nba2* mice compared with B6 mice (19), the expression of CD69 on splenic B cells was determined. The percentage of CD69^{+} B cells in B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice (mean of 5 mice ± SD, 6.6 ± 0.7%) were intermediate when compared with B6.*Nba2 Tlr7*$^{+}$*/Yaa* mice (10.6 ± 1.3%, $p < 0.01$) and B6.*Nba2 Tlr7*$^{+}$*/y* mice (4.7 ± 0.6%, $p < 0.05$; Fig. 5*A*). The number of splenic B cells in B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice (34.3 ± 8.2 × 10^{6}) appeared to be slightly reduced compared with B6.*Nba2.Tlr7*$^{+}$*/Yaa* mice

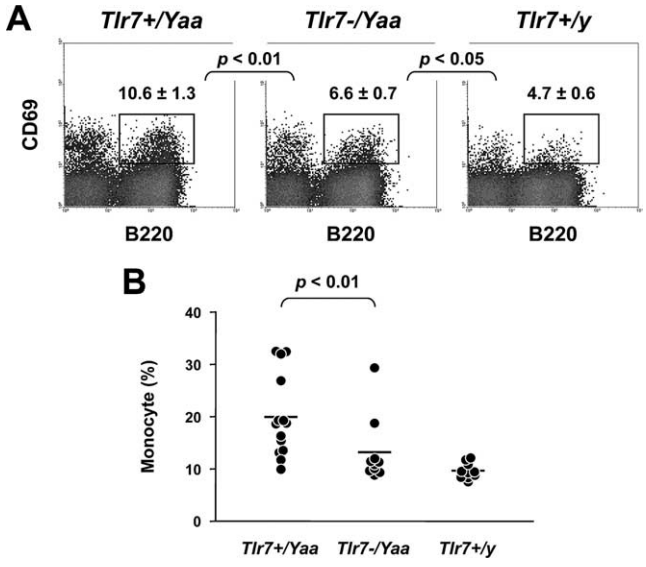

FIGURE 5. Lower incidence of CD69^{+} B cells and of monocytosis in male B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice compared with male B6.*Nba2 Tlr7*$^{+}$*/Yaa* mice. *A*, Spleen cells from 2-mo-old B6.*Nba2 Tlr7*$^{+}$*/Yaa*, *Tlr7*$^{-}$*/Yaa*, and *Tlr7*$^{+}$*/y* mice were stained with a combination of anti-B220 and anti-CD69 mAb. Percentages of CD69^{+} cells in the B220^{+} population (means of five mice ± SD), with gates based on an irrelevant isotype-matched control conjugate, are shown. Differences between B6.*Nba2 Tlr7*$^{+}$*/Yaa* and *Tlr7*$^{-}$*/Yaa* mice were significant ($p < 0.05$). *B*, PBMC from 8-mo-old B6.*Nba2 Tlr7*$^{+}$*/Yaa*, *Tlr7*$^{-}$*/Yaa* and *Tlr7*$^{+}$*/y* mice were stained with a combination of anti-CD11b and F4/80 mAb. Percentages of CD11b^{+}F4/80^{+} monocytes in PBMC were determined by flow cytometric analysis. Each symbol represents an individual animal (10–14 mice in each group), and mean values are indicated by horizontal lines. Differences between B6.*Nba2 Tlr7*$^{+}$*/Yaa* and *Tlr7*$^{-}$*/Yaa* males were significant ($p < 0.01$).

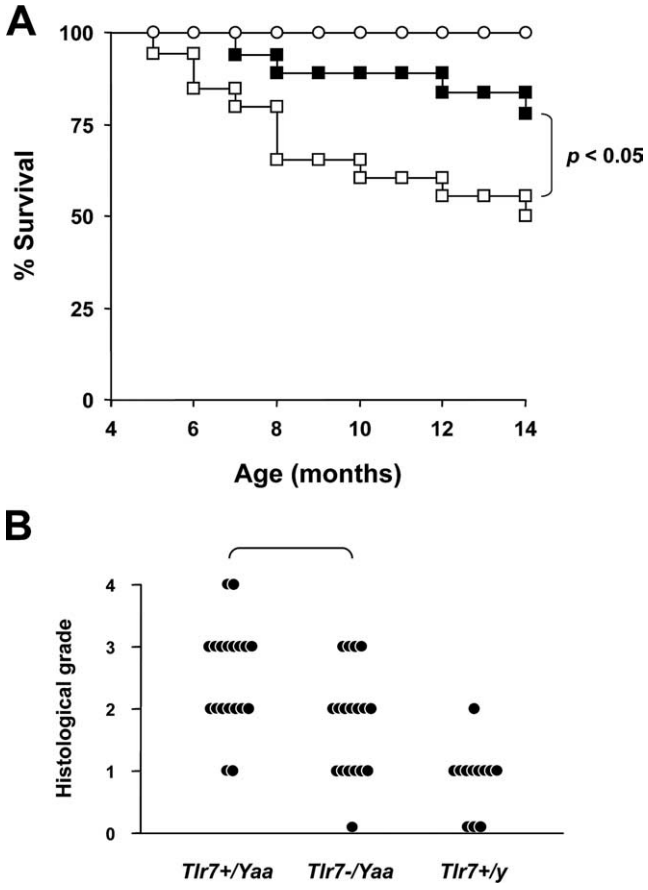

FIGURE 6. Cumulative rates of mortality due to glomerulonephritis in male *Tlr7*$^+$*/Yaa*, *Tlr7*$^-$*/Yaa* and male *Tlr7*$^+$*/y* B6.*Nba2* mice. *A*, 19 *Tlr7*$^+$*/Yaa* (□), 18 *Tlr7*$^-$*/Yaa* (■) and 12 *Tlr7*$^+$*/y* B6.*Nba2* (○) mice were followed for mortality due to glomerulonephritis. The difference in mortality between B6.*Nba2 Tlr7*$^+$*/Yaa* and *Tlr7*$^-$*/Yaa* mice was significant ($p < 0.05$). *B*, The intensity of glomerular lesions was scored on a 0 to 4 scale. Results from individual mice, sacrificed either moribund or at the end of a 14-mo observation period, are shown. The cumulative incidence of severe glomerulonephritis (grade ≥3) in B6.*Nba2 Tlr7*$^+$*/Yaa* mice was significantly increased, as compared with B6.*Nba2 Tlr7*$^-$*/Yaa* mice ($p < 0.05$), and the incidence in B6.*Nba2 Tlr7*$^-$*/Yaa* mice vs *Tlr7*$^+$*/y* mice was significantly different ($p < 0.005$).

($52.2 \pm 12.2 \times 10^6$), although the differences were not statistically different.

Contribution of Tlr7 *gene duplication to the development of monocytosis in male B6.*Nba2 Tlr7$^+$/Yaa *mice*

B6.*Nba2 Tlr7*$^+$*/Yaa* male mice develop monocytosis, a unique cellular abnormality associated with the *Yaa* mutation (20), in association with autoantibody production and subsequent development of lupus nephritis (21). Indeed, 10 of 14 (71%) of B6.*Nba2 Tlr7*$^-$*/Yaa* mice displayed significantly increased percentages of monocytes at 8 mo of age, compared with 2 of 10 (20%) of B6.*Nba2 Tlr7*$^-$*/Yaa* mice (Fig. 5*B*). The mean value obtained with B6.*Nba2 Tlr7*$^-$*/Yaa* mice (means ± SD: 13.2 ± 6.3%) was significantly lower than that of B6.*Nba2 Tlr7*$^-$*/Yaa* mice (19.9 ± 7.9%, $p < 0.01$) and not statistically different from that of B6.*Nba2 Tlr7*$^+$*/y* mice (9.7 ± 1.5%; Fig. 5*B*). Together, these data indicated that the development of monocytosis in B6.*Nba2 Tlr7*$^+$*/Yaa* mice was mostly dependent on the *Tlr7* gene duplication.

Partial contribution of Tlr7 *gene duplication to accelerated development of lupus nephritis in male B6.*Nba2 Tlr7$^+$/Yaa *mice*

Because male B6.*Nba2 Tlr7*$^+$/Yaa mice developed a lethal form of lupus nephritis with a 50% mortality rate at 14 mo of age (13), we followed the development of lupus nephritis in male B6.*Nba2 Tlr7*$^-$*/Yaa* mice and also in male B6.*Nba2 Tlr7*$^+$*/y* non-*Yaa* mice. Although the cumulative rate of mortality due to glomerulonephritis in B6.*Nba2 Tlr7*$^-$*/Yaa* male mice was significantly less than that of B6.*Nba2 Tlr7*$^+$*/Yaa* male mice ($p < 0.05$), approximately one-fourth of B6.*Nba2 Tlr7*$^-$*/Yaa* males died of lupus nephritis (grade ≥3) by 14 mo of age (Fig. 6). In contrast, none of the male B6.*Nba2 Tlr7*$^+$*/y* non-*Yaa* mice succumbed to lupus nephritis by 14 mo of age, and their glomerular lesions were limited as compared with those observed with male B6.*Nba2 Tlr7*$^-$*/Yaa* mice ($p < 0.005$). These data again indicated that the accelerated development of lupus nephritis in B6.*Nba2 Tlr7*$^+$*/Yaa* mice could not be totally explained by the *Tlr7* gene duplication alone.

Discussion

The present study was designed to define the contribution of the *Tlr7* gene duplication to *Yaa*-linked accelerated development of SLE, because the *Tlr7* gene duplication was proposed as a cause of the *Yaa* mutation, as a consequence of a translocation from the telomeric end of the X chromosome (which contains the *Tlr7* gene) onto the Y chromosome (6, 7). Our analysis of male B6.*Nba2* mice of three different genotypes (*Tlr7*$^+$*/Yaa*, *Tlr7*$^-$*/Yaa,* and *Tlr7*$^+$*/y*) demonstrated that the accelerated development of lupus-like autoimmune syndrome in male B6.*Nba2 Tlr7*$^+$*/Yaa* mice was partially but not completely prevented by the introduction of the *Tlr7* null mutation on the X chromosome. Our data thus indicate that additional gene(s) separate from *Tlr7* and present in the translocated X chromosome contribute to the *Yaa* abnormality.

Our analysis of the development of lupus-like disease demonstrated a significant contribution of the *Tlr7* gene duplication to *Yaa*-linked accelerated development of SLE, as documented by substantial decreases in anti-dsDNA and anti-RNP autoantibody titers and by a lower incidence of lupus nephritis in male B6.*Nba2 Tlr7*$^-$*/Yaa* mice. This is consistent with the recent results obtained with FcγRIIB$^{-/-}$ B6 *Tlr7*$^-$*/Yaa* mice and TLR7-overexpressing B6 mice (12). However, the contribution of the *Tlr7* gene duplication is partial, not complete. Indeed, serum levels of anti-chromatin autoantibodies and of retroviral gp70 IC in B6.*Nba2 Tlr7*$^-$*/Yaa* males were essentially identical with levels in B6.*Nba2 Tlr7*$^+$*/Yaa* males and much higher than those in B6.*Nba2 Tlr7*$^+$*/y* males. This is consistent with the finding that one-fourth of B6.*Nba2 Tlr7*$^-$*/Yaa* male mice still developed a lethal form of glomerulonephritis by 14 mo of age, as anti-chromatin and anti-gp70 autoantibodies have been implicated in the development of murine lupus nephritis (22, 23). It should be stressed that the expression level of *Tlr7* in B cells from male *Tlr7*$^-$*/Yaa* mice was comparable with that of male *Tlr7*$^+$*/y* mice, excluding possible dysregulated expression of the *Tlr7* gene translocated onto the Y chromosome. In addition, the absence of the 129-derived chromosome 1 interval implicated in antinuclear autoantibody production and lupus nephritis (18) excludes the possible genetic contribution of this 129-derived susceptibility gene(s) to the development of the residual lupus-like autoimmune syndrome in B6.*Nba2 Tlr7*$^-$*/Yaa* mice. Our B6.*Nba2 Tlr7*$^-$*/Yaa* mice still carry a ~6-Mb segment from 129 flanking the *Tlr7* mutant gene. Because the presence of lupus susceptibility genes on the X chromosome has not yet been analyzed, we cannot completely exclude a putative contribution of 129-derived gene(s) present in this segment to anti-chromatin and

anti-gp70 autoimmune responses in these mice. However, our preparatory analyses have shown that the production of gp70 IC was completely prevented in female B6.*Nba2 Tlr7*$^{-/-}$ mice (unpublished observations), thus arguing against this possibility at least for the anti-gp70 response.

Serological analysis of different autoantibodies characteristic of murine SLE in different *Tlr7* genotypes of B6.*Nba2* mice also defined the differential contribution of TLR7 on autoimmune responses against nuclear Ags. As suggested by the previous studies in *Yaa*-bearing B6 mice (6, 7), reduced levels of anti-RNP autoantibodies in B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice support the idea that TLR7 is implicated in the autoimmune response against RNA-related autoantigens (8–11). In our studies, serum levels of anti-dsDNA autoantibodies were also diminished in B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice, whereas anti-chromatin autoantibody production was not affected by the TLR7 deficiency. These data suggest that autoimmune responses against chromatin and dsDNA are likely to be controlled by different innate immune receptors. It is also significant that serum levels of gp70 IC were not different between *Tlr7*$^{+}$*/Yaa* and *Tlr7*$^{-}$*/Yaa* B6.*Nba2* mice. Because retroviral gp70 is a gene product of endogenous retroviruses, we expected that this autoimmune response would be mediated through TLR7.

The present results cannot rule out the role of TLR7 in the development of anti-chromatin and anti-gp70 autoimmune responses because of the expression of a single copy of the *Tlr7* gene in B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice. Importantly, these studies also do not exclude a contribution from the *Tlr8* gene, located next to *Tlr7*, to the autoantibody production and disease, because murine TLR8 was recently shown to be activated by a combination of a TLR7/8 agonist (imidazoquiniline) and oligonucleotides (24), although it is inactive in response to any known human TLR7/8 agonist alone (15, 25, 26). Further analysis in lupus-prone non-*Yaa* mice deficient in TLR7 or TLR8 would help elucidate the precise role of these innate immune receptors in the development of autoimmune responses and lupus-like disease.

A unique cellular abnormality associated with *Yaa*-mediated lupus-like autoimmune disease is monocytosis (20). We recently demonstrated that monocytosis is strongly associated with autoantibody production and subsequent development of lupus nephritis and that an NZB-derived susceptibility locus overlapping with *Nba2* promoted monocytosis (21). In the current studies, the development of monocytosis was almost completely suppressed in B6.*Nba2 Tlr7*$^{-}$*/Yaa* mice, indicating that the *Tlr7* gene duplication is critically involved in the *Yaa*-associated monocytosis. In addition, other ongoing studies have shown that the development of monocytosis was strongly suppressed in BXSB mice deficient in activating FcγRs, despite a high-level production of autoantibodies (unpublished data). This is consistent with the idea that *Yaa*-mediated monocytosis may result from an excessive production of monocyte-specific growth factor(s) through macrophages activated by FcγRs in response to IgG IC (27). Thus, it is possible that IgG IC, containing endogenous nuclear Ags and internalized through FcγRs, could excessively activate *Yaa*-bearing macrophages through subsequent interaction with TLR7, which is expressed at increased levels in endosomes of these mice.

A final point that merits emphasis is that the *Tlr7* gene duplication is not responsible for a selective loss of MZ B cells in the spleen occurring in *Yaa*-bearing mice (5). This is not totally surprising because it has never been reported that TLR7 is implicated in the development and maturation of MZ B cells. Although the precise molecular basis of the loss of MZ B cells in mice bearing the *Yaa* mutation is still unknown, our preliminary studies revealed that the MZ B cell loss was almost completely abrogated in B6 mice deficient in the Flt3 ligand, in which the development of dendritic cells was markedly compromised (28). In view of a hyperreactive phenotype of *Yaa*-bearing B cells, as judged by a markedly increased spontaneous IgM secretion associated with an accumulation of plasma cells in the red pulp (5, 29), the loss of MZ B cells in *Yaa* mice may result from their excessive activation by environmental Ags and apoptotic cells presented by dendritic cells in the MZ (30–32). Because activated MZ B cells can migrate into the red pulp and B cell follicles (30, 33–35), it is possible that the continuous activation of *Yaa*-expressing MZ B cells may lead to its depletion in the MZ compartment. If so, the *Yaa*-dependent hyperactive phenotype of B cells must be determined by gene(s) other than *Tlr7* present in the translocated X chromosome, and these gene(s) may contribute to the increased production of autoantibodies. A recent study claimed no significant loss of MZ B cells in male B6 *Tlr7*$^{-}$*/Yaa* mice (12). The difference in results remains unexplained at this time.

In conclusion, our current results suggest that the *Yaa*-mediated acceleration of SLE includes contributions from the duplication of one or several genes, in addition to *Tlr7*, present in the translocated part of the X chromosome. An interesting candidate is the *Tlr8* gene, which might be implicated in anti-chromatin and anti-gp70 autoimmune responses. In addition, another gene responsible for the possible activation of MZ B cells and hence the hyperreactive phenotype of *Yaa*-bearing B cells could additionally contribute to the enhancement of overall autoimmune responses. Clearly, further studies to identify the molecular basis of the *Yaa* mutation will help to determine the target molecules central to the development of SLE, thereby facilitating the design of novel therapeutic strategies in human SLE.

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Disclosures

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