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The Lbw2 Locus Promotes Autoimmune Hemolytic Anemia

John C. Scatizzi,* Maria K. Haraldsson,* K. Michael Pollard, † Argyrios N. Theofilopoulos,* and Dwight H. Kono*

The lupus-prone New Zealand Black (NZB) strain uniquely develops a genetically imposed severe spontaneous autoimmune hemolytic anemia (AIHA) that is very similar to the corresponding human disease. Previous studies have mapped anti-erythrocyte Ab (AEA)-promoting NZB loci to several chromosomal locations, including chromosome 4; however, none of these have been analyzed with interval congenics. In this study, we used NZB.NZW-Lbw2 congenic (designated Lbw2 congenic) mice containing an introgressed fragment of New Zealand White (NZW) on chromosome 4 encompassing Lbw2, a locus previously linked to survival, glomerulonephritis, and splenomegaly, to investigate its role in AIHA. Lbw2 congenic mice exhibited marked reductions in AEAs and splenomegaly but not in anti-nuclear Abs. Furthermore, Lbw2 congenics had greater numbers of marginal zone B cells and reduced expansion of peritoneal cells, particularly the B-1a cell subset at early ages, but no reduction in B cell response to LPS.

Analysis of a panel of subinterval congenic mice showed that the full effect of Lbw2 on AEA production was dependent on three subloci, with splenomegaly mapping to two of the subloci and expansions of peritoneal cell populations, including B-1a cells to one. These results directly demonstrated the presence of AEA-specific promoting genes on NZB chromosome 4, documented a marked influence of background genes on autoimmune phenotypes related to Lbw2, and further refined the locations of the underlying genetic variants. Delineation of the Lbw2 genes should yield new insights into both the pathogenesis of AIHA and the nature of epistatic interactions of lupus-modifying genetic variants. The Journal of Immunology, 2012, 188: 000–000.

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utoimmune hemolytic anemia (AIHA) occurs in 7–22% of patients with systemic lupus erythematosus (SLE) (1–5) and often precedes the diagnosis of SLE. Moreover, 50–91% of patients initially diagnosed with idiopathic AIHA subsequently develop SLE (4, 6, 7). The anti-erythrocyte Abs (AEAs) are usually of the warm (IgG and IgA) or mixed warm and cold (IgM) isotypes and target several different RBC Ags, most commonly the anion channel exchanger, band 3 (SLC4A1, solute carrier family 4, and member 1), glycoporphin A, and others (8–10). Although it is likely that genetic susceptibility contributes to this disease, support is limited to a single genomewide family study that identified two loci of “suggestive” significance at 1q24 and 11q14 (11).

The lupus-prone New Zealand Black (NZB) mouse is unique among inbred strains in developing severe autoimmune hemolytic anemia that, similar to human disease, is associated with primarily warm-type AEAs and band 3 as the major target (12–16). NZB mice also exhibit elevated serum IgM, autoantibodies to nuclear Ags, expansions of B-1 and marginal zone (MZ) B cell populations, and mild late-onset glomerulonephritis (GN), although the relationship of these manifestations to autoimmune hemolytic anemia is not known (13, 17). Mapping studies have identified NZB loci associated with the production of AEAs on several chromosomes, consistent with polygenic inheritance (18–22). Of these, those on chromosomes 4 (named Aia1 and Aem1), 7 (Aem2), and 10 (Aem3) were detected in more than one study. However, none of the loci have been verified with interval congenic mice.

Crossing NZB and the mildly lupus-prone New Zealand White (NZW) strains yields offspring (BWF1) that develop early-onset lupus with high levels of IgG autoantibodies and fatal membranoproliferative GN, but not AIHA, consistent with recessive transmission of AEAs (13). To define lupus-predisposing genes in NZB mice, we previously conducted a genomewide mapping study of BWF1 intercrosses and identified several susceptibility loci (Lbw1–8), among which Lbw2, a NZB locus on chromosome 4 that overlaps with the aforementioned loci linked to AEA production, was associated with early mortality, GN, and splenomegaly (23). We further investigated the role of Lbw2 in BWF1 mice using Lbw2 congenic mice and documented that the NZB Lbw2 interval promoted GN and early mortality but did not affect serum IgG anti-DNA Abs or splenomegaly (24). The presence of the NZB Lbw2 allele also increased B cell response to LPS, the number of spontaneous and anti-DNA Ab-secreting cells in the spleen, and immune complex deposits in the kidney, suggesting that the NZB Lbw2 locus might promote autoimmunity by enhancing B cell activation.

To investigate the role of the NZB Lbw2 interval in AIHA, we replaced the Lbw2 locus with the NZW genome in NZB mice and showed that this reduces AEAs and splenomegaly. Furthermore, this resulted in fewer peritoneal B-1a cell numbers but did not alter either serum anti-nuclear Ab levels or B cell activation. By analyzing a panel of subinterval specific Lbw2 congenic mice, we further showed that the AEA-affecting Lbw2 interval contains three nonoverlapping subloci.

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Abbreviations used in this article: AEA, anti-erythrocyte Ab; AIHA, autoimmune hemolytic anemia; B6, C57BL/6; BWF1, (NZB × NZW)F1; GN, glomerulonephritis; Lbw2, NZB.NZW-Lbw2; MFI, mean fluorescence intensity; MZ, marginal zone; NZB, New Zealand Black; NZW, New Zealand White; SLE, systemic lupus erythematosus.

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Materials and Methods

Mice

NZB/B1ScCr (NZB), C57BL/6 (B6), and NZB/NZW-Lbw2 (Lbw2) congenic and subcongensics were bred and maintained at The Scripps Research Institute Animal Facility. The Lbw2 congenic was generated by marker-assisted backcrossing for seven generations. Chromosomal markers used to define congenic intervals are listed in Supplemental Table I. Markers corresponding NZB and NZW genomic regions. Female mice were exclusively used. Experiments were approved by The Scripps Institutional Animal Care and Use Committee.

AEAs

Direct Coombs’ tests were performed as described previously (25). Briefly, washed RBCs diluted in 1.5% BSA PBS were incubated in 96-well round-bottom plates with anti-mouse anti-IgG Ab (1:100 and 1:1000 dilution; MP Biomedicals, Solon OH) for 4 h at 37 C and then scored for agglutination. In addition, flow cytometry detection of bound AEAs was performed by incubating washed RBCs first with biotin-conjugated goat anti-mouse IgG (MP Biomedicals) and then with streptavidin-PE (BioLegend, San Diego CA). Data were acquired on an LSRII using FACSDiva software (BD Biosciences, San Jose, CA). Coombs’ test was considered positive with either agglutination at both dilutions or a positive agglutination at one dilution plus a mean fluorescence intensity (MFI) > 250 as determined by flow cytometry.

Polycyclonal Ig and anti-chromatin

Ab concentrations were determined by ELISA as described previously (24). Briefly, wells in 96-well plates were coated with goat anti-mouse Ab (Southern Biotechnology Associates, Birmingham, AL) or chromatin in PBS and sequentially incubated after washes with appropriately diluted sera, alkaline phosphate-conjugated goat anti-mouse secondary Abs (Southern Biotechnology Associates), and detecting substrate (p-nitrophenol phosphate in ethanolamine; Thermo Fisher Scientific, Rockport, IL). OD values were obtained on a plate reader, and IgG and IgM concentrations were extrapolated from standard curves generated using polyclonal mouse reference sera (Bethyl Laboratories, Montgomery, TX).

Fluorescent anti-nuclear Ab

Indirect immunofluorescence was performed on sera, as described previously (26). Briefly, monolayers of HEp-2 cells on slides (Bion Enterprises, Sunnyvale CA) were first incubated with biotin-conjugated goat anti-mouse IgG (MP Biomedicals) and then with streptavidin-PE (BioLegend). Data were acquired on a plate reader, and IgG and IgM concentrations were determined by ELISA as described previously (24).

Cells and cell cultures

Splenocytes or purified B cells (3 × 10^5/well) in RPMI 1640 supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine in 5% CO2 were stimulated with serial concentrations of LPS (O55:B5; Sigma-Aldrich, St. Louis, MO) for up to 5 d. A total of 0.5 μCi [3H]Tdr were added to the media 18 h prior to harvesting, and counts per minute were normalized by subtracting media-alone values.

Statistical analysis

Data were analyzed using the GraphPad Prism software program (GraphPad, La Jolla, CA). Mann–Whitney U test was used to compare

FIGURE 1. The NZB Lbw2 locus promotes AEAs but not anti-nuclear Abs. (A) Serial IgG direct Coombs’ test results for NZB and Lbw2 congenic mice (n = 7–38/group). Percentage of mice positive for Coombs’ test from 7 to 12 mo of age is shown. (B) MFIs of IgG AEAs in all mice (n = 13–38 mice/group). Mean ± SEM MFI of only Coombs’ positive NZB and Lbw2 congenic mice were 2895 ± 565 and 436 ± 35, respectively, at 9 mo (p < 0.001) and at 12 mo 2429 ± 485 and 293 ± 6 (p < 0.001). MFI was measured by flow cytometry. (C and D) Total Ig and anti-chromatin autoantibody concentrations. Serum Abs were measured by ELISA (n = 5–12 mice/group). (E) Fluorescence intensity scores for 9-mo-old NZB and Lbw2 congenic mice (n = 6 mice/group). Statistical significance for Lbw2 or B6 compared with NZB is indicated by asterisks alone, while Lbw2 compared with B6 is indicated by a bar and asterisks. In addition to Mann–Whitney U’ test, data were analyzed by Fisher’s exact (A). Horizontal bars in (B)–(E) indicate mean values. *p < 0.05, **p < 0.01, ***p < 0.001.
groups unless indicated otherwise. Fisher’s exact test was used in Coombs’ test results and linear regression analysis of slope and intercept/elevation was performed to compare changes in spleen weights with age. \( p < 0.05 \) was considered significant.

**Results**

The **NZB Lbw2 locus promotes AEs but not anti-nuclear Abs**

To define the role of the *Lbw2* locus in AEA production, NZB and *Lbw2* congenic mice were assessed for Coombs’ positivity monthly for a total of 1 y. This revealed a striking reduction in AEs in *Lbw2* congenic mice compared with NZB mice that was clearly evident at the earliest 7-mo time point (Fig. 1A). Hence, at 9 mo of age, 95% of the NZB mice were Coombs’ positive compared with only 26% of the *Lbw2* congenic mice, and at 12 mo of age, all NZB mice were affected, less than ~40% of the congenics were positive (Fig. 1A). Furthermore, the amount of AEs on RBCs was also lower in *Lbw2* congenics even when comparing only Coombs’ positive mice (Fig. 1B).

In contrast, NZB and *Lbw2* congenic mice showed no differences in total IgM and IgG serum Ab levels, which, at 12 mo, were comparable to the nonautoimmune B6 mice (Fig. 1C). Similarly, there were no differences in anti-chromatin Abs or ANA were comparable to the nonautoimmune B6 mice (Fig. 1C).

**Splenomegaly is reduced in Lbw2 congenic mice**

Because of the initial association of *Lbw2* with splenomegaly, we investigated whether *Lbw2* affected spleen weights in the NZB background (20, 23). Indeed, 1-yr-old *Lbw2* congenic NZB mice displayed substantially lower spleen weights that were strikingly only slightly greater than those of nonautoimmune B6 mice (Fig. 2A). Hence, by 11–14 mo, <50% (7 of 16) of *Lbw2* congenics exhibited splenomegaly (mean + 4 SD of B6 spleen weights) compared with 97% (31 of 32) of NZB mice. Furthermore, when spleen weights were plotted against age, an extreme difference in the slopes between the NZB and *Lbw2* congenic mice was apparent (Fig. 2B). Thus, *Lbw2* had a profound influence on splenomegaly in NZB mice, in sharp contrast to its lack of effect in the BWF1 background (24).

To investigate the reason for the lower spleen weights in *Lbw2* congenics, histologic and cellular characteristics of spleens were analyzed. Sections of 1-yr-old NZB spleens revealed a marked accumulation of RBCs within the red pulp and severe distortion of the normal architecture, which was much less evident for *Lbw2* congenics (Fig. 2C). Because this suggested that splenomegaly was mainly attributable to RBC sequestration, we compared the total number of RBCs or RBC precursors (extramedullary erythropoiesis) in NZB and *Lbw2* congenic spleens by staining for erythroid lineage (Ter119\(^+\)) cells prior to (unlysed) or after depletion (lysed) of mature RBCs. Consistent with the histological findings, NZB spleens had a much greater number of spleen cells than the *Lbw2* congenics, and this was due to a marked increase in Ter119\(^+\) erythrocytes (Fig. 2D). In contrast, when spleens depleted of mature RBCs were examined, no significant differences in either the total or Ter119\(^+\) immature erythrocyte cell numbers were observed between the NZB and *Lbw2* congenic groups, suggesting that the larger spleens in NZB mice were almost entirely attributable to the accumulation of mature RBCs (Fig. 2E). Thus, it can be concluded that, in the NZB background, *Lbw2* is associated with splenomegaly resulting from AEA-related accumulation of RBCs.

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**FIGURE 2.** The *Lbw2* locus promotes splenomegaly in NZB mice. (A) Spleen weights from mice between the ages of 11 and 14 mo (\( n = 32 \) NZB, 18 *Lbw2*, and 8 B6). Tick marks on either side of the graph indicate mean + 4 SD of B6 spleens (0.166 g), which was used to define splenomegaly. (B) Spleen weights from 4 to 16 mo of age. Lines were analyzed by linear regression (\( n = 73 \) NZB, 46 *Lbw2*). (C) Representative histological sections of spleens from B6, NZB, and *Lbw2* congenic mice described below. Samples were stained with H&E. Arrow indicates a representative area of RBC sequestration. (D) and (E) Total and Ter119\(^+\) spleen cell numbers in unlysed or lysed samples from 11- to 14-mo-old mice. Spleens were cut into three equal sections and either fixed for histology, used directly for flow cytometry or lysed prior to flow cytometry (\( n = 13 \) NZB, 14 *Lbw2*, and 10 B6). Statistical significance for *Lbw2* or B6 compared with NZB is indicated by asterisks alone, whereas *Lbw2* compared with B6 is indicated by a bar and asterisks. Horizontal bars for (A), (D), and (E) indicate mean values. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).
It has been suggested that the CD5+ peritoneal B-1a subset is responsible for the production of AEAs, which is further supported by the greater expansion of these cells in NZB mice (28–31). Consistent with this notion, we found that the AEA-resistant Lbw2 congenic mice had lower percentages and numbers of B-1a cells than NZB mice at early ages (2 and 5 mo) before the onset of AIHA, with the percentages and numbers of B-1a cells in Lbw2 congenics similar or lower than those in the nonautoimmune B6 strain (Fig. 4B). In addition, Lbw2 congenic mice did not develop the marked increase in peritoneal cell numbers and expansion of B-1b/B2 cells and T cells seen in NZB mice at later ages (9 and 12 mo; Fig. 4). Taken together, these findings document a modest, but significant, effect of the Lbw2 interval on splenic MZ B cells and certain peritoneal cell populations, including B-1a cells.

The Lbw2 locus on the NZB background does not affect B cell proliferation

Previously, we and others (24, 32) have shown that the NZB chromosome 4 interval that encompasses the Lbw2 locus is associated with B cell hyperactivity. However, when total splenocytes or isolated B cells from Lbw2 congenic mice of different ages were stimulated with LPS, there was no decrease in proliferative response (Fig. 5A, 5B). These data suggest that the Lbw2 allele affecting AEA production is not related to B cell hyperactivity.

AEA production is controlled by multiple Lbw2 subloci

To more precisely map the location of the Lbw2 gene responsible for the suppression of AEAs, a panel of Lbw2 interval-specific subcongenic mice were created and screened for the presence of AEAs (Fig. 6A). All subcongenic strains demonstrated suppression of AEA production compared with NZB mice, however, the subcongenic containing the largest interval, designated SA, showed the same degree of suppression as the full Lbw2 congenic, indicating it probably contains all relevant genes (Fig. 6B). In contrast, the remaining three subcongenics (designated SB, SC, and SD) demonstrated only partial suppression of AEAs. These findings indicate Lbw2 contains at least three subloci, designated as Lbw2-1, -2, and -3 (Fig. 7). The centromeric most Lbw2-1 sublocus (62.0–89.9 Mb) can be defined by the region of SA shared with the other subcongenics, Lbw2-2 (86.0–115.7 Mb) by the SC interval and Lbw2-3 (118.9–134.2 Mb) by the centromeric end of SD and the telomeric end of SA. Thus, the additive contributions of at least three genetic variants on chromosome 4 are required for full susceptibility to AEA production in NZB mice.

Splenomegaly is modulated by at least two Lbw2 subloci

The same panel of subcongenic mice was then analyzed to localize splenomegaly. Similar to the reduction in AEA, all subcongenics exhibited lower spleen weights than NZB and, based on the severity of splenomegaly, could be segregated into two groups (Fig. 6C). One group, the SC and SD subcongenics, exhibited spleen weights of intermediate severity between those of NZB and the full Lbw2 congenic mice. Because there was no overlap of the SC and SD intervals, this indicated that the two AEA-promoting subloci, Lbw2-2 and Lbw2-3, were also linked to splenomegaly, a finding consistent with the association of AEA and splenomegaly (Fig. 2). In the other group, composed of SA and SB subcongenics, spleen weights were much lower than those in NZB mice and indistinguishable from those of the original Lbw2 congenic. This suggested that the Lbw2-2 and Lbw2-3 subloci, contained within the SA and SB subloci, accounts for the bulk of the genetic contribution to splenomegaly with little, if any, augmentation from Lbw2-1. Thus, splenomegaly, in contrast to AEA, can be definitely mapped to only two of the three Lbw2 subloci (Fig. 7).

MZ B cell and B-1a cell phenotypes map to different Lbw2 subloci

We also sought to determine the specific sublocus responsible for the greater number of MZ B cells in the full Lbw2 congenic, reasoning that localization of this trait to a single locus would provide a clue to the function of the corresponding susceptibility gene. Unexpectedly, however, all Lbw2 subcongenic mice had higher numbers of MZ B cells than NZB mice (Supplemental Fig. 2). Likewise, as was found for the full Lbw2 congenic, no differences in the total numbers of B cells or the follicular B cells compared with NZB mice at 5 or 9 mo of age were seen (data not shown). This suggests that the effects of the Lbw2 locus on MZ B cell numbers are likely a secondary phenomenon and not a primary cause of AIHA.

Lbw2 subcongenic mice were also used to more precisely map the location of the gene affecting total and B-1a cell numbers in the peritoneum. In contrast to the MZ B cell phenotype, we found that the B-1a and peritoneal cell phenotype mapped to SA, SB, and...
but not SD subcongenic mice, and furthermore, the phenotype in these subcongenics was similar in degree to mice containing the entire Lbw2 locus (Fig. 6D). In particular, the smallest of the three overlapping subcongenics, SC, consistently demonstrated a significant decrease in both total peritoneal and B-1a cells at most ages. These findings localize genetic control of this phenotype to only one of the AEA-promoting subloci, Lbw2-2 (Fig. 7).

Discussion

We show in this article that the NZB chromosome 4 interval encompassing the Lbw2 locus promoted AEAs and splenomegaly but did not alter serum levels of IgM and IgG, or autoantibodies to nuclear Ags. Furthermore, it was determined that B cell response to LPS was not affected by the Lbw2 locus, although lack of the NZB allele was associated with higher numbers of MZ B cells and reduced expansion of peritoneal cells, including B-1a cells. Last, using several subinterval congenic mice, we documented that Lbw2 includes at least three distinct AEA-promoting subloci with individual and additive effects, of which two also affected splenomegaly and one the expansion of peritoneal B-1a cells. Thus, these data provide direct evidence for AEA-specific predisposing genes on NZB chromosome 4, document the profound influence of ge-

FIGURE 4. NZB Lbw2 locus promotes expansion of the peritoneal B-1a cell population. (A) Total numbers of peritoneal cells from NZB, Lbw2 congenic, and B6 mice. (B-E) Percentages (left panels) and total counts (right panels) of peritoneal B-1a, B-1b/B2, T cells, and macrophages. Peritoneal cells were isolated by lavage, and cell populations were identified by flow cytometry. Peritoneal cells were defined as follows: B-1a B cells (F480^{neg} B220^{+} CD5^{+} IgM^{+}), B-2 and B-1b B cells (F480^{neg} B220^{+} CD5^{neg} IgM^{+}), T cells (F480^{neg} B220^{neg} CD5^{high} IgM^{neg}), and macrophage (F480^{+}). Data compiled from multiple individual experiments with n = 4-17 mice/time point. Comparisons of Lbw2 or B6 compared with NZB are indicated by asterisks alone, whereas Lbw2 compared with B6 are indicated by a bar and asterisks. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 5. B cells from NZB and Lbw2 congenic mice demonstrate similar B cell proliferation. (A) Proliferation of total splenocytes from 6-mo-old NZB and Lbw2 congenic mice cultured in the presence of LPS or media alone. Data are from a pool of two mice and representative of four separate experiments. (B) Proliferation of isolated B cells (B220^{+}) from three 6-mo-old Lbw2 and NZB mice and two 6-mo-old B6 mice cultured in the presence of LPS or media alone. Data are from a representative of two independent experiments. Cell proliferations were determined by [3H]Tdr uptake. Error bars represent the SE of triplicate wells.
These observations are consistent with our initial genomewide BWF2 study, which did not map IgG anti-chromatin Ab production to chromosome 4 (23), and the lack of reduction in IgG anti-nuclear Abs in subsequently generated BWF1.NZW-Lbw2 mice (BWF1 hybrids lacking the NZB Lbw2 allele) (24). Other studies have also examined the contribution of the NZB chromosome 4 region in lupus using B6 background congenic mice containing a large introgressed NZB fragment (32.2–150.0 Mb). This resulted in B-1a and NKT cell expansions but no functional alterations in NKT cells or autoimmune disease including AIHA (33, 34). Furthermore, the addition of this same chromosome 4 interval to another lupus-prone B6.NZBc1 congenic line significantly reduced anti-nuclear autoantibody production, kidney disease, and early mortality (34). These findings are consistent with our conclusion that Lbw2 primarily promotes AEA and that this is dependent on additional NZB genes.

Our findings also help clarify the effects of Lbw2 on splenomegaly. This trait was initially localized to chromosome 4, but later studies of Lbw2 in BWF1 background congenics showed no association with spleen size, thereby questioning the accuracy of the mapping result (23, 24). We now show that the NZB Lbw2 locus does indeed confer enhanced susceptibility to splenomegaly in the NZB background and that this is secondary to exacerbation of AIHA and the large accumulation of RBCs within the spleen. The lack of association of Lbw2 with splenomegaly in BWF1 mice is, therefore, consistent with the absence of significant AEA in this background and further suggests that the splenomegaly trait used in the initial mapping study was likely a surrogate phenotype for AIHA. In addition, these findings illustrate a potential limitation of using a single background to characterize the effects of a locus. Thus, the contributions of Lbw2 to splenomegaly in NZB mice as with AEA production are dependent on the epistatic contributions of other AEA-affecting genes.

An unexpected result was the lack of significant effect of Lbw2 on B cell proliferation, which contrasted sharply with our previous observation of enhancement in BWF1 mice. The most straightforward explanation is that certain NZW background genes in the BWF1 background are required for the Lbw2-associated B cell hyperactivity, presumably in an epistatic manner similar to the requirement for additional NZB genes in the development of AEA.

FIGURE 6. Dissection of the Lbw2 locus with subcongenic mice. (A) Genotype map of Lbw2 and subcongenic mice on chromosome 4. Thick black lines represent NZB genome, thick white lines NZW genome, and thin solid lines regions that remain undefined and could be either NZB or NZW. Markers used to define the intervals are further described in Supplemental Table 1 and Materials and Methods. (B) Percentage of NZB, Lbw2 congenic, and subcongenic mice positive for direct Coombs’ test. n = 6–38 mice/time point. (C) Spleen weights of NZB, Lbw2, and Lbw2 subcongenic mice with age. On the basis of linear regression analysis, subcongens can be grouped into two phenotypes when compared with parental NZB mice: highly resistant (Lbw2, SA, and SB) or moderately resistant (SC and SD). Statistical significance for slope and intercept/elevation is listed separately when there is a difference. (D) Total and B-1a peritoneal cell numbers for NZB, Lbw2 congenic, and subcongenic mice at indicated ages. Data were compiled from multiple experiments with 6–17 mice/group. Comparisons are indicated for NZB versus Lbw2 congenic or Lbw2 subcongenic mice. *p<0.05, **p<0.01, ***p<0.001, NS p>0.05.

FIGURE 7. Maps and phenotypes of Lbw2 subcongens and Lbw2 subloci. For comparison, the genotypes of B6.Sle2 and Sle2c are included at the top of the map (39, 43). Phenotypes include AEA, spleen weight (Spl), numbers of peritoneal B-1a cells, and MZ B cell numbers (MZ B) (right side of panel). Severity is graded from + to +++ with B6 negative (−). Subloci and corresponding phenotypes are at the bottom of the figure. ND indicates not determined and blank spaces represent where phenotype could not be mapped to the interval.
AEAs. It should be noted, however, that B cells from both NZB and Lbw2 congenic mice had significantly greater proliferation than B cells from B6 mice, indicating the presence of genes in NZB mice other than those in Lbw2 that promote B cell hyperactivity (Fig. 5). Regardless of the explanation, we can conclude that suppression of AEAs in Lbw2 congenics is not caused by a change in intrinsic B cell hyperactivity.

Another finding was lower numbers and/or percentages of MZ B cells in older (≥5 mo) NZB mice compared with Lbw2 congenic and all subcongenic mice. This reduction of MZ B cells in the parental NZB strain could be due to enhanced activation of MZ B cells, as described for Yaa⁺ lupus-prone mice (35) and chronic inflammation (36, 37), or the possibility that the altered architecture and increased phagocytosis associated with splenomegaly might lead to loss of MZ B cell niches. Notably, although lupus has been associated with both expansions (38, 39) and reductions (35) in the MZ B cell subset, their role in disease pathogenesis remains uncertain (40–42). In our study, however, the data suggest that the decrease in MZ B cells in NZB mice is secondary to the severity of AIHA and not directly related to Lbw2 susceptibility genes.

Lbw2 congenic mice also demonstrated an early age (at 2–5 mo) decrease in peritoneal B-1a cells followed later (9–12 mo) by lower numbers of peritoneal cells, which corresponded to reduced production of AEAs. Further dissection of this B-1a/peritoneal cell phenotype in subcongenic mice localized it to the SC subcongenic line (Lbw2-2 sublocus; Fig. 6). In support of this finding, expansion of B-1a cells was also reported in Sle2c1 mice, a B6 subcongenic containing an introgressed chromosome 4 fragment of NZB genome that overlaps with the SC sublocus (Fig. 7) (39, 43, 44). Additionally the Sle2c1 locus has been shown to promote lupus-like disease on the B6.Ipr background through alteration of T cell differentiation by increasing Th17 polarization (45). These findings support the possibility that the early expansion of B-1a promotes AIHA by either enhancing Ag presentation or AEA production. The former is also consistent with the later expansion of peritoneal T cells (Fig. 4).

We also showed that Lbw2 contains three subloci (Lbw2-1, -2, and -3) that function in an additive manner to promote AEA production (Fig. 7). The presence of multiple subloci have similarly been documented for loci on other chromosomes identified by genewide screening, including Sle1, Nba2, Sle2, and Sle3, which likely facilitated their initial detection (reviewed in Ref. 46). The Lbw2-1 sublocus extends from 62.0 to 89.9 Mb (~28 Mb) and was deduced by considering the larger SA interval had a greater effect on AEAs than the smaller SB fragment contained within SA (Fig. 7). Lbw2-1 contains ~85 known genes that include several immunologically relevant genes such as the type I IFN gene family, Tlr4, and Tnfsf8, which encodes CD30L. Of interest, high serum levels of the soluble form of CD30, the receptor for CD30L, were reported in patients with SLE (47). The Lbw2-2 sublocus spans ~30 Mb (86.0–115.7 Mb) and contains ~191 unique protein coding genes, of which only a few are associated with the immune system, including Jak1 and Jun. In addition, the Lbw2-2 sublocus contains the Cdkn2c gene in which a polymorphism within the promoter of the NZB allele has been associated with increased proliferation and subsequent accumulation of peritoneal B-1a cells (44). The Lbw2-3 sublocus, defined by the overlap of SA/SA and SD, is ~13.5 Mb in size (118.9–132.2 Mb) and contains ~243 unique protein coding genes, among which are Tlr12 and Lck and two RBC membrane protein genes, Ermap and Ebp4l.1.

Finally, the finding that Lbw2 primarily affects AEA and not anti-nuclear Abs implies that it promotes loss of tolerance to specific self-Ags rather than enhancement of overall autoimmunity. Moreover, the absence of compelling immunological phenotypes or obvious candidate genes that would account for this suggests that the underlying genes are likely to promote AIHA by novel mechanisms. Hence, the elucidation of the Lbw2 genes and their roles should reveal new and significant insights relevant to both AIHA and autoimmunity.

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