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Dissociation of the Genetic Loci Leading to B1a and NKT Cell Expansions from Autoantibody Production and Renal Disease in B6 Mice with an Introgressed New Zealand Black Chromosome 4 Interval

Christina Loh,*† Yong-Chun Cai,* Gabriel Bonventi,* Ginette Lajoie,‡¶ Ralph MacLeod,* and Joan E. Wither2*†§

Previous mapping studies have linked New Zealand Black (NZB) chromosome 4 to several lupus traits, including autoantibody production, splenomegaly, and glomerulonephritis. To confirm the presence of these traits, our laboratory introgressed homozygous NZB chromosome 4 intervals extending from either 114 to 149 Mb or 32 to 149 Mb onto the lupus-resistant C57BL/6 background (denoted B6.NZBc4S and B6.NZBc4L, respectively). Characterization of aged cohorts revealed that B6.NZBc4L mice exhibited a striking increase in splenic B1a and NKT cells in the absence of high titer autoantibody production and significant renal disease. Tissue-specific expansion of these subsets was also seen in the peritoneum and liver for B1a cells and in the bone marrow for NKT cells. Staining with CD1d tetramers loaded with an α-galactosylceramide analog (PBS57) demonstrated that the expanded NKT cell population was mainly CD1d-dependent NKT cells. The lack of both cellular phenotypes in B6.NZBc4S mice demonstrates that the genetic polymorphism(s) that result in these phenotypes are on the proximal region of NZB chromosome 4. This study confirms the presence of a locus that promotes the expansion of B1a cells and newly identifies a region that promotes CD1d-restricted NKT cell expansion on NZB chromosome 4. Taken together, the data indicate that neither an expansion of B1a cells and nor NKT cells is sufficient to promote autoantibody production and ultimately, renal disease. The Journal of Immunology, 2007, 178: 1608–1617.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder primarily characterized by production of autoantibodies directed against nuclear Ags. Similar to other autoimmune conditions, SLE is well-established to have a strong genetic basis. However, the majority of the susceptibility alleles remain to be defined. Murine models of lupus have been particularly valuable in this connection, leading to elucidation of disease mechanisms and identification of a number of candidate genes (1–2). A commonly used model of lupus is the New Zealand Black (NZB) mouse, because it spontaneously develops anti-RBC, -lymphocyte, and -ssDNA Abs that clinically result in hemolytic anemia and late-onset mild glomerulonephritis (GN) (3). Although mapping studies have demonstrated that multiple genetic loci contribute to lupus susceptibility in NZB mice, loci on chromosome 4 have been repeatedly identified as contributing to the abnormal cellular, serologic, GN, and mortality phenotypes. Manifestations of lupus mapped to NZB chromosome 4 include: anti-nuclear Abs (ANA; *Imh1), anti-RBC production (Aia1, *Aem1), IgM hypergammaglobulinemia (*Imh1), GN (Lbw2/Nba1), splenomegaly (*Imh2), elevated levels of splenic IgMhighIgDlow cells and CD23low/− B cells, and thymic medullary abnormalities (4–13).

Congenic mice in which NZB chromosome 4 intervals have been added or subtracted have also been produced. In one congenic model, the NZB Lbw2 locus was replaced in (NZB × NZW)F1 mice with a New Zealand White (NZW) interval confirming the importance of Lbw2 in GN, mortality, and Ab production (14). In another model, chromosome 4 intervals of various lengths from the NZM2410 mouse strain, that contain both NZB and NZW regions, were introgressed onto the B6 background (15). This revealed the presence of several lupus susceptibility loci, some of which were derived from the NZB mouse strain, and dispelled the notion that B1a cell expansion is required for development of autoimmunity and nephritis (15–17). Although characterization of these congenic mouse strains has been informative, they have not addressed the contribution of a homozygous NZB chromosome 4 interval, which encompasses all mapped traits, on lupus development. In particular, chromosomal intervals from the NZM2410 mouse strain contained only a portion of the mapped lupus susceptibility loci for NZB mice (15). As a consequence, *Imh1, thymic medullary abnormalities, and the impact of a region in Lbw2/Nba1 on distal chromosome 4, linked to renal disease and mortality, have not been explored.

In this study, we have sought to further investigate the involvement of NZB chromosome 4 on the generation of the abnormal
serologic, cellular, and clinical phenotypes in these mice by creating congenic mice bearing homozygous NZB chromosome 4 intervals. Two strains were generated with differing chromosome 4 lengths that encompassed or overlapped with Sle2, Lbw2/Nba1, and/or mapped abnormalities indicative of lupus. Phenotypic analysis demonstrated that many of the lupus traits originally mapped to chromosome 4 and located within our congenic intervals, could not be fully recapitulated in our mice. In particular, there was a lack of high titer autoantibody production and renal disease in both congenic strains. At a cellular level, mice with a long NZB chromosome 4 interval (B6.NZBc4L) encompassing all of the mapped phenotypes, exhibited marked age-related increases in the proportions of splenic, peritoneal, and hepatic B1a cells. Surprisingly, a significant increase in CD1d-restricted NKT cells within the spleen and bone marrow was also detected in these mice. The NKT cell phenotype was striking as a recent study proposed a pathogenic role of an expanded NKT cell population in lupus development. Thus, we have confirmed the presence of an NZB genetic locus that contributes to B1a cell expansion and demonstrate for the first time the presence of a NZB chromosome 4 polymorphism that increases CD1d-restricted NKT cell numbers. Our results indicate that neither a B1a nor an NKT cell expansion is sufficient to produce a lupus phenotype.

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 4 interval onto the B6 genetic background, using the speed congenic technique. Mice were typed at each successive generation using polymorphic microsatellite markers that discriminated 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 B6.NZBc4S and B6.NZBc4L mice were obtained in 11 and 9 generations, respectively, and intercrossed to produce congenic mice that were homozygous for the NZB interval. Previously characterized lupus susceptibility loci, Lbw2, and Sle2, overlap or are encompassed by our B6.NZBc4S and B6.NZBc4L congenic lines, respectively (Fig. 1) (14–15). Both congenic strains also contain the region maximally linked to increased IgM/hIgG phenotype cells and CD23−/low B cells that were originally identified in our prior genetic mapping study (11). Only female B6.NZBc4S and B6.NZBc4L mice were used in this study. All of the mice were housed in microisolators in the animal facility at the Toronto Western Hospital (Toronto, Ontario, Canada) and were specific pathogen free.

Flow cytometry staining and analysis
A total of 5 × 10⁶ RBC-depleted spleen cells was incubated on ice with 10 μg/ml mouse IgG (Sigma-Aldrich) for 15 min to block FcRs 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 using a dual laser FACS Calibur (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-CD11c (HL3), biotin anti-NK1.1 (PK136), PE anti-B220 (RA3-6B2), biotin anti-CD62L (OX-85), biotin anti-CD11b (M1/70), FITC anti-CD21 (7G6), FITC anti-CD23 (PC61.5.3), and FITC anti-IgM (AF6-78). Biotin- and PE-conjugated anti-B220 (RA3-6B2), biotin anti-CD62L (OX-85), FITC anti-CD8 (CT-8D8a), FITC anti-CD44 (KM81), and FITC anti-CD62L (MEL-14) mAbs were purchased from Cedarlane Laboratories. Allophycocyanin-conjugated mCD1d-empty and -PBS57-filled tetramers were provided by the National Institute of Allergy and Infectious Disease MHC Tetramer Core Facility (Atlanta, GA). All isotype controls were purchased from Cedarlane Laboratories except for hamster IgG controls, which were obtained from BD Pharmingen.

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FIGURE 1. Genetic map of chromosome 4 congenic mice. Comparison of the chromosomal intervals in this report, B6.NZBc4S and B6.NZBc4L, with those of congenic mice previously reported, BWF1.NZW-Lbw2 and B6.Sle2. Thick and thin black lines denote NZB and B6 regions, respectively. □ and □ indicate NZW and (NZB/NZW)F1, genetic regions, respectively. A dashed line indicates intervals of undefined origin. Genetic mapping reported in both BWF1.NZW-Lbw2 and B6.Sle2 studies were used to establish the representations of their congenic intervals (14, 17). Polymorphic microsatellite markers used to discriminate NZB and B6 genetic contributions to B6.NZBc4S and B6.NZBc4L congenic intervals are shown to the right of the scale and each interval representation. Markers were mapped using the National Center for Biotechnology Information m37 mouse genome assembly (www.ensembl.org).

Measurement of Ab production
IgM and/or IgG anti-ssDNA, -dsDNA, -chromatin, -thylglobulin, -trinitrophenyl (TNP), -hen egg-white lysozyme (HEL) and -OVA 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 (18). ELISA plates were coated overnight with Ag diluted in PBS (dsDNA, 40 μg/ml; ssDNA, 20 μg/ml; chromatin, 8 μg/ml/thyroglobulin, 50 μg/ml; TNP, 25 μg/ml (Biosearch Technologies)); HEL, 50 μg/ml; OVA, 50 μg/ml washed with PBS/Tween 20 (0.05%), and blocked with PBS-BSA (2%). After repeated washing, serum samples, diluted 1/100 in PBS/BSA/Tween 20, were added. Bound IgG 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, IgG, and IgA, plates were coated with goat anti-mouse IgM, IgG (Jackson ImmunoResearch Laboratories) or IgA (BD Pharmingen), respectively, and the serum was diluted at 1/3,000 for IgM, 1/500–1/5,000 for IgA or 1/10,000 for IgG. Standard curves were performed using class-specific controls (Southern Biotechnology Associates) and the Ab concentration was calculated from a log-log plot of concentration vs absorbance. The presence of ANAs was determined by immunofluorescence microscopy with HEp-2 cells as per the manufacturer’s protocol (Antibodies Incorporated). Serum from 4-, 8-, and 12-mo mice were assayed at a 1/40 dilution.
FIGURE 2. Lack of autoantibody production and renal disease. A. Serum isolated from 8-mo-old B6.NZBc4S, B6.NZBc4L, and age-matched control B6 and NZB mice were examined by ELISA for the presence of IgM and IgG anti-chromatin, -sDNA, and -dsDNA Abs. B. Kidneys from 12-mo-old B6.NZBc4S, B6.NZBc4L, and age-matched control B6 and NZB mice were either: fixed in formalin, paraffin embedded, sectioned, and stained with PAS or frozen in OCT, sectioned and stained with anti-IgM or -IgG, as indicated. Kidneys stained with PAS were graded using glomerular scores on the following scale: grade 0, normal glomeruli; grade 1, mesangial expansion and/or proliferation; grade 2, focal segmental proliferative GN; grade 3, diffuse proliferative GN; and grade 4, diffuse proliferative GN with crescents. Frozen kidney sections stained for immunofluorescence 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. Each point represents the determination from an individual mouse as indicated. Horizontal lines indicate the mean for each population examined.

ELISPOT assay
Splenic IgM-producing cells were detected by ELISPOT assay, as previously described (11). Briefly, 96-well Millititer HA plates (Millipore) were coated with goat anti-mouse IgM (Jackson ImmunoResearch Laboratories). Following blocking with 5% FCS in PBS, freshly isolated splenocytes were plated in triplicate at 10^5 cells and 10^4 cells/well, and incubated for 16 h at 37°C. The plates were repeatedly washed with PBS/Tween 20 (0.05%) and 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 terminated with distilled water. Individual spots, reflecting Ab-producing cells, were visualized using a stereomicroscope.

Hematocrit and Coombs test
The proportion of RBCs was determined by centrifuging capillary tubes containing heparinized blood obtained from saphenous vein bleeds and measuring the hematocrit using a Critocaps microhematocrit capillary tube reader (Oxford Labware). Anti-RBC Ab production was assessed by flow cytometry and the direct Coombs test. For flow cytometry, heparinized blood was washed twice in 2% FCS in PBS and stained with PE anti-B220 (to exclude B cells) and FITC anti-IgM (Caltag Laboratories), FITC anti-IgG (Caltag Laboratories) or FITC anti-hamster IgG (BD Pharmingen). For the direct Coombs tests, cells were diluted in 2% FCS in PBS and plated in 0–10 µl/ml goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) in round-bottom 96-well plates. Plates were incubated for 4 h at 37°C and evaluated for the presence of agglutination using a light microscope.

Immunofluorescence staining and grading of kidney sections
For each mouse, one kidney was fixed in formalin, paraffin embedded, sectioned (3 µm), and stained with periodic acid-Schiff (PAS) for light microscopy and the other was snap-frozen in OCT compound (Sakura Finetek) for immunofluorescence microscopy. Cryostat sections (6 µm) of frozen kidneys were fixed in acetone, washed with PBS, and blocked with PBS/5% normal goat serum, before staining with FITC-conjugated anti-IgM F(ab')2 (Caltag Laboratories) or FITC-conjugated anti-IgG F(ab')2 (Caltag Laboratories), and mounting with Mowiol (Calbiochem). All 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 GN; grade 3, diffuse (endocapillary) proliferative GN; and grade 4, diffuse proliferative GN with crescents. Immunofluorescence 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, the χ² test was used.

Results
Serologic phenotype of B6.NZBc4S and B6.NZBc4L congenic mice
To determine whether B6.NZBc4S and B6.NZBc4L mice have serologic hallmarks of lupus, cohorts of congenic mice were aged up to 12 mo. Serum from 8-mo B6.NZBc4L demonstrated an increase in total serum IgM and IgA production, whereas both congenic strains had elevated levels of total serum IgG Abs as compared with B6 mice (total serum IgM (mg/ml): B6 = 1.74 ± 0.66,
Contribution of NZB chromosome 4 to lupus nephritis

Renal disease can develop in the absence of anti-dsDNA or -chromatin Ab production (19). Therefore, congenic mice were monitored regularly for development of proteinuria by urinary dipstick and kidney pathology was assessed in mice sacrificed at 12 mo. Both congenic strains exhibited at most low-grade proteinuria, which was comparable to that seen in control B6 mice (data not shown). Ig deposition and light microscopic changes in kidney sections of sacrificed mice were graded on a 4-point scale as outlined in Materials and Methods. Although there was a trend to increased IgM and IgG deposition and kidney damage in B6.NZBc4L mice, this did not reach statistical significance (Fig. 2B). Thus, the NZB chromosomal interval contained in B6.NZBc4S or B6.NZBc4L mice is insufficient in itself to produce extensive renal disease.

Increased spleen weight and expansion of splenic CD21low/intCD23+ B cells in B6.NZBc4L mice

To determine whether the congenic mouse strains displayed any of the abnormal cellular phenotypes seen in NZB mice, B6.NZBc4S and B6.NZBc4L were aged to 4, 8, and 12 mo and their spleens were analyzed. Increased spleen weight was observed in B6.NZBc4L congenic mice at all age points examined as compared with B6 mice, but was not as marked as that seen in NZB mice (Table I). This was not accompanied by an increase in the number of splenocytes suggesting an increase in splenic tissue or nonlymphocyte populations in B6.NZBc4L mice (Table I). To examine B cell control, and congenic splenocytes were stained and analyzed by flow cytometry. Expression of B220, CD21, and CD23 was used to discriminate the various peripheral B cell subsets within the spleen. Although NZB mice have reduced proportions of CD21lowCD23+ B cells in B6.NZBc4L congenic (Table I, data not shown). Thus, B6.NZBc4S and B6.NZBc4L exhibit a nonautoactive serological phenotype with slightly elevated total serum IgM, IgG, and/or IgA production.

Sera from both congenic strains were also tested for polyreactivity with a variety of Ags, including, thyroglobulin, TNP, HEL, and OVA. Congenic sera were not reactive to the aforementioned Ags, except for a minor increase in IgM anti-thyroglobulin Abs in B6.NZBc4L mice (OD: B6 = 0.029 ± 0.026, B6.NZBc4S = 0.032 ± 0.034, B6.NZBc4L = 0.043 ± 0.033, and NZB = 0.073 ± 0.050, p = 0.0495 for B6.NZBc4L as compared with B6, n = 11–12; data not shown).
was significantly larger in B6.NZBc4L mice than in NZB mice (Table I, 8 mo, \( p = 0.0011 \); 12 mo, \( p = 0.0097 \)). Additional stains confirmed that the B220\(^{low}\)CD5\(^{low}\) cells were splenic B1a cells as they were CD21\(^{low/int}\), CD23\(^{hi}\), IgM\(^{hi}\), IgD\(^{-}\), and B7.1\(^{-}\) (data not shown). Examination of the proportion of CD5\(^{-}\) cells that contributed to the CD21\(^{low/int}\) B cell population revealed an expansion similar to the increase in CD21\(^{low/int}\)CD23\(^{hi}\)B cells (percentage of CD5\(^{-}\)CD21\(^{low/int}\)B cells: 8 mo, B6 = 5.73 ± 0.90 and B6.NZBc4L = 7.87 ± 1.92, \( p = 0.003 \); 12 mo, B6 = 6.09 ± 1.36 and B6.NZBc4L = 15.72 ± 8.30, \( p = 0.0019 \); \( n = 5–12 \)). This suggested that the expanded splenic B1a cell population was primarily responsible for this increase. In further support of this conclusion, there was no increase in the proportion of preplasmablast (CD138\(^{hi}\)), T1 (AA4.1\(^{hi}\)), or memory/germinal center (IgM\(^{hi}\)) cells in the CD21\(^{low/int}\) B cell population (data not shown). Consistent with an expansion of B1a cells, which are the primary producers of natural serum IgM, an almost 2-fold increase in the numbers of splenic IgM-producing cells in 4- and 8-mo B6.NZBc4L congenic mice was observed compared with B6 mice (Table I) (21). Nevertheless, values obtained did not reach levels observed in NZB mice.

B1a cells also reside in the peritoneal cavity (perC) (21). We therefore performed flow cytometric analysis on perC cells.

### FIGURE 3.
Expansion of the splenic and perC B1a cell compartment in B6.NZBc4L mice. Freshly isolated splenocytes or perC cells from 8-mo female B6 and B6.NZBc4L mice were stained with anti-B220 and -CD5. A. Shown are representative dot plots gated on PI-excluding cells and, in the case of peritoneal cells, lymphocytes, defined by forward and side scatter. Regions used to determine the proportions of B220\(^{low}\)CD5\(^{low}\) (B1a) and B220\(^{high}\)CD5\(^{low}\) (B2) cells are indicated. Numbers within the regions indicate the percentage of cells in the total splenocyte or lymphocyte-gated peritoneal cell population. B. Scatterplots of 4, 8, and 12 mo B6, B6.NZBc4S, B6.NZBc4L, and NZB peritoneal B cell populations. The frequency of B220\(^{low}\)CD5\(^{low}\) (B1a) and B220\(^{high}\)CD5\(^{low}\) (B2) cells were calculated as a percentage of the total lymphocyte population. Cells were stained with anti-CD5 and -B220, and gated as shown in A. In all scatterplots, only the \( p \) values for significant differences between B6 and B6.NZBc4S or B6.NZBc4L, and between NZB and B6.NZBc4L, are shown. Each point represents the determination from an individual mouse. Horizontal lines indicate the mean for each population examined.
within the total CD3
B6 mice:

B220lowCD5
ined, an impressive age-related increase in the frequency of perC
was less pronounced by 8 mo. In contrast, at all age points exam-
for each population examined.

Although results were pooled from three independent experiments, the findings were consistent between experiments. Horizontal lines indicate the mean
Table II. Splenic T cell phenotype in B6.NZBc4S and B6.NZBc4L congenic mice at 4, 8, and 12 mo old

<table>
<thead>
<tr>
<th>Age (mo)</th>
<th>Strain</th>
<th>% CD4+CD3+ Cells</th>
<th>% CD4+CD3+ NK1.1+ Cells</th>
<th>% CD4+CD3+ NK1.1+ Cells</th>
<th>% CD4+CD3+ NK1.1+ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>B6</td>
<td>18.3 ± 2.3 (21)</td>
<td>1.2 ± 0.3 (23)</td>
<td>1.0 ± 0.5 (23)</td>
<td>17.0 ± 2.3 (23)</td>
</tr>
<tr>
<td></td>
<td>B6.NZBc4Sb</td>
<td>20.5 ± 2.9** (15)</td>
<td>1.1 ± 0.3 (15)</td>
<td>0.9 ± 0.2 (15)</td>
<td>19.3 ± 2.7* (15)</td>
</tr>
<tr>
<td></td>
<td>B6.NZBc4L</td>
<td>23.1 ± 4.2*** (20)</td>
<td>2.1 ± 0.4*** (20)</td>
<td>1.3 ± 0.2*** (20)</td>
<td>21.0 ± 4.1** (20)</td>
</tr>
<tr>
<td></td>
<td>NZB</td>
<td>24.3 ± 3.2*** (5)</td>
<td>1.6 ± 0.2* (5)</td>
<td>0.9 ± 0.2 (5)</td>
<td>22.6 ± 3.2** (5)</td>
</tr>
<tr>
<td>8</td>
<td>B6</td>
<td>16.6 ± 3.3 (24)</td>
<td>1.0 ± 0.2 (13)</td>
<td>1.3 ± 0.4 (13)</td>
<td>15.4 ± 3.2 (24)</td>
</tr>
<tr>
<td></td>
<td>B6.NZBc4Sb</td>
<td>18.8 ± 3.1* (22)</td>
<td>1.0 ± 0.2 (13)</td>
<td>1.1 ± 0.2 (13)</td>
<td>17.5 ± 3.3* (22)</td>
</tr>
<tr>
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<td>B6.NZBc4L</td>
<td>22.8 ± 3.9*** (16)</td>
<td>2.3 ± 0.4*** (16)</td>
<td>1.5 ± 0.2* (16)</td>
<td>20.1 ± 3.8** (16)</td>
</tr>
<tr>
<td></td>
<td>NZB</td>
<td>19.0 ± 4.4 (7)</td>
<td>0.5 ± 0.1** (5)</td>
<td>0.3 ± 0.1 (5)</td>
<td>18.2 ± 4.3 (7)</td>
</tr>
<tr>
<td>12</td>
<td>B6</td>
<td>15.8 ± 2.5 (6)</td>
<td>0.8 ± 0.2 (6)</td>
<td>1.1 ± 0.3 (6)</td>
<td>14.9 ± 2.5 (6)</td>
</tr>
<tr>
<td></td>
<td>B6.NZBc4Sb</td>
<td>16.4 ± 1.9 (8)</td>
<td>0.7 ± 0.1 (8)</td>
<td>1.2 ± 0.3 (8)</td>
<td>15.6 ± 1.7 (8)</td>
</tr>
<tr>
<td></td>
<td>B6.NZBc4L</td>
<td>19.1 ± 3.2 (13)</td>
<td>2.1 ± 0.2** (13)</td>
<td>1.6 ± 0.3* (13)</td>
<td>16.6 ± 3.1 (13)</td>
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<tr>
<td></td>
<td>NZB</td>
<td>14.9 ± 4.6 (9)</td>
<td>0.5 ± 0.1* (9)</td>
<td>0.4 ± 0.2 (9)</td>
<td>14.2 ± 4.3* (5)</td>
</tr>
</tbody>
</table>

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

b Results for B6.NZBc4S mice highlighted in bold are significantly different (p < 0.05) from those obtained for age-matched B6.NZBc4L mice.

Although only B6.NZBc4L mice had an expanded splenic B1a cell population, both congenic strains had an increase in perC B220lowCD5+ cells (Fig. 3B). In B6.NZBc4S mice, an increased proportion of perC B220lowCD5+ cells was observed at 4 mo, but was less pronounced by 8 mo. In contrast, at all age points exam-
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with a perC B1a cell phenotype, the expanded B220lowCD5+ subset was IgD−, IgM−, and primarily Mac-1+. Although the propor-
tion of perC conventional B2 cells was lower in both congenic strains, the total absolute number of B6.NZBc4L perC B2 cells
was similar to that seen in B6 mice (Fig. 3B, data not shown).

In (NZB × NZW)F1 mice, B1a cells are not only expanded but
also traffic abnormally to other organs, such as the thymus (22–23).

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ined, an impressive age-related increase in the frequency of perC B220lowCD5+ cells was observed in B6.NZBc4L mice. Consistent
with a perC B1a cell phenotype, the expanded B220lowCD5+ subset was IgD−, IgM−, and primarily Mac-1+. Although the propor-
tion of perC conventional B2 cells was lower in both congenic strains, the total absolute number of B6.NZBc4L perC B2 cells
was similar to that seen in B6 mice (Fig. 3B, data not shown).

In (NZB × NZW)F1 mice, B1a cells are not only expanded but
also traffic abnormally to other organs, such as the thymus (22–23).
Assessment of the thymus and bone marrow of our congenic and control mice revealed no differences in the proportions of B1a cells at 4 and 8 mo (data not shown). However, an accumulation of B1a cells in the liver was observed in B6.NZBc4L mice at 8 mo (percentage of B220lowCD5− cells: B6: 5.81 ± 1.47, B6.NZBc4L: 11.62 ± 3.09 (p = 0.0002), n = 10–13). As in the perC, a proportion of liver B1a cells was Mac-1+ (data not shown). Collectively, these results show that B6.NZBc4L mice exhibit an accumulation of splenic, peritoneal, and hepatic B1a cells, as found in NZB mice.

Tissue-specific expansion of NKT cells in B6.NZBc4L congenic mice

In addition to B cell phenotypic differences, NZB mice demonstrate a number of other splenic cellular abnormalities (20). To determine whether any non-B cell abnormalities were seen in our congenic mice, the proportions of various splenic cell subsets were analyzed by flow cytometry. Assessment of non-T cell populations (NK, macrophage, and dendritic cells) and γδ and CD8+ T cells revealed no difference in the frequency or activation status of these cells (data not shown). In contrast, as seen in NZB mice, there was an age-related expansion of CD4+CD3− cells (CD4+ T cells) in both congenic mouse strains, which was most prominent in B6.NZBc4L mice (Table II). To determine the subset(s) of CD4+ T cells contributing to this expansion, splenocytes were stained to measure the frequency of NKT, T regulatory and memory T cells. No changes in the proportions of these populations or differences in activation status (CD69 expression) were found in B6.NZBc4S mice, suggesting a small increase in naive, conventional CD4+ T cells (data not shown). In 4-mo B6.NZBc4L mice, a transient increase in memory T cell (CD4+CD44hi/CD62Llo) proportions (p = 0.0026) and a small reduction in T regulatory (CD4+CD25+CD62Llo) proportions (p = 0.0104) was observed, but was not maintained at 8 or 12 mo (data not shown). In contrast, a consistently >2-fold increase in CD4+CD44hi/CD62Llo proportions was observed, and was not maintained at 8 or 12 mo (data not shown). In addition, a small increase in CD4− NKT cells was also observed, demonstrating an overall increase in splenic NKT cells (Table II).

The NKT cell population can be further subdivided by CD1d restriction. Therefore, freshly isolated splenocytes were stained with CD1d tetramers (CD1d-Tet) loaded with an α-galactosylceramide (α-galcer) analog (PBS57) to specifically identify CD1d-dependent NKT cell precursors (www.yerkes.emory.edu). This revealed a significantly increased frequency of CD1dTet−CD3+ cells in B6.NZBc4L mice (Fig. 4, A and B). This population can be further divided into CD4+ and CD4− (double-negative (DN)) subsets, both of which were expanded in B6.NZBc4L mice (Fig. 4B and data not shown). In contrast to B6 mice, where the majority of NKT cells are CD1d independent, the majority of B6.NZBc4L splenic NKT cells were CD1d dependent (Fig. 4E, Ref. 24).

Immature and recently activated NKT cells are NK1.1+ (25–26). Therefore, to determine whether the expansion of CD4−CD3−NK1.1− cells in B6.NZBc4L mice was due to this population, cells were stained with the CD1d tetramer. Only a small population of NK1.1− cells was CD1d-Tet+, suggesting that the increase in CD4−CD3−NK1.1− cells in B6.NZBc4L mice was primarily due to an increase in conventional CD4+ T cells (percentage of NK1.1− CD1d-Tet− cells at 4 mo: B6, 0.38 ± 0.04, and B6.NZBc4L, 0.60 ± 0.09, n = 3–4). No significant differences in the proportion of CD1d-independent NKT cells (CD1dTet−CD3−NK1.1+) were observed in B6.NZBc4L mice (data not shown).

Mature NKT cells also localize to the liver, thymus, and bone marrow (26). Trends for an increased proportion and number of mature NKT cells were observed in the liver and thymus, but did not achieve statistical significance (data not shown). In contrast, a marked increase in NKT cells was detected in the bone marrow (Fig. 4, C–E). As in the spleen, the majority of the expanded CD1d-dependent NKT cells were CD4+, with a small frequency expressing neither CD4 nor CD8 (DN; Fig. 4D and data not shown). Thus, B6.NZBc4L mice display a profound NKT cell abnormality in the spleen and bone marrow suggesting the presence...
of a locus affecting NKT cell frequencies in the proximal region of NZB chromosome 4.

Thymic abnormalities in B6.NZBc4L congenic mice

To determine whether the NKT cell expansion in B6.NZBc4L mice could be traced back to abnormalities in T cell development, thymi of 4-mo B6.NZBc4L mice were examined. Compared with B6 mice, congenic B6.NZBc4L thymi were visually larger with a trend to increased number of thymocytes (data not shown). Examination of the CD4+CD8– (DN) thymocyte subsets, based on CD44 and CD25 expression, showed a marked increase in the frequency and number of DN CD44+CD25– (DN1) and a slight decrease in the proportion but not number of DN CD44+CD25+ (DN4) thymocytes in B6.NZBc4L mice (Fig. 5A and data not shown). No difference in the proportions of DN CD44+CD25+ (DN2), DN CD44–CD25+ (DN3), CD4+ (single-positive (SP)), CD8+ (SP) or CD4+CD8+ (double-positive (DP)) cells was observed in B6.NZBc4L mice, as compared with B6 mice (Fig. 5A, data not shown). Thus, although the B6.NZBc4L thymus contains more DN1 thymocytes, normal proportions of DP and SP thymocytes are maintained.

Because developing immature NKT cells do not express NK1.1, CD1d tetramers were used to examine CD1d-restricted NKT cell development in the thymus. Early thymic CD1d-restricted NKT precursors are CD4+ or DN HSAlow cells (27). In B6.NZBc4L mice, a trend for increased thymic CD1d-Tet+ HSAlow cells with or without CD4 expression was detected (Fig. 5B). This observation suggests that the NKT cell expansion in the periphery may originate, at least in part, from altered thymocyte development.

Discussion

To further investigate the contribution of NZB chromosome 4 genetic loci to the generation of the abnormal B cell phenotypes and lupus susceptibility in NZB mice, we produced B6 congenic mice with introgressed homozgyous NZB chromosome 4 intervals extending from either 114 to 149 Mb, denoted B6.NZBc4S, or 32 to 149 Mb, denoted B6.NZBc4L. We show that congenic mice with an interval containing only the distal portion of chromosome 4 have a serologic and cellular profile almost indistinguishable from B6 control mice. In contrast, B6.NZBc4L mice, with an interval that encompasses all previously mapped chromosome 4 cellular and lupus traits, exhibited several serologic and cellular abnormalities. Most striking were the differences in total serum Ig, the expansion of B1a cells, and an unexpected increase in NKT cells. Interestingly, these phenotypes were observed in the absence of high titer autoantibody production and renal disease. Thus, the results provide strong evidence that genetic loci leading to expanded B1a and/or NKT cell populations are insufficient to promote lupus development.

Of all the phenotypes mapped to chromosome 4, only elevated total serum IgM production, increased spleen weight, and an expanded IgM+IgD–CD23– B cell population were evident in our congenic mice. Although Inhl1, an NZB locus linked to increased total serum IgM levels, showed peak linkage in a region contained in both congenic intervals, only B6.NZBc4L mice demonstrated this phenotype (4–5). Similarly, the increased splenic IgM+IgD–CD23– B cell population that our laboratory previously mapped to NZB chromosome 4, with peak linkage at 123.3 Mb, was restricted to B6.NZBc4L mice (11). In this study, we show that this phenotype is due to an expansion of the splenic B1a cell compartment. As B1a cells are the primary producers of natural serum IgM, it is likely that these two phenotypes result from the same genetic polymorphism. The absence of these phenotypes in B6.NZBc4S mice suggests that this locus is located closer to the centromere than previously thought (17). Splenomegaly was also only seen in B6.NZBc4L mice, consistent with the maximal linkage of a NZB splenomegaly locus, Sbw2, to a region that excludes the B6.NZBc4S interval (10). It is important to note that these abnormalities did not reach levels characteristic of NZB mice, indicating that other loci may additively contribute to the generation of these phenotypes. This possibility is supported by mapping studies that linked these traits to different chromosomes and our previous demonstration that B6.NZBc13 congenic mice, with an introgressed NZB chromosome 13 interval, share these phenotypes (10, 20).

Chromosome 4 intervals from NZM2410 mice, a recombinant inbred strain derived from NZB and NZW mouse strains, have been introgressed onto a B6 background (15, 17, 28). These intervals contain homozygous NZW (55–99 Mb) and NZB (99–127 Mb) regions. Consistent with the phenotype of our congenic mice, B6.Sle2 congenic mice demonstrated an expansion of B1a cells in the absence of autoantibody production and renal disease (15). Further analysis of Sle2 subcongenic mice showed that an NZB locus, Sle2c, that overlaps with or is encompassed within our NZBc4S or NZBc4L intervals, respectively, leads to expansion of perC B1a cells (17). Our results confirm and extend this finding, as comparison of the Sle2c locus with our congenic intervals successfully narrows down the location of the polymorphism(s) that produce the B1a cell phenotype to a region that is ~11 Mb (103.2–114.5 Mb). Within this region there are ~60 known genes, and of these genes we have identified three candidate genes: Rab3b (RAB3B, 107.8–107.9 Mb), Cdkn2c (cyclin-dependent kinase inhibitor 2C, also known as p18INK4c; 108.6 Mb), and Fas (Fas-associated factor 1; 108.6–108.9 Mb). Our laboratory is currently investigating these genes.

In B6.Sle2 mice, Sle2c is not associated with expansion of the splenic B1a cell subset. Another locus, Sle2a, also located within the NZBc4L interval, but NZW derived, was found to produce this phenotype. Thus, it is unlikely that the expansion of splenic B1a cells in our congenic mouse strain arises from the same locus that drives the increase in perC B1a cells. Instead, NZB and NZW mice may share the same Sle2a genetic polymorphism. However, unlike B6.Sle2, B6.NZBc4L mice do not demonstrate an enhanced thymus-independent Ag response to TNP-LPS immunization suggesting that B6.NZBc4L mice lack the polymorphism responsible for this phenotype (our unpublished observations).

In a subtraction congenic mouse strain that was generated by replacing the chromosome 4 Lbw2 locus (62–141 Mb) in NZB mice with an NZW interval (NZB.NZW-Lbw2), comparison of (NZB.NZW-Lbw2 × NZW)F1, with (NZB × NZW)F1, mice revealed that Lbw2 contributes to the increased serum levels of IgM, number of total IgM- and IgG-producing and IgM anti-dsDNA-producing cells, GN, and mortality in (NZB × NZW)F1 mice (14). Our findings of elevated serum Igs and increased numbers of IgM ELISPOTs in B6.NZBc4L mice confirm the presence of genetic loci that impact on Ab production in this region. Previous mapping studies indicate that the chromosomal region associated with GN and mortality is located within the B6.NZBc4S interval and the positioning of increased mortality to this region has been confirmed using subtraction congenic mice (15). These observations suggest that the GN susceptibility locus is distinct from those producing the cellular abnormalities in B6.NZBc4L mice.

The expansion of B1a cells in B6.NZBc4L mice represents one of the most penetrant and consistent phenotypes of our congenic mice. In fact, by 12 mo, we observed an increased number of B1a cells in B6.NZBc4L mice, which exceeded that seen in NZB mice. This difference may be explained by the absence of other NZB...
genetic polymorphisms in B6.NZBc4L mice, which may negatively impact on the proportion of B1a cells by impairing cellular production/renewal or increasing their recruitment into the autoimmune response.

Studies of genetically altered mice indicate that increases in the size of the B1a compartment arise with mutations that increase the strength of BCR signaling or affect expression of growth factors used by B1a cells (21). In support of a genetic defect in B cell signaling, perC B1a from B6.Sle2 displayed enhanced proliferation and resistance to apoptosis (16). Because our B6.NZBc4L mouse shares an NZB region in common with Sle2, it is possible that the same defect will be found in B6.NZBc4L perC B1a cells. Ongoing functional studies are addressing this issue. Alternatively, that the same defect will be found in B6.NZBc4L perC B1a cells.

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...lupus development (59–60). In contrast, (NZB × NZWF1)F1 mice have an expansion of CD1d-restricted NKT cells at the onset of disease and blockade of CD1d interactions using a mAb inhibits disease progression (45, 53). The CD1d-restricted NKT cells in (NZB × NZWF1)F1 mice demonstrate reduced NK1.1 expression, which has been linked to chronic activation and increased secretion of IFN-γ upon α-galcer stimulation (45). As the expanded NKT cell population in B6.NZBc4L mice does not express lower levels of NK1.1, it is likely that their activation differs from those in (NZB × NZWF1)F1 mice (our unpublished observations). Ongoing studies are addressing the cytokine response of the CD1d-restricted NKT cells in B6.NZBc4L mice that is induced by α-galcer.

...data suggest that not only the expansion but also the activation status of NKT cells dictates their role in the autoimmune process.

Disclosures

The authors have no financial conflict of interest.

References