The Yaa Locus and IFN-α Fine-Tune Germinal Center B Cell Selection in Murine Systemic Lupus Erythematosus

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The Yaa Locus and IFN-α Fine-Tune Germinal Center B Cell Selection in Murine Systemic Lupus Erythematosus

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Male NZW/BXS B Yaa (W/B) mice express two copies of TLR7 and develop pathogenic autoantibodies, whereas females with only one copy of TLR7 have attenuated disease. Our goal was to analyze the regulation of the autoantibody response in male and female W/B mice bearing the autoreactive site-directed H chain transgene 3H9. Serum anti-dsDNA Abs appeared in males at 12 wk, and most had high-titer IgG anti-dsDNA and anti-cardiolipin Abs and developed >300 mg/dl proteinuria by 8 mo. Females had only low-titer IgG anti-cardiolipin Abs, and none developed proteinuria by 1 y. Males had a smaller marginal zone than females with a repertoire that was distinct from the follicular repertoire, indicating that the loss of marginal zone B cells was not due to diversion to the follicular compartment. Vk5-43 and Vk5-48, which were rare in the naive repertoire, were markedly overrepresented in the germinal center repertoire of both males and females, but the VJ junctions differed between males and females with higher-affinity autoreactive B cells being selected into the germinal centers of males. Administration of IFN-α to females induced anti-cardiolipin and anti-dsDNA autoantibodies and proteinuria and was associated with a male pattern of junctional diversity in Vk5-43 and Vk5-48. Our studies are consistent with the hypothesis that presence of the Yaa locus, which includes an extra copy of Tlr7, or administration of exogenous IFN-α relaxes the stringency for selection in the germinal centers resulting in increased autoreactivity of the Ag-driven B cell repertoire. The Journal of Immunology, 2012, 189: 4305–4312.

B cells and APCs express intracellular nucleic acid-sensing TLRs that may be accessed by self-antigens when apoptotic debris or immune complexes are internalized by B cell receptors or Fc receptors, respectively. (1). Ligation of these TLRs by nucleic acids results in rapid cell activation and release of inflammatory mediators including type I IFNs. Genome-wide association studies of systemic lupus erythematosus (SLE) in humans have highlighted the importance of the TLR and type I IFN signaling pathways in the genetic risk for SLE (2). TLR7 is an innate sensor of ssRNA, helping to protect against RNA viruses; it is also required for the expression of anti-RNA autoantibodies in lupus models (3). Two-fold TLR7 overexpression induces SLE in susceptible mouse strains (4, 5), and 4- to 8-fold TLR7 overexpression induces spontaneous disease even in non-autoimmune C57BL/6 mice (6).

Male BXS B mice bearing the Yaa locus have a duplication of part of the X chromosome that includes the Tlr7 gene onto the Y chromosome (4, 5) and therefore have a 2-fold increase in TLR7 expression. Although there are at least 16 genes in the Yaa locus, recent studies suggest that the Tlr7 duplication is the dominant genetic contributor to the Yaa phenotype (6–8) including the production of Abs to dsDNA, organomegaly, and the development of severe SLE nephritis. More recent studies show that 4- to 8-fold overexpression of TLR7 is sufficient to induce spontaneous onset of SLE even in non-autoimmune strains (6). NZW/BXS B F1 (W/B) male mice bearing the Yaa locus spontaneously develop high titer anti-cardiolipin and anti-Sm/RNP Abs that are associated with both anti-phospholipid syndrome and renal failure, whereas females that express only one copy of Tlr7 develop a much later and milder disease (9). When we administered a small dose of IFN-α-expressing adenovirus (Ad) to female W/B mice, they developed high-titer anti-cardiolipin and anti-Sm/RNP IgG autoantibodies within 6 wk followed by the onset of nephritis and early mortality (10).

To analyze the mechanisms for dysregulation of the autoantibody response in TLR7-overexpressing W/B males and for the loss of B cell tolerance after exogenous IFN-α administration in females, we generated W/B mice bearing the site-directed anti-cardiolipin/DNA autoantibody V3H3 transgene 3H9 (11). V3H3H9 is an H chain isolated from an anti-DNA Abs spontaneously produced in MRL/lpr mice; it pairs with a wide variety of V L chains to generate DNA and non-DNA binding Abs as well as low-affinity anti-cardiolipin Abs (12). Previous elegant studies by the Weigert laboratory have shown that both genetic background and strength of BCR signaling influence the stringency of selection of 3H9 B cells (13, 14). We found that loss of tolerance to cardiolipin (CL) and DNA is broken in 3H9 H/B mice as they age but occurs much earlier in males than in females. Analysis of the naïve L chain repertoire associated with the 3H9 transgene suggests an increase in stringency of negative selection of naïve B cells in males resulting in depletion of marginal zone (MZ) B cells. In contrast, B cell...
for IgM and IgG isotype as previously described (9). Thirty-two autoreactive IgG hybridomas randomly selected from three male and one female fusion were successfully subcloned by limiting dilution, and the H and L chains were subjected to PCR and sequenced as above.

Ig expression studies

The 3H9 H chain was cloned into the Nhel and EcoRI restriction sites of mouse IgG2a expression vector (pFUSE-CHIg-mG2a; Invivogen, San Diego, CA) using the manufacturer’s instructions. Germline-encoded L chain variable regions were synthesized (Genscript, New Piscataway, NJ) or generated by PCR from purified FO or MZ B cells and were cloned into the BstAPI and BseEII restriction sites of mouse k expression vector (pFUSE2-CLig-mk; Invivogen). H and L chain combinations were cotransfected into 293T cells using Lyovek kit (Invivogen) according to the manufacturer’s instructions, and supernatants were harvested after 48 h. Supernatants were normalized to 1 μg/ml and tested for binding to CL (9), dsDNA (21), ssDNA, histones (10 μg/ml), and chromatin (22) and to phosphatidyl serine, phosphatidyl choline, insulin, chymotrypsin A, cytochrome c, and keyhole limpet hemocyanin all plated at 10 μg/ml.

Because it has been reported that the 3H9 Ab does not bind to dsDNA when purified away from associated nuclear material that may contaminate the cell supernatants (23), supernatants were diluted 3-fold in 0.01 M Tris pH 7.8 and incubated with DNase I (1 μg/ml; Worthington Biochemical, Lakewood, NJ) for 90 min at 37°C in the presence of 2 mM MgCl2. After DNase treatment, the Ab was diluted 4-fold in PBS and passed through a protein A column. Before elution, the column was washed extensively with 1 M NaCl in PBS) to disrupt immune complexes by dissociating histones associated with DNA. Bound Ab was eluted with Na citrate pH 4, immediately neutralized, and dialyzed against PBS. Eluates were normalized to an IgG2a concentration of 2 μg/ml by ELISA and tested in serial dilutions for reactivity to CL, dsDNA, ssDNA, histones, and chromatin as described earlier. Purified hybridoma Abs were similarly subjected to DNase treatment and purification prior to testing for antigenic specificity.

Statistics

Comparisons shown in Figs. 1 and 3 were performed using Wilcoxon rank sum test. Proteinuria and survival data shown in Fig. 2 were analyzed using Kaplan–Meier curves and log rank test. Comparisons in Fig. 5 were performed using χ2 analysis. Only significant p values are shown. Statistical analysis of the Vκ repertoire data were performed as previously described (19).

Results

Clinical phenotype of 3H9 W/B mice

Male mice developed high-titer IgG autoantibodies to both CL and DNA by 6 mo of age (Fig. 1A, 1C), whereas females expressed significantly lower titers of autoantibodies than males until 1 y of age (Fig. 1B, 1D). Significantly increased titers of anti-CL and anti-DNA autoantibodies were induced in Ad–IFN-α–treated mice compared with age-matched untreated or Ad-LacZ controls (Fig. 1B, 1D). Male mice developed proteinuria starting as young as 16 wk of age, whereas females remained proteinuria free until sacrificed at the age of 56 wk (Fig. 2). Proteinuria in the males persisted for many weeks and in some cases was relapsing and remitting. Survival rate for the males was 64% at 1 y. As we have reported for wild-type W/B females, a small dose of Ad–IFN-α, but not of control virus Ad-LacZ, induced proteinuria in female 3H9 W/B mice within 6–8 wk; these mice had a high mortality rate (Fig. 2).

B cell phenotypic of 3H9 W/B mice

Flow cytometric analysis of cell subsets was performed on spleens of 8 wk, 22–28 wk, and 56 wk male and female mice (Fig. 3). As often observed in Ig transgenic mice, there was an increase in the percentage of MZ B cells in the 3H9 mice; males, however, consistently had a lower percentage of MZ B cells than female mice (9.9 ± 1.5 versus 16.1 ± 3.5% and 9.1 ± 6.7 versus 16.7 ± 3.4% at 4 wk and 56 wk, respectively; Fig. 3A), and several male mice had lost their MZ B cells by 56 wk of age. By the age of 22 wk, male spleens were three to four times larger than female
Of these 80 genes, 32 contributed to the repertoire associated with use of the V<sub>k</sub>5-43/5-45 family, particularly V<sub>k</sub>5-43/5-45 (which differ by only two amino acids, one in FR1 and one in CDR1) and V<sub>k</sub>5-48 (Fig. 4). Subsequent PCR and sequencing of the full-length L chains from GC B cells showed that all of the V<sub>k</sub>5-43/5-45 encoded L chains used V<sub>k</sub>5-43. V<sub>k</sub>5 encoded L chains were rare in the naive B cell compartment of both males and females, indicating strong positive selection/clone expansion of B cells expressing these L chains in the GC. Because V<sub>k</sub>5-43, V<sub>k</sub>5-45, and V<sub>k</sub>5-48 were also represented among autoreactive hybridomas, we examined the J<sub>k</sub> regions of GC B cells and hybridomas using these L chains; the J<sub>k</sub> contributes variability to the L chain CDR3. For V<sub>k</sub>5-43, we found that males predominantly used J<sub>k</sub>5, whereas females used J<sub>k</sub>4 and J<sub>k</sub>2. Importantly, 13 of 14 autoreactive V<sub>k</sub>5-43 encoded hybridomas as well as the single V<sub>k</sub>5-45 encoded autoreactive hybridoma used the “male” J<sub>k</sub>5 with the germline Leu at the CDR3/J<sub>k</sub> junction as in the GCs (Fig. 5A). For V<sub>k</sub>5-48, males used either J<sub>k</sub>2 or J<sub>k</sub>4 whereas females used J<sub>k</sub>5 almost exclusively. The few autoreactive V<sub>k</sub>5-48 hybridomas generated from males used either J<sub>k</sub>2 or J<sub>k</sub>4, whereas those from females used J<sub>k</sub>5 (Fig. 5B).

The L chain repertoire of GC B cells from IFN-treated females was more diverse than that of untreated females but still included V<sub>k</sub>5 family members. Importantly, most GC-derived V<sub>k</sub>5-43 encoded genes from IFN-treated females used the “male” J<sub>k</sub>5 and GC-derived V<sub>k</sub>5-48 genes predominantly used the “male” J<sub>k</sub>2 and J<sub>k</sub>4 (Fig. 5). Thus, the administration of IFN-α to females results in altered GC selection.

**Autospecificity of reconstituted Abs**

To analyze the DNA binding characteristics of the germline encoded 3H9 encoded Abs that were being selected into the various B cell subsets and into the GCs, we cotransfected the 3H9 H chain using an IgG2a Fc together with germline encoded L chains of interest and the most commonly associated J<sub>k</sub> into 293T cells. Twelve L chains from the 32 displayed in Fig. 4 and one additional L chain represented among hybridomas from males were chosen for the transfection experiments. For some L chains, including V<sub>k</sub>5-43, V<sub>k</sub>5-45, and V<sub>k</sub>5-48, more than one J<sub>k</sub> region
was used. On screening, 7 of the 13 L chains (Vk1-110, 4-57, 5-43, 5-45, 5-48, 10-94, 12-46) conferred autoreactivity in the germline configuration, with variable binding to the three autoantigens; Vk1-117, 3-4, 3-12, 4-55, 13-85, 16-104 displayed no reactivity with any of the Ags tested. The autoreactive transfecteds were then subjected to treatment with DNAse and purification on protein A using high salt to dissociate immune complexes.

Several of the autoreactive L chains were of particular interest (Fig. 6). Vk12-46*01/Jk2, which was found frequently in the T2-MZ precursor and MZ compartments but was negatively selected in the FO compartment and was only rarely found in the GCs, conferred weak binding to chromatin and histones. Vk4-57*01, which reacted only with CL, was found in the GCs of females but not of males. Vk1-110*01, which was strongly reactive to both DNA and CL, was found rarely in the mature repertoire and in GCs but was captured among hybridomas derived from males. Only one of the transfecteds, 3H9/Vk10-94, displayed polyreactivity (data not shown).

Strikingly, for Abs using Vk5 encoded L chains, we found that the antigenic specificity was highly dependent on the Jk used. For Vk5-43, Jk5, the most common Jk in males, conferred low-titer anti-CL activity and anti-histone activity and high-titer anti-chromatin activity but not anti-DNA activity, whereas the “female” Jk2 and Jk4 did not bind histone and conferred lower titers of anti-CL and anti-chromatin specificity (Fig. 7A). Because most of the Vk5-43 encoded hybridomas were DNA binding, acquisition of this autoreactive specificity as well as of high-titer anti-CL activity can therefore be attributed to somatic mutation (Supplemental Table III). For Vk5-48, the highest-titer autoreactivity to chromatin was conferred by the “male” Jk4 with weak autoreactivity to dsDNA conferred by the “male” Jk2 and only minimal autoreactivity conferred by the “female” Jk5 (Fig. 7B). Vk5-45, which was selected only rarely, was highly autoreactive with dsDNA and chromatin regardless of the Jk, but had the highest binding to CL when associated with Jk5 (Fig. 7C). The one autoreactive hybridoma encoded by this L chain had only a single somatic mutation in the H chain (Supplemental Table III). Thus, the male mice have a defect in tolerance in the GC that allows both entry and expansion of autoreactive clones that recognize dsDNA, chromatin, and CL. Survival of clones that acquire autoreactivity as a result of somatic mutation is also dysregulated in the males. Tolerance to DNA at both these checkpoints is maintained in females <1 y of age, but eventually females lose tolerance as a result of failure to regulate B cells that have acquired autoreactivity as a result of somatic mutation. Finally, tolerance at the GC entry checkpoint can be broken in females by the administration of IFN-α.

Of the non-autoreactive L chains, Vk3-4*01 and Vk3-12-01/Jk2 were found in the GCs, particularly in IFN-α–treated females, but they were not as frequent as Vk5-43 encoded L chains and were not represented among the hybridoma panel.

Hybridomas

Hybridomas were generated from seven males (>8 mo of age) and four females (>12 mo of age). Approximately two times as many hybridomas were generated per fusion from males as from females (p < 0.02), and the percentage of autoreactive hybridomas per fusion was higher in males than in females (12.4 ± 4.8 versus 6.9 ± 3.2%; p = 0.07). Forty-two and 187 stable autoreactive hybridomas were generated from female and male mice, respectively. Twenty-one percent of the hybridomas bound only dsDNA, 10% bound only CL, and 69% bound to both Ags; this distribution was similar in hybridomas from males and females, however IgG hybridomas were more commonly found in males (72 versus 30% p < 0.0001). Thirty-two anti-dsDNA and/or anti-CL IgG randomly selected hybridomas from three different male fusions and one aged female fusion were subcloned and the H and L chain sequences obtained using PCR. Six of these used VH1 genes other
than the 3H9 encoded VH1-82, and three used the 3H9 H chain but expressed more than one L chain after multiple subcloning attempts. As observed in GCs, the remaining 23 hybridomas had a highly restricted repertoire with predominant use of V\kappa L chains, consistent with that of the GCs. Mutation analysis revealed that both the H and L chains of the GC B cells and the hybridomas were highly mutated, accounting for the diverse binding characteristics of hybridomas using the same H and L chain genes (Supplemental Table III). After treatment with DNAse and purification under high salt conditions, antigenic specificity was retained, and binding characteristics of hybridomas using a wide variety of L chains are detected among spontaneous autoantibodies earlier and in higher titer than females but that pathogenic anti-dsDNA Abs are permissive for autoreactivity to DNA or CL (12). Tolerance in non-autoimmune 3H9 mice is maintained by receptor editing of both the H (24) and L chains to yield a less autoreactive naive repertoire (12, 25), by follicular exclusion of autoreactive B cells (26), and by negative selection of autoreactive B cells in the GCs (27). In contrast, pathogenic anti-dsDNA Abs using a wide variety of L chains are detected among spontaneous IgG hybridomas from 3H9 transgenic lupus-prone mice (13).

In non-autoimmune C57BL/6 site-directed transgenic 3H9 mice, in which class switching and somatic mutations can occur, V\kappa12-46 rearranged to J\kappa2 is the dominant L chain used by LPS-induced hybridomas (28). One of the problems with using LPS induction to study the naive L chain repertoire is that hybridomas may preferentially reflect the repertoire of MZ B cells that are more rapidly activated by 3 d of treatment with LPS than are follicular B cells in the GCs (27). In contrast, pathogenic anti-dsDNA Abs using a wide variety of L chains are detected among spontaneous IgG hybridomas from 3H9 transgenic lupus-prone mice (13).

### Discussion

The purpose of these experiments was to analyze mechanisms for loss of tolerance to CL and DNA in NZW/BXSB mice that develop anti-phospholipid Abs and coronary artery thromboses and nephritis as part of their disease phenotype. We show here that male 3H9 NZW/BXSB mice that carry the Yaa locus develop autoantibodies earlier and in higher titer than females but that females can be induced to produce these Abs by a low dose of Ad secreting IFN-\alpha.

The V\kappa3H9 H chain used in these studies was isolated from an anti-DNA Ab spontaneously produced in MRL/lpr mice; the H chain is a member of the J778 family and the accompanying L chain is from the V\kappa4 gene family (V\kappa4-81*01). 3H9 can also pair with a wide variety of V L chains; ∼60% of L chains that associate with 3H9 are permissive for autoreactivity to DNA or CL. Tolerance in non-autoimmune 3H9 mice is maintained by receptor editing of both the H (24) and L chains to yield a less autoreactive naive repertoire (12, 25), by follicular exclusion of autoreactive B cells (26), and by negative selection of autoreactive B cells in the GCs (27). In contrast, pathogenic anti-dsDNA Abs using a wide variety of L chains are detected among spontaneous IgG hybridomas from 3H9 transgenic lupus-prone mice (13).
C57BL/6. A similar skewing of the L chain repertoire away from V\(\kappa\)19-93 has been reported in 3H9 C57BL/6 mice that are transgenic for BAFF (30).

Despite the enlarged MZ in males and females and the relative loss of the MZ in males with age, the MZ-dominant L chain V\(\kappa\)12-46/J\(\kappa\)2, which confers weak chromatin binding, was not significantly represented in follicles or in the GCs or among hybridomas from male NZW/BXSB mice. These data are consistent with previous studies suggesting that presence of the Yaa locus causes an intrinsic developmental defect in MZ B cell development (31).

Furthermore our findings conclusively show that autoreactive effector cells that are generated in the GCs of male mice as a result of the Yaa translocation, or are captured as hybridomas, are not derived from the major subpopulation of autoreactive B cells in the MZ.

We further show that the loss of tolerance in NZW/BXSB males is due predominantly to GC expansion of B cells bearing V\(\kappa\)5-encoded L chains. In non-autoimmune mice, germline encoded autoreactive B cells are usually excluded from participating in the GC reaction (32) or are regulated within the GC before they clonally expand (33). B cells that newly acquire self-reactivity within the GC are removed from the effector repertoire by engagement with soluble self-antigen, by failure to obtain cognate help from T cells, by other unidentified checkpoints within the GC, or by post-GC receptor editing (34, 35).

We show here that a significant proportion of 3H9/V\(\kappa\)5 encoded B cells is autoreactive in the germline configuration; these cells are uncommon in the naive B cell repertoire of both males and females. They are, however, selected in the GC with exquisite specificity at the level of the V region and the V\(\kappa\)-J\(\kappa\) junction such that higher-affinity autoreactive B cells are positively selected in males, but only low-affinity autoreactive B cells are selected in females. Thus, abnormal GC entry and expansion of rare high-affinity germline encoded autoreactive B cells is conferred by the presence of the Yaa locus and/or the consequent activated state of lymphocytes. In the case of the male W/B mouse, this tolerance defect occurs spontaneously but, as we show here, a similar loss of tolerance in females with a shift in germinal selection from the V\(\kappa\)5 encoded “female” pattern of V\(\kappa\)-J\(\kappa\) genes to the “male” pattern of V\(\kappa\)5-43/J\(\kappa\)5 and V\(\kappa\)5-48/J\(\kappa\)4 can result from the extrinsic administration of IFN-\(\alpha\), which has multiple effects on innate and adaptive immune responses including GC responses.

**Figure 4.** Repertoire analysis of 3H9-associated V\(\kappa\)-chains in aged male (A) and female (B) NZW/BXSB mice. Genes that contribute >5% of the \(\chi^2\) value and constitute >2.5% of the repertoire in any of the comparisons are shown. Thirty-nine to 93 sequences from three to six mice were analyzed per subset. The y axis shows percentage of the total repertoire for each subset. The complete data set is shown in Supplemental Tables I and II.

**Figure 5.** Analysis of the J\(\kappa\) regions associated with (A) V\(\kappa\)5-43*01 and (B) V\(\kappa\)5-48*01 L chains in GC B cells and hybridomas of male and female 3H9 mice. The amino acid present at the V-J junction (position 116) for each J\(\kappa\) is shown on the x axis. J\(\kappa\) usage of GC B cells from males and IFN-treated females or of autoreactive hybridomas was compared with that of female GC B cells: *\(p<0.0001\), †\(p<0.002\) (compared with females), NS (compared with males). (C) Amino acid sequences of the CDR3 region of V\(\kappa\)5-43*01 and each of the relevant J\(\kappa\) regions.

**Figure 6.** Binding characteristics of reconstituted germline encoded H and non-V\(\kappa\)5 L chain pairs compared with five representative autoreactive hybridomas (see Supplemental Table III). Reconstituted Abs were adjusted to a concentration of 2 \(\mu\)g/ml and hybridomas to 1 \(\mu\)g/ml and were tested for binding to the Ags shown by ELISA. The L chain V\(\kappa\) genes associated with the 3H9 H chain in each H/L pair are shown on the x axis.
(36–42). In addition, the Vα5 encoded Abs found in the GCs and among hybridomas have accumulated somatic mutations that can be associated with acquisition of DNA binding and an increase in affinity for other autoantigens. Thus, the Yaa translocation is also associated with early failure of regulation of high-affinity somatically mutated autoantibody-producing B cells. This defect also eventually occurs in aged females.

Most of the SLE phenotype of the Yaa locus is associated with 2-fold overexpression of the Trl7 gene (7, 8) and is conferred by the expression of Yaa in B cells (43). Exogenous administration of IFN-α can also enhance expression of TRL7 in B cells, and this is required for initiation of SLE by pristane in non-autoimmune mouse strains (44). Our data in sum show that both expression of the Yaa locus and exogenous administration of IFN-α result in failure of exclusion of high-affinity autoreactive B cells from the GC, with subsequent clonal expansion of these cells, and failure to regulate autoreactive cells generated as a consequence of somatic mutation. Our data suggest that inhibition of IFN-α signals or of TLR7 signaling in B cells could regulate selection of the Ag-activated B cell repertoire and prevent the generation of high-affinity pathogenic autoantibodies. This hypothesis can be tested in the context of human clinical trials.

Disclosures

The authors have no financial conflicts of interest.

References


