Colocalization of Expansion of the Splenic Marginal Zone Population with Abnormal B Cell Activation and Autoantibody Production in B6 Mice with an Introgressed New Zealand Black Chromosome 13 Interval

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Colocalization of Expansion of the Splenic Marginal Zone Population with Abnormal B Cell Activation and Autoantibody Production in B6 Mice with an Introgressed New Zealand Black Chromosome 13 Interval

Joan E. Wither,*†Christina Loh,*† Ginette Lajoie,§ Svinda Heinrichs,* Yong-Chun Cai,* Gabriel Bonventi,* and Ralph MacLeod*

Polyclonal B cell activation is a prominent feature of the lupus-prone New Zealand Black (NZB) mouse strain. We have previously demonstrated linkage between a region on NZB chromosome 13 and increased costimulatory molecule expression on B cells. In this study we have produced C57BL/6 congenic mice with an introgressed homozygous NZB interval extending from ∼24 to 73 cM on chromosome 13 (denoted B6.NZBc13). We show that B6.NZBc13 female mice not only have enhanced B cell activation but also share many other B cell phenotypic characteristics with NZB mice, including expansion of marginal zone and CD5+ B cell populations, increased numbers of IgM ELISPOTs, and increased serum levels of total IgM and IgM autoantibodies. In addition these mice have increased T cell activation, increased numbers of germinal centers, mild glomerulonephritis, and produce high-titer IgM and IgG anti-chromatin Abs. Male B6.NZBc13 mice have a less pronounced cellular phenotype, lacking expansion of the marginal zone B cell population and IgG anti-chromatin Ab production, indicating the presence of gender dimorphism for this locus. Thus, we have identified a genetic locus that recapitulates with fidelity the B cell phenotypic abnormalities in NZB mice, and we demonstrate that this locus is sufficient to induce an autoimmune phenotype. The data provide further support to the contention that immune abnormalities leading to altered B cell activation and selection contribute to the development of autoimmunity in NZB mice. The Journal of Immunology, 2005, 175: 4309–4319.

New Zealand Black (NZB)3 mice develop a lupus-like autoimmune disease that is characterized by production of anti-RBC, -lymphocyte, and -ssDNA Abs leading to hemolytic anemia and a mild glomerulonephritis that develops later in life (reviewed in Ref. 1). Although NZB mice do not develop rapidly progressive glomerulonephritis, they appear to possess most of the immunological defects required, because backcrossing H-2bm12 onto the NZB background is sufficient to produce mice that develop both high-titer anti-dsDNA Abs and early onset nephritis (2).

The precise immune defects that lead to production of autoantibodies in NZB mice have not been characterized. However, these mice possess a number of B cell phenotypic and functional abnormalities that suggest B cell signaling defects play an important role in this process. These include the following: polyclonal B cell activation with increased serum IgM levels, increased numbers of IgM-producing cells, and increased number of activated-B cells on a percoll gradient (3–9); an abnormal distribution of B cells into peripheral B cell subsets with an increased proportion of marginal zone and CD5+ B cells (1, 9); and an increased proportion of B cells expressing elevated levels of costimulatory molecules (9). To determine the relationship between these B cell phenotypic abnormalities and autoimmunity in NZB mice, we performed a genetic mapping study of (B6 × NZB)F2 mice (10). This revealed two independently segregating clusters of B cell traits that were linked to chromosomal regions previously associated with the autoimmune phenotype. The first, consisting of an increased proportion of B cells expressing elevated levels of costimulatory molecules, elevated serum IgM levels and an increased number of IgM-secreting cells, mapped to NZB regions on chromosomes 1 and 13, and a B6 region close to the MHC locus. The second cluster was characterized by an increased proportion of IgMhighIgDlow cells and CD23low/− B cells, and showed suggestive linkage to NZB chromosome 4.

To further dissect the role of these B cell abnormalities in the development of autoimmunity, we have produced C57BL/6 (B6) congenic mice with introgressed NZB intervals corresponding to the chromosomal regions linked to the clusters of B cell traits. We have recently published the results of our study of B6 congenic mice with a NZB interval extending from 35 to 106 cM on chromosome 1 (B6.NZBc1(35–106)) (11). Although B6.NZBc1(35–106) mice had an increased proportion of B cells with elevated levels of costimulatory molecules, as expected based upon the results of our mapping study, the B cell phenotype in these mice differed in several important respects from that seen in NZB mice.

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1 Abbreviations used in this paper: NZB, New Zealand Black; PI, propidium iodide; int, intermediate; PNA, peanut agglutinin; Treg, regulatory T; NZW, New Zealand White; gp, glycoprotein; gp70IC, gp70-anti-gp70 immune complex.

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In contrast to NZB mice, the increased proportion of B cells expressing elevated levels of costimulatory molecules was predominantly in the CD21<sup>low</sup> B cell subset. Additionally, the distribution of B cells in peripheral B cell subsets was different from that seen in NZB mice, with a reduced rather than increased proportion of marginal zone B cells. Finally, B6.NZBc1c3 (35–106) mice did not produce IgM autoantibodies, a prominent feature of the autoimmune phenotype in NZB mice.

In the present study, we have generated and characterized B6 congenic mice with a NZB chromosome 13 interval (B6.NZBc13), the second non-MHC interval linked to increased expression of costimulatory molecules in our mapping study. We show that the B cell phenotype in these mice, in contrast to B6.NZBc13 (35–106) mice, recapitulates many of the cardinal features of NZB mice. In particular, B6.NZBc13 mice have similar increased proportions of cells with elevated levels of costimulatory molecules in diverse B cell subsets and similar distribution of peripheral B cell subsets, with expansion of the marginal zone and CD5<sup>+</sup> compartments, to NZB mice. Additionally, B6.NZBc13 mice have an increased number of splenic IgM-producing cells, increased serum IgM levels, and increased production of IgM autoantibodies. Surprisingly, B6.NZBc13 mice also produced high-titer IgM and IgG anti-chromatin Abs, and had evidence of increased T cell activation. The data provide further support to the contention that immune abnormalities leading to altered B cell activation and selection contribute to the development of autoimmunity in NZB mice.

Materials and Methods

Mice

B6 and NZB mice were purchased from The Jackson Laboratory and Harlan Sprague Dawley, respectively. Congenic mice were produced by backcrossing mice with a NZB chromosome 13 interval onto the B6 genetic background, using the speed congenic technique. Mice were typed at each successive generation using polymorphic microsatellite markers that discriminate between NZB and B6 DNA, spaced at ~20 cM intervals throughout the genome except for regions containing lupus susceptibility genes where more densely spaced markers were used. Fully backcrossed mice were obtained in seven generations and intercrossed to produce congenic mice that were homozygous for the NZB interval. All of the mice were housed in microisolators in the animal facility at the Toronto Western Hospital (Toronto, Canada) and were specific pathogen free.

Flow cytometry staining and analysis

A total of 5 × 10<sup>6</sup> RBC-depleted spleen cells were incubated with 10 µg/ml mouse IgG (Sigma-Aldrich) for 15 min to block Fc receptors and then stained with various combinations of directly conjugated mAbs. Following washing, allophycocyanin-conjugated streptavidin (BD Pharmingen) was used to reveal biotin-conjugated Ab staining. Dead cells were excluded by staining with propidium iodide (PI; Sigma-Aldrich), 0.6 µg/ml. Flow cytometry of the stained cells was performed by using a dual laser FACScalibur (BD Biosciences) and analyzed using CellQuest (BD Biosciences) software. The following directly conjugated mAbs were purchased from BD Pharmingen: biotin anti-CD4 (L3T4), biotin anti-CD8 (53-6.7), biotin anti-N418, PE anti-B7.1 (16-10A1), PE anti-B7.2 (GL1), PE anti-ICAM-1 (3E2), PE anti-CD23 (B3B4), PE anti-CD69 (H1.2F3), PE anti-CD44 (IM7), PE anti-MHC (1-A<sup>+</sup>), PE anti-NK1.1 (PK136), PE anti-CD5, PE anti-CD38, PE anti-CD44, FITC anti-CD21 (76G6), and FITC anti-CD35 (53-7.3). Biotin, PE, and FITC-conjugated anti-B220, as well as FITC-conjugated anti-CD62L mAbs were purchased from Cedarlane Laboratories. FITC-conjugated anti-IgM and Mac-1 Abs were purchased from Jackson ImmunoResearch Laboratories and Serotec, respectively. All of the isotype controls were purchased from Cedarlane Laboratories except for hamster IgG controls, which were obtained from BD Pharmingen.

Measurement of Ab production

IgM and IgG anti-sDNA, -dsDNA, -chromatin, and -histone Abs were measured by ELISA. dsDNA was prepared from calf thymus DNA (Sigma-Aldrich), and ssDNA was prepared by boiling dsDNA for 10 min and quick cooling on ice. H1-stripped chromatin was prepared from chicken RBC, as described previously (12). Bovine histones (a mixture of H1, H2A, H2B, H3, and H4) were purchased from Roche. ELISA plates were coated overnight with Ag diluted in PBS (dsDNA, 20 µg/ml; ssDNA, 10 µg/ml; chromatin, 8 µg/ml; histones, 2.5 µg/ml), washed with PBS/Tween 20 (0.05%), and blocked with PBS-BSA (2%). After further washing, serum samples, diluted 1/100 in PBS/BSA/Tween 20, were added. Bound IgM or IgG Abs were detected using alkaline-phosphatase-conjugated anti-IgM or -IgG as a secondary reagent (Caltag Laboratories). For measurement of total serum IgM and IgG, plates were coated with goat anti-mouse IgM or IgG (Jackson ImmunoResearch Laboratories), respectively, and the serum was diluted at 1/3000 for IgM or 1/10,000 for IgG. Standard curves were performed using class-specific controls, and the Ab concentration was calculated from a log-log plot of concentration vs absorbance.

ELISPOT

Splenic IgM-producing cells were detected by ELISPOT assay, as described previously (10). Briefly, 96-well Millititer HA plates (Millipore) were coated with goat anti-mouse IgM. Following blocking with 5% FCS in PBS, freshly isolated splenocytes were plated at 10<sup>5</sup> cells and 10<sup>4</sup> cells/ well and incubated for 48 h at 37°C. The cells were washed away with PBS/Tween 20 (0.05%), and the plates were incubated with alkaline phosphatase-conjugated goat anti-mouse IgM (Caltag Laboratories) diluted in 0.1% BSA/PBS/Tween 20 for 2 h at room temperature. Following further washing substrate was added (Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; Sigma-Aldrich), and the reaction was terminated by washing with distilled water. Individual spots, reflecting Ab-producing cells, were visualized using a stereomicroscope.

Immunofluorescence staining of tissue sections

Spleens and kidneys were snap frozen in OCT compound (Sakura Finetek) at the time of sacrifice. Cryostat sections (6 µm) were fixed in acetone, washed with PBS, and blocked with PBS/5% normal goat serum. Kidney sections were stained with FITC-conjugated anti-IgG (Fla<sup>+</sup>), (Caltag Laboratories). Spleen sections were stained with FITC-conjugated anti-IgM (Jackson ImmunoResearch Laboratories) and biotinylated-PNA (to reveal germinal centers; Sigma-Aldrich). Biotin staining was detected using rhodamine-conjugated streptavidin as a secondary reagent (Molecular Probes). Stained sections were mounted with Mowiol (Calbiochem), and tissue fluorescence was visualized using a Zeiss Axioplan 2 imaging microscope. Digital images were obtained using the manufacturer’s imaging system.

Grading of kidney sections

Kidneys were fixed in formalin, paraffin embedded, sectioned (3 µm), and stained with periodic acid-Schiff. Grading was performed by a renal pathologist (G. Lajoie) who was blinded as to the strain of origin of the tissue section. The grading scale used for light microscopy was as follows: grade 0, normal glomeruli; grade 1, mesangial expansion and/or proliferation; grade 2, focal segmental (endocapillary) proliferative glomerulonephritis; grade 3, diffuse (endocapillary) proliferative glomerulonephritis; and grade 4, diffuse proliferative glomerulonephritis with crescents. Glomerular staining of kidney sections stained with FITC anti-IgG was graded by immunofluorescence microscopy. Sections with no or only trace deposits were graded as 0; those with mesangial deposits, grade 1; those with mesangial and segmental capillary wall deposits, grade 2; those with diffuse mesangial and capillary wall deposits, grade 3; and those with crescents, grade 4.

Statistical analysis

Comparisons of differences between groups of mice for continuous data were performed using the Mann-Whitney nonparametric test. For comparison of differences between groups with discontinuous data, such as renal scores, a Fisher’s exact test was used.

Results

Increased B cell activation in B6.NZBc13 congenic mice

A NZB chromosome 13 interval extending from D13Mit318 (24 cM) to D13Mit77 (73 cm) was backcrossed onto the B6 genetic background using the speed congenic technique. Mice were then intercrossed to produce congenic mice that were homozygous for the NZB chromosome 13 interval (denoted B6.NZBc13). As shown in Fig. 1, this interval overlaps with the region of linkage...
individual experiments revealed that the proportion of CD44high B cells showed the most significant association with chromosome 13 in our mapping study, B cell staining with anti-CD44 yielded variable results, which precluded pooling the data from individual experiments. Nevertheless, analysis of IgM ELISpots are described previously (10). LOD scores were obtained using MAPMAKER/QTL version 1.1 with an additive genetic model for all traits examined except ICAM-1 where a dominant model was used. Superimposed on the top of the figure is a map of chromosome 13 showing the NZB interval in B6.NZBc13 mice. Thin lines indicate regions of the chromosome that are B6, thick lines regions that are NZB, and the gaps between lines are areas of undefined origin.

for increased expression of costimulatory molecules and CD44 on B cells, as defined by our previous mapping study of (B6 × NZB)F2 mice (10). Cohorts of B6.NZBc13 and corresponding B6 control mice were then aged up to 8 mo, and splenic cellular phenotypes were assessed at 4 and 8 mo of age. As shown in Table I, consistent with the results of our previous mapping experiment, at 4 and 8 mo of age female B6.NZBc13 mice had a significantly increased proportion of B cells expressing elevated levels of the costimulatory molecules B7.1, B7.2, and ICAM-1. Although the proportion of CD44high B cells showed the most significant association with chromosome 13 in our mapping study, B cell staining with anti-CD44 yielded variable results, which precluded pooling the data from individual experiments. Nevertheless, analysis of individual experiments revealed that the proportion of CD44high B cells was elevated in some experiments comparing B6.NZBc13 and B6 splenocytes. Assessment of other activation markers on B cells revealed a significant increase in the proportion of CD69+ B cells in B6.NZBc13 mice (Table I). Thus, the NZB chromosome 13 interval in B6.NZBc13 mice appears to contain the susceptibility locus defined in our previous mapping study. Of interest, male 8-mo-old B6.NZBc13 mice had a B cell activation phenotype intermediate between B6 and B6.NZBc13 mice, suggesting that there is gender dimorphism for this locus.

**B6.NZBc13 mice share several other B cell phenotypic characteristics with NZB mice**

Further characterization of the splenic cellular phenotype in B6.NZBc13 mice revealed a number of additional phenotypic differences (Table II). Four-month-old female B6.NZBc13 mice had significantly larger spleens than their B6 counterparts. As observed for NZB mice, this was not accompanied by a significant increase in cellularity in young mice. Nevertheless, splenic size and cellularity increased progressively with age and at 8 mo of age was significantly increased for both B6.NZBc13 and NZB mice (Table II, and data not shown). Flow cytometric analysis of splenocyte populations revealed an ~2-fold increase in the proportion of Mac-1high cells and significant decreases in the proportions of B (B220+) and CD8+ T cells in B6.NZBc13 mice as compared with B6 controls. In 8-mo-old mice, changes in the Mac-1high and B cell populations were seen in both male and female mice. Despite the increase in cellularity of B6.NZBc13 spleens with age, the altered distribution of the cellular subsets remained stable with age.

To further investigate the source of the Mac-1high cell expansion in B6.NZBc13 mice, additional stains were performed on a subset of mice. Stains together with B220 confirmed that the Mac-1high population excluded Mac-1lowB220+ B1 cells. Further stains in combination with N418 showed that in young mice (≤4 mo) the majority of Mac-1high cells were N418− macrophages. In older mice (5–10 mo), the expanded Mac-1high population contained both N418− and N418+ cells, resulting in an increased proportion of N418high dendritic cells (B6, 2.52 ± 0.57; B6.NZBc13, 13.07 ± 6.47; p = 0.004; n = 4–8 mice). In young mice, the proportion of splenic N418high dendritic cells was normal, as was the size of the plasmacytoid dendritic cell subset (N418− B220+ NK1.1−) in both the spleen and bone marrow (data not shown).

In NZB mice the reduced proportion of splenic B cells is accompanied by alterations in the distribution of splenic B cells into peripheral B cell subsets (Ref. 9, and Table III). To determine whether B6.NZBc13 mice have a similarly altered distribution of their peripheral B cells, we stained freshly isolated splenocytes with a combination of anti-B220, -CD21, and -CD23 mAb to discriminate between distinct peripheral B cell subsets (Fig. 2A), and the proportion of cells in each subset was determined by flow cytometry (Table III). At all ages examined (2, 4, and 8 mo), B6.NZBc13 mice had significant increases in the proportion of CD21highCD23− marginal zone and decreases in the proportion of CD21lowCD23+ follicular B cells (Fig. 2B, Table III, and data not shown). At 4 and 8 mo of age, there was also a significant increase

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**Table I. Abnormal B cell activation in B6.NZBc13 congenic mice**

<table>
<thead>
<tr>
<th>Trait</th>
<th>Female</th>
<th>Male</th>
<th>B6</th>
<th>B6.NZBc13</th>
<th>NZB</th>
<th>B6</th>
<th>B6.NZBc13</th>
<th>Male B6.NZBc13</th>
</tr>
</thead>
<tbody>
<tr>
<td>% B7.1</td>
<td>13.6 ± 1.4 (15)</td>
<td>18.7 ± 3.3*** (11)</td>
<td>38.1 ± 5.3*** (13)</td>
<td>19.1 ± 3.2 (14)</td>
<td>27.4 ± 6.2*** (15)</td>
<td>21.6 ± 6.8 (11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MFI B7.2</td>
<td>37.0 ± 6.9 (15)</td>
<td>50.4 ± 13.7*** (15)</td>
<td>67.54 ± 16.7*** (5)</td>
<td>51.0 ± 7.2 (14)</td>
<td>80.4 ± 17.5*** (15)</td>
<td>66.0 ± 13.0*** (11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MFI ICAM-1</td>
<td>244 ± 20 (15)</td>
<td>397 ± 77*** (15)</td>
<td>472 ± 97*** (5)</td>
<td>357 ± 43 (14)</td>
<td>617 ± 125*** (15)</td>
<td>494 ± 114*** (11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CD69+</td>
<td>4.9 ± 1.1 (15)</td>
<td>7.5 ± 1.9*** (15)</td>
<td>6.7 ± 2.2 (13)</td>
<td>7.9 ± 2.2 (9)</td>
<td>16.5 ± 3.5*** (10)</td>
<td>13.2 ± 2.1*** (11)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Results are mean ± SD. Numbers in parentheses denote number of mice tested. Significance level was determined by Mann-Whitney nonparametric test for comparison with B6 mice. MFI, Mean fluorescence intensity. * p < 0.05, ** p < 0.005, *** p < 0.0005.

* Results for male B6.NZBc13 mice highlighted with bold font are significantly different (p < 0.05) from those obtained for female age-matched B6.NZBc13 mice.
in the proportion of CD21\textsuperscript{low}CD23\textsuperscript{−} B cells. These changes were similar to, but not as pronounced as, those seen in NZB mice. As for the B cell activation markers, the B cell phenotypes in male B6.NZBc13 mice were intermediate between that of female B6.NZBc13 and B6 mice (Fig. 2B, and data not shown).

Transitional 1, plasmablast, centroblast, and CD5\textsuperscript{−} B cell populations can all be found in the CD21\textsuperscript{low}/CD23\textsuperscript{−} B cell population of 4- to 8-mo-old NZB mice (Fig. 2C, and data not shown). To determine which of these populations contributes to the expansion of the CD21\textsuperscript{int}/CD23\textsuperscript{−} B cell population in B6.NZBc13 mice, splenocytes from 5- to 12-mo-old female mice were stained with anti-B220 and -CD21, together with anti-CD138, anti-CD5, peanut agglutinin (PNA), or AA4.1. As shown in Fig. 2, C and D, B6.NZBc13 mice had increased proportions of CD21\textsuperscript{low}/CD5\textsuperscript{+}, CD21\textsuperscript{int}/PNA\textsuperscript{high}, and CD21\textsuperscript{int}/CD138\textsuperscript{+} B cells as compared with B6 mice. For CD21\textsuperscript{int}/CD5\textsuperscript{+} B cells, the proportion of these cells was similar to that seen in NZB mice. Staining with AA4.1, a marker specific for immature B cells (13), confirmed that the proportion of transitional 1 cells was comparable in 4- to 6-mo-old B6 and B6.NZBc13 mice (data not shown). Thus, the expansion of CD21\textsuperscript{low}/CD23\textsuperscript{−} B cells in B6.NZBc13 mice reflects increased numbers of CD5\textsuperscript{−} B cells, centroblasts, and to a lesser extent plasmablasts in this population.

As can be seen in Fig. 2C, the CD5\textsuperscript{−} and PNA\textsuperscript{high} B cell populations are not restricted to the CD21\textsuperscript{low} compartment but are also found in the CD21\textsuperscript{int} population. Therefore, to determine whether the total proportions of CD5\textsuperscript{−} and PNA\textsuperscript{high} B cells are increased in B6.NZBc13 mice, cells were gated irrespective of their CD21 expression. This analysis confirmed that B6.NZBc13 mice had increased proportions of both these populations (the percentage of B cells CD5\textsuperscript{−}: B6, 3.35 ± 0.93 and B6.NZBc13, 9.40 ± 2.18 (p = 0.0095); the percentage of B cells PNA\textsuperscript{high}: B6, 3.45 ± 1.15 and B6.NZBc13, 9.92 ± 2.85 (p = 0.0095)). Additional stains indicated that the CD5\textsuperscript{−} population was IgM\textsuperscript{low}/B220\textsuperscript{−} and the PNA\textsuperscript{high} population was IgM\textsuperscript{high}/B220\textsuperscript{−}, consistent with B1a and centroblast phenotypes, respectively. As was seen for the CD21\textsuperscript{low} subset the total proportion of CD5\textsuperscript{−}, but not PNA\textsuperscript{high} B cells, approximated that seen in NZB mice (the percentage of B cells CD5\textsuperscript{−}: NZB, 7.91 ± 1.84 (p = NS); the percentage of B cells PNA\textsuperscript{high}: NZB, 19.08 ± 3.85 (p = 0.012) as compared with B6.NZBc13). However, in contrast to NZB mice, staining of peritoneal cells revealed no expansion of the CD5\textsuperscript{−} B cell subset (the percentage of B220\textsuperscript{−}CD5\textsuperscript{−} peritoneal cells: B6, 25.30 ± 3.91; B6.NZBc13, 21.58 ± 6.83; NZB, 49.23 ± 20.82; p = NS and 0.036 for B6 as compared with B6.NZBc13 and NZB, respectively; n = 3-5 mice aged 8–12 mo).

The increased PNA staining of a CD21\textsuperscript{low}/int B cell population in B6.NZBc13 mice suggested that these mice might have an increased number of germinal centers. To further explore this possibility, spleens from 8-mo-old B6.NZBc13 mice were stained with anti-IgM and PNA. As shown in Fig. 3A, there was a marked increase in the number and size of germinal centers in 8-mo-old female B6.NZBc13 mice as compared with B6 mice, with large ill-defined germinal centers essentially filling all of the lymphoid follicles. This was accompanied by a relative expansion of the white pulp and infiltration of the remaining red pulp with IgM\textsuperscript{bright} cells, similar to but not as pronounced as that seen in NZB mice (Fig. 3B). Although the majority of the cells appeared to be plasmablasts, some of the cells had eccentric nuclei suggesting that they were plasma cells. The number and size of germinal centers in male mice was intermediate between those observed for female B6.NZBc13 and B6 mice.

### Table II. Splenic phenotype in B6.NZBc13 mice

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
<th>4 mo</th>
<th>8 mo</th>
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<tbody>
<tr>
<td></td>
<td>B6</td>
<td>B6.NZBc13</td>
<td>NZB</td>
<td>B6</td>
</tr>
<tr>
<td>Spleen weight (mg)</td>
<td>84.8 ± 13.2 (15)</td>
<td>119.2 ± 12.9** (15)</td>
<td>132.5 ± 36.3*** (13)</td>
<td>84.1 ± 8.3 (14)</td>
</tr>
<tr>
<td>No. splenocytes per spleen × 10\textsuperscript{6}</td>
<td>98.3 ± 36.7 (15)</td>
<td>84.8 ± 72.0 (12)</td>
<td>56.2 ± 20.7* (15)</td>
<td>73.5 ± 18.4 (14)</td>
</tr>
<tr>
<td>% B220\textsuperscript{*} cells</td>
<td>59.4 ± 2.4 (15)</td>
<td>53.0 ± 3.5*** (15)</td>
<td>35.8 ± 5.4*** (13)</td>
<td>56.1 ± 5.4 (14)</td>
</tr>
<tr>
<td>% CD4\textsuperscript{−} cells</td>
<td>14.9 ± 2.1 (15)</td>
<td>16.4 ± 4.1 (15)</td>
<td>26.0 ± 2.9*** (13)</td>
<td>16.4 ± 3.0 (14)</td>
</tr>
<tr>
<td>% CD8\textsuperscript{−} cells</td>
<td>13.5 ± 1.4 (12)</td>
<td>12.0 ± 1.6** (15)</td>
<td>12.8 ± 1.9* (13)</td>
<td>13.2 ± 1.2 (14)</td>
</tr>
<tr>
<td>% Mac-1\textsuperscript{high} cells</td>
<td>4.7 ± 0.96 (12)</td>
<td>7.6 ± 3.3* (11)</td>
<td>8.0 ± 2.0*** (11)</td>
<td>4.6 ± 1.1 (14)</td>
</tr>
</tbody>
</table>

\* Results are mean ± SD. Numbers in parentheses denote number of mice tested. Significance level was determined by Mann-Whitney nonparametric test for comparison with B6 mice. *: p < 0.05, **: p < 0.005, ***: p < 0.0005.

\# Results for male B6.NZBc13 mice highlighted with bold font are significantly different (p < 0.05) from those obtained for female age-matched B6.NZBc13 mice.

Enhanced B cell activation in diverse B cell subsets in B6.NZBc13 mice

Although the majority of B cells expressing elevated levels of costimulatory molecules in young (<4 mo old) NZB mice are found within the marginal zone B cell subset, increased proportions of B cells with elevated levels of costimulatory molecules are seen in virtually all peripheral B cell subsets (Ref. 9, and our unpublished observations). To determine whether the increased expression of costimulatory molecules on B6.NZBc13 B cells results from expansion of B cell populations that normally express high levels of these molecules, such as the CD5\textsuperscript{−} and marginal zone populations, or altered expression of costimulatory molecules within these subsets, costimulatory molecule expression was assessed on splenic B cells categorized by the levels of CD21 expression, as in our previous studies (9, 11). As shown in Fig. 4, increased expression of costimulatory molecules was seen in all three B cell subsets, indicating that costimulatory molecule expression is elevated in diverse B cell populations in female B6.NZBc13 mice. Results shown are for mice 8 mo of age; however, similar results were observed for 4-mo-old mice. Expression of costimulatory molecules for all of the CD21-defined B cell subsets in male B6.NZBc13 mice was intermediate between female B6 and B6.NZBc13 mice (Table I). Staining with anti-B7.1, together with anti-CD21 and -CD23, anti-CD21 and -CD24, anti-B220 and -CD5, or anti-B220 and -AA4.1 confirmed an...
increased proportion of B.7.1+ cells in the transitional, follicular, marginal, and marginal zone, and CD5+B cell subsets of female B6.NZBc13 mice (data not shown).

**Enhanced T cell activation in B6.NZBc13 mice**

We next sought to determine whether the abnormal lymphocyte activation in B6.NZBc13 mice was restricted to the B cell subset. To this end, freshly isolated splenocytes from 4- and 8-mo-old mice were stained with anti-CD4 or -CD8 together with anti-CD69 mAb to identify recently activated T cells, or anti-CD44 and -CD62L to identify memory (CD44hiCD62Llow) and naive (CD44lowCD62Lhigh) populations. As shown in Table IV, both CD4+ and CD8+ T cell subsets had an increased proportion of recently activated and memory cells, with a corresponding decrease in naive cells. Enhanced CD4+ T cell activation was first seen in B6.NZBc13 mice at 10 wk of age (data not shown) and increased progressively thereafter.

CD8+ and regulatory T (Treg) cell subsets have been proposed to inhibit generation of autoantibody responses in (NZB × New Zealand White (NZW))F1 (NZB/W) mice (14, 15). To quantitate Treg cells in both CD4+ and CD8+ T cell subsets had an increased proportion of recently activated and memory cells, with a corresponding decrease in naive cells. Enhanced CD4+ T cell activation was first seen in B6.NZBc13 mice at 10 wk of age (data not shown) and increased progressively thereafter.

**Polyclonal B cell activation in B6.NZBc13 mice is accompanied by high-titer anti-chromatin Ab production and mild renal disease**

One of the hallmarks of polyclonal B cell activation in NZB mice is an increased number of IgM-producing cells in the spleen. Increased numbers of IgM-producing cells were seen in both 4- and 8-mo-old B6.NZBc13 mice, and in contrast to other B cell phenotypes, were similar in both male and female mice (number of ELISPOTs per 10^5 splenocytes: 4 mo: B6, 35.5 ± 30.0 and B6.NZBc13, 250.4 ± 166.0 (p = 0.046); 8 mo: B6, 71.34 ± 28.26; female B6.NZBc13, 315.7 ± 76.3 and male B6.NZBc13, 371.6 ± 207.0 (both p < 0.0001)). However, the number of ELISPOTs remained significantly lower than seen in age-matched NZB mice (number of ELISPOTs per 10^5 splenocytes in 8-mo-old mice, 1100.0 ± 465.4 (p < 0.0001 as compared with female and male B6.NZBc13 mice)). These findings validate the results of our previous mapping study in which we demonstrated suggestive linkage for IgM ELISPOTs to the same region on chromosome 13 as altered costimulatory molecule expression.

Although total serum IgM levels were only minimally increased in B6.NZBc13 mice at 4 mo of age (serum concentration IgM (milligrams per milliliters): B6, 0.92 ± 0.60 and B6.NZBc13, 1.77 ± 1.31 (p = 0.020); and NZB, 6.86 ± 1.73), they were increased 3- to 4-fold in 8-mo-old B6.NZBc13 mice and were similar to levels seen in NZB mice (serum concentration IgM (milligrams per milliliters): B6, 2.13 ± 0.53; female B6.NZBc13, 7.74 ± 2.12 and male B6.NZBc13, 5.90 ± 1.14 (both p < 0.0001); and NZB, 5.46 ± 2.76). Total serum IgG was also elevated in 8-mo-old mice (serum concentration IgG (milligrams per milliliters): B6, 2.32 ± 0.35; female B6.NZBc13, 3.32 ± 0.85 and male B6.NZBc13, 4.04 ± 1.35 (p = 0.0005 and 0.0017, respectively)).

Given the evidence of increased spontaneous B and T cell activation, and germinal center formation in B6.NZBc13 mice, we next investigated whether these mice produced autoantibodies. As shown in Fig. 5A, both male and female 8-mo-old mice produced IgM Abs directed at an array of nuclear Ags including histone, chromatin, ssDNA, and dsDNA. However, with the exception of chromatin these autoantibodies were of relatively low titer, as compared with those seen in NZB mice. IgG autoantibody production was also restricted in B6.NZBc13 mice, with significant titers of IgG autoantibodies demonstrated only for chromatin, and to a minimal extent, histone. Although both male and female mice produced high-titer IgM anti-chromatin Abs, only female mice produced IgG anti-chromatin Abs, suggesting that loss of T cell tolerance to chromatin is likely restricted to or markedly accelerated in female mice. Of note, the kinetics of anti-chromatin Ab production also appeared to be accelerated in female B6.NZBc13 mice, as compared with NZB mice, because high titers of anti-chromatin Abs were present in B6.NZBc13 mice, but not NZB mice, at 4 mo of age (Fig. 5B).

We next addressed whether the autoantibody production in B6.NZBc13 mice was associated with renal disease. Deposition of Ig in the kidneys of 8-mo-old mice was assessed by immunofluorescence microscopy following staining with FITC anti-IgG and graded using a four-point scale, as outlined in Materials and Methods (Fig. 6A). This revealed that the majority of 8-mo-old B6 and

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**Table III. Splenic B cell populations in B6.NZBc13 mice**

<table>
<thead>
<tr>
<th></th>
<th>4 mo</th>
<th>8 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>B6</td>
</tr>
<tr>
<td>% CD21lowCD23+</td>
<td>8.3 ± 1.6 (15)</td>
<td>10.2 ± 2.1* (11)</td>
</tr>
<tr>
<td>% CD21hiCD23+</td>
<td>73.4 ± 1.9 (15)</td>
<td>65.8 ± 3.5*** (11)</td>
</tr>
<tr>
<td>% CD24hiCD23+</td>
<td>4.0 ± 2.1 (15)</td>
<td>3.2 ± 1.3 (11)</td>
</tr>
<tr>
<td>% CD5hiCD23+</td>
<td>9.1 ± 1.3 (15)</td>
<td>12.9 ± 1.9*** (11)</td>
</tr>
</tbody>
</table>

* Results are mean ± SD. Numbers in parentheses denote number of mice tested. Significance level was determined by Mann-Whitney nonparametric test for comparison with B6 mice. p < 0.05. **, p < 0.005. ****, p < 0.0005.

**Results for male B6.NZBc13 mice highlighted with bold font are significantly different (p < 0.05) from those obtained for female age-matched B6.NZBc13 mice.**
FIGURE 2. Splenic B cell populations in B6.NZBc13 mice. A, Freshly isolated splenocytes from 8-mo-old B6 and B6.NZBc13 mice were stained with anti-B220, -CD21, and -CD23. Shown are dot plots gated on PI excluding B220$^+$ cells. Regions used to determine the proportion of CD21$^{lo}$CD23$^+$, CD21$^{hi}$CD23$^+$, CD21$^{lo}$CD23$^+$, and CD21$^{lo}$CD23$^+$ populations are indicated. Numbers within the boxes indicate the proportion of B cells in each population. B, Scatter plot showing the proportion of B cells in each B cell subset, gated as indicated in A. Each symbol represents the determination for an individual 8-mo-old mouse. Horizontal lines indicate the mean for each population examined. The p values for significant differences between female B6 and B6.NZBc13 mice, or male and female B6.NZBc13 mice are shown.

FIGURE 3. Immunofluorescence microscopy of spleen sections from B6.NZBc13 mice. Spleens from 8-mo-old B6 and B6.NZBc13 mice were stained for dual-color immunofluorescence. Green and red colors depict staining for IgM and PNA, respectively. A, Splenic germinal centers. There is a marked increase in both the number and size of germinal centers in female B6.NZBc13 mice, with less pronounced changes in male B6.NZBc13 mice. A spleen from a 4-mo-old NZB mouse is shown for comparison. All of the photomicrographs were taken at ×2.5 magnification. Note the increased infiltration of the red pulp with IgM$^{bright}$ cells in B6.NZBc13 and NZB mice. B, Higher power magnification (×20) showing the IgM$^{bright}$ cells within the red pulp of female B6.NZBc13 and NZB mice. Some of the IgM$^{bright}$ cells have eccentric nuclei suggesting that they are plasma cells.
B6.NZBc13 mice had mesangial deposits. However, there was a trend to an increased proportion of male and female B6.NZBc13 mice with segmental and diffuse capillary wall deposits. On light microscopy, both male and female B6.NZBc13 mice had significantly more severe renal disease than control B6 mice (Fig. 6B; \( p = 0.0023 \) female and \( p = 0.0081 \) male as compared with B6, Fisher’s exact test). Although in B6.NZBc13 mice there was a trend to more severe renal disease in female as compared with male mice, this did not achieve statistical significance. Despite changes on light microscopy, none of the B6.NZBc13 mice developed high-grade proteinuria, and only one mouse died before 8 mo of age. It is not known whether this mouse died of renal disease.

### Discussion

Results of our previous gene mapping study suggested that a genetic locus that contributed to the abnormal B cell activation in NZB mice was located on chromosome 13. In this study we have produced congenic mice bearing a NZB chromosome 13 interval encompassing the region of peak linkage, and we have shown that these mice share not only abnormal B cell activation, but also many of the other distinctive B cell characteristics of NZB mice. These characteristics include the following: an altered distribution of cells in splenic B cell compartments; increased serum levels of IgM; increased numbers of IgM-producing cells; and increased IgM autoantibody production. Of these phenotypes, only the increased number of IgM-producing cells was detected in our previous mapping study. This discordance likely results from differences in the age of the mice that were examined in this study and the mapping study as well as the lack of specific stains for the CD5 and marginal zone B cell subsets in the mapping study. In our linkage analysis, B cell phenotypes were assessed at 12 wk of age. Although increased numbers of IgM ELISPOTs are readily detected in 4-mo-old B6.NZBc13 mice, serum IgM levels were only minimally elevated. Similarly, IgM autoantibodies, with the exception of antichromatin Ab, develop later in B6.NZBc13 mice (data not shown). These findings suggest that the phenotype in B6.NZBc13 mice does not mirror precisely that seen in NZB mice, a conclusion that is compatible with our previous observation that NZB loci on chromosomes 1, 4, and 7 also contribute to the altered B cell phenotype in NZB mice (10).

Of the phenotypes that were mapped to NZB chromosome 13, only increased expression of CD44 was less marked than predicted by our linkage study. CD44 expression was linked to both chromosomes 1 and 13 in our mapping study, with significant epistasis.
demonstrated between these loci. In the absence of an NZB chromosome 1 allele, two NZB chromosome 13 alleles resulted in marginally elevated levels of CD44 expression. It is likely that detection of this minimal elevation was precluded by the variablility in CD44 staining between individual experiments, which prevented pooling of results.

Although B cell phenotypic abnormalities are a prominent feature of NZB mice, the immune processes leading to the development of these abnormalities and their role in the autoimmune disease has been controversial. In this study we have found a major NZB chromosomal region that contributes to the development of many of these B cell phenotypes and show that this region is sufficient to produce high-titer IgM and IgG anti-chromatin Abs, a hallmark of lupus, and glomerulonephritis. The presence of glomerulonephritis in B6.NZBc13 confirms the results of a previous linkage study indicating the presence of a susceptibility locus for glomerulonephritis on NZB chromosome 13 (16).

High serum levels of endogenous retroviral envelope glycoprotein (gp70) and high gp70-anti-gp70 immune complexes (gp70IC) have also been mapped to NZB chromosome 13 (16, 17). Indeed, the peak linkage for elevated CD44 and costimulatory molecule expression in our mapping study was in the same area where peak linkage to glomerulonephritis (~35 cM), gp70 levels (~43 cm), and gp70IC (~35 cM) were seen for a B6 × (NZB × B6.Yaa)F1 cross (16), and peak linkage to serum gp70 (~40–55 cM) and gp70IC (~35 cM) was seen for (B6.H2b × NZB)F1 × NZB and (B6 × NZB)F2 crosses (17). A region on chromosome 13 between ~30 and 50 cM has also been linked to gp70, gp70IC, and nephritis in NZW and BXSB mice (18–20). We have not measured the serum levels of gp70 in our congenic mice; however, it is likely that they are elevated. Previous work indicates that the major source of gp70 production is in the liver (21), and we have found elevated levels of transcripts for gp70 in the livers of B6.NZBc13 mice (data not shown).

At present, it is unclear whether the genetic locus associated with elevated levels of gp70 is the same as that leading to the altered immune function in B6.NZBc13 mice. Congenic mice with chromosome 13 intervals containing the gp70 susceptibility alleles from NZB and NZW mice have been produced. Male mice with a ~20 cM NZB interval that is encompassed within our congenic interval have elevated levels of gp70 and slightly increased titers of gp70IC but do not produce IgG anti-DNA or -chromatin Abs (16). However, in this study only results for male mice with the congenic interval in combination with Yaa, the Y-linked autoimmune acceleration gene, were reported, and cellular phenotypes were not examined. Therefore, it is not possible to determine whether the two congenic mouse strains have comparable phenotypes. A series of congenic mice with NZW chromosome 13 intervals has also been reported, and the genetic locus associated with elevated levels of gp70 was localized to an interval extending from ~37 to 40 cM (22). Elevated levels of IgG anti-DNA or chromatin Abs were not seen in either male or female mice, suggesting that NZB mice have an additional or different susceptibility allele than NZW mice.

It is likely that the elevated levels of serum IgM, and IgM anti-ssDNA, -dsDNA, and -histone Abs, are related in part to the expansion of CD5− and marginal zone B cells. Both cell subsets contribute to the levels of natural IgM Abs that are found in the serum of normal individuals, and these Abs have been shown to bind with low affinity to autoantigens (23–25). Because the marginal zone and CD5− B cell populations are positively selected during development and are enriched for cells with intermediate/low affinity for self Ags (26–29), the expansion of these populations in B6.NZBc13 mice indicates a disturbance in B cell selection. Altered B cell selection leading to changes in the size of marginal zone and/or CD5− compartments can result from B cell signaling abnormalities, as in the SHP-1-deficient motheaten mouse model (30); increased survival of autoreactive B cells, as in BAFF-transgenic mice (31); or increased abundance of self-Ags, as in mer mice with a disrupted mer tyrosine kinase domain (32). Although it is unclear at this time which of these processes is involved in the expansion of these cell subsets in B6.NZBc13 mice, our data clearly link immune mechanisms leading to altered B cell selection in NZB mice with the generation of pathogenic autoantibodies.

A particularly interesting aspect of the autoimmune phenotype in B6.NZBc13 mice is the presence of gender dimorphism, with female but not male mice producing high-titer IgG anti-chromatin Abs. It is unlikely that this simply reflects delayed kinetics of autoantibody production in male mice due to a less pronounced cellular phenotype because female B6.NZBc13 mice already have high levels of IgG anti-chromatin Abs at 4 mo of age, and with the exception of marginal zone B cell expansion, cellular phenotypic changes were more advanced in 8-mo-old male mice than 4-mo-old female B6.NZBc13 mice.

It has been previously demonstrated that estrogen plays an important role in gender dimorphism in (NZB × NZW)F1 mice (34). Estrogen has pleomorphic effects on the immune system and has been shown to affect both B and T cell activation. Administration of exogenous estrogen to nonautoimmune mice expressing an anti-DNA Ig H chain leads to expansion of the marginal zone and development of autoimmunity (34). Furthermore, estrogen has been shown to alter the threshold for B cell activation and apoptosis (35). The expansion of the marginal zone in female, but not...
male, B6.NZBc13 mice is compatible with potential estrogen-mediated effects on B cell selection. In female mice, this altered B cell selection could lead to an increased proportion of anti-chromatin-reactive marginal zone B cells and enhanced activation of the autoreactive T cells that provide support for IgG anti-chromatin Ab production. Alternatively, the increased IgG anti-chromatin Ab production in female B6.NZBc13 mice could reflect direct effects of estrogen on T cell function. Estrogen has been shown to alter the cytokine profile of T cells leading to increased generation of a Th1 phenotype (36). IFN-γ plays an important role in the generation of pathogenic autoantibodies in NZB and (NZB × NZW)F₁ mice (37, 38), and female (NZB × NZW)F₁ mice have been shown to have increased levels of IFN-γ relative to male mice (39).

In summary, we have identified a genetic locus that recapitulates most of the B cell phenotypic abnormalities in NZB mice and is sufficient to induce an autoimmune phenotype. Characterization of the precise genetic polymorphism(s) that lead to this phenotype will provide important insights into the immune mechanisms that lead to altered B cell selection and autoimmunity in spontaneously arising lupus.

FIGURE 5. Autoantibody levels in B6.NZBc13 mice. A, Serum samples from 8-mo-old B6.NZBc13 and age-matched control B6 and NZB mice were assayed for the presence of IgM and IgG anti-chromatin, -ssDNA, -dsDNA, and -histone Abs by ELISA. B, IgM and IgG anti-chromatin Abs in 4-mo-old mice. Each symbol represents the determination from an individual mouse, with open circles indicating female B6, filled circles female B6.NZBc13, filled triangles male B6.NZBc13, and open triangles female NZB mice. Horizontal lines indicate the mean for each population examined. The p values for significant differences between female B6.NZBc13 mice and B6, male B6.NZBc13, and NZB mice are shown.
We thank Robert Inman for critical reading of the manuscript and Andrew Paterson for assistance with preparation of the LOD score plot and statistical analysis.

Disclosures
The authors have no financial conflict of interest.

References

FIGURE 6. Renal involvement in 8-mo-old B6.NZBc13 mice. Each symbol represents the score for an individual mouse, with open circles indicating female B6, filled circles female B6.NZBc13, filled triangles male B6.NZBc13, and open triangles female NZB mice. A. Immunofluorescence scores of frozen kidney sections stained with anti-IgG. Sections were graded as follows: grade 0, no or only trace deposits; grade 1, mesangial deposits; grade 2, mesangial and segmental capillary wall deposits; grade 3, diffuse mesangial and capillary wall deposits, grade 4, crescents. B. Glomerular scores of kidneys fixed in formalin, paraffin embedded, sectioned, and stained with periodic acid-Schiff. Sections were graded as follows: grade 0, normal glomeruli; grade 1, mesangial expansion and/or proliferation; grade 2, focal segmental proliferative glomerulonephritis; grade 3, diffuse proliferative glomerulonephritis; and grade 4, diffuse proliferative glomerulonephritis with crescents.


