Published in final edited form as:

Autoimmunity. 2017 February ; 50(1): 42–51. doi:10.1080/08916934.2016.1272597.

Alterations in B cell development, CDR-H3 repertoire and dsDNA binding antibody production among C57BL/6 *D-iD* mice congenic for the lupus susceptibility loci *sle1*, *sle2*, or *sle3*

Mohamed Khass^{1,2}, Robert L Schelonka^{3,a}, Cun Ren Liu¹, Ada Elgavish¹, Laurence Morel⁴, Peter D Burrows⁴, and Harry W Schroeder Jr^{1,b}

¹Department of Medicine, University of Alabama at Birmingham, Birmingham, AL

²Genetic Engineering and Biotechnology division, National Research Center, Cairo, Egypt

³Department of Pediatrics, University of Alabama at Birmingham, Birmingham, AL

⁴Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL

⁴Department of Pathology, University of Florida, Gainesville, FL

Abstract

Systemic lupus erythematosus is an autoimmune disease that reflects a failure to block production of self-reactive antibodies, especially those that bind double stranded DNA (dsDNA).

Backcrossing the lupus-prone NZM2410 genome onto C57BL/6 led to the identification of three genomic intervals, termed *sle1*, *sle2*, and *sle3*, which are associated with lupus susceptibility. We previously generated a C57BL/6 strain congenic for an immunoglobulin D_H locus (*D-iD*) that enriches for arginine at dsDNA binding positions. We individually introduced the *D-iD* allele into the three *sle* strains to test whether one or more of these susceptibility loci could affect the developmental fate of B cells bearing arginine enriched CDR-H3s, the CDR-H3 repertoire created by the D_H, and the prevalence of dsDNA binding antibodies. We found that the combination of the *D-iD* allele and the *sle1* locus led to a decrease in mature, recirculating B cell numbers and an increase in marginal zone cell numbers while maintaining a highly charged CDR-H3 repertoire.

D-iD and *sle2* had no effect on peripheral B cell numbers, but the CDR-H3 repertoire was partially normalized. *D-iD* and *sle3* led to an increase in marginal zone B cell numbers, with some normalization of hydrophobicity. Mice with *D-iD* combined with either *sle1* or *sle3* had increased production of dsDNA binding IgM and IgG by twelve months of age. These findings indicate that the peripheral CDR-H3 repertoire can be categorically manipulated by the effects of non-immunoglobulin genes.

Keywords

Systemic lupus erythematosus; Sle; B cell repertoire; Third complementary determining region heavy chain; CDR-H3 and Diversity gene segments; D_H

^bCorresponding Author: hschroeder@uabmc.edu.

^aCurrent Address: Department of Pediatrics, Oregon Health & Science University, Portland, OR

Introduction

Systemic lupus erythematosus (SLE) is characterized by failure to block production of self-reactive antibodies, most notably those that bind double stranded DNA (dsDNA) (1). Elucidation of the mechanisms that control the composition of the antibody repertoire and promote the production of protective antibodies while preventing, or limiting, the production of ineffective or pathogenic antibodies is thus critical to our understanding of the pathogenesis and treatment of SLE and other autoimmune diseases.

Studies over the past thirty years have shown that although V(D)J rearrangement, N region addition and somatic hypermutation provide the opportunity to produce a vast, essentially random repertoire of antigen binding sites; the initial μ H chain repertoire is categorically constrained by natural selection of germline antibody sequence (2, 3), with a preference for tyrosine and a bias against charged or hydrophobic amino acids (4–7). The third complementarity determining region of the H chain, CDR-H3, is the direct product of VDJ rearrangement and N region addition. It lies at the center of the antigen binding site where it often plays a key role in defining the binding activity of the antibody. The non-random use of amino acids in CDR-H3 reflects natural selection of the amino acids encoded by germline D_H gene segments, somatic regulation of reading frame preference, and somatic selection of CDR-H3 content as B cells pass through sequential quality control and tolerance checkpoints in the primary and secondary lymphoid organs (3, 7–9). Amino acid diversity introduced by N addition resulting in CDR-H3 content that lies outside a preferred range of lengths, average hydrophobicity, or specific amino acids at particular structural positions is often eliminated in a stepwise fashion as B cells pass through sequential developmental checkpoints

Prototypic dsDNA binding antibodies contain arginine residues in CDR-H3 that are positioned to bind to the negatively charged phosphate groups on the DNA backbone. For example, many dsDNA binding antibodies display a preference for arginines in Kabat CDR-H3 positions 95–98 (99–102 in this work) and 100–100A (10, 11). B cells with such highly charged CDR-H3 intervals are normally selected against by both evolutionary and somatic mechanisms. This observation has led to the hypothesis that the development of dsDNA binding antibodies in SLE might reflect a failure to properly control the amino acid composition of the CDR-H3 repertoire. However, this issue has been difficult to address experimentally in unmodified mice.

To test the role of arginine in CDR-H3 in the production of dsDNA binding antibodies, we previously used Cre-loxP methodologies to manipulate the content of the D_H locus. We deleted all but one D_H , and modified that remaining D_H to preferentially rearrange into an inverted reading frame sequence enriched for arginine (12). We termed this D_H allele *D-iD*. Introduction of the *D-iD* allele altered the initial composition of CDR-H3, enriching for arginine and depleting tyrosine (3, 13). This change in CDR-H3 content persisted throughout early B cell development, generating in mature, recirculating B cells an antigen binding site repertoire enriched for arginine CDR-H3 positions 95–98 (99–102 in this work) and 100–100A (10, 11). In both homozygous and heterozygous otherwise unmanipulated *D-iD*

BALB/c mice, increased production of dsDNA binding IgG antibodies occurred with increasing age (13).

The NZM2410 mouse is a New Zealand Black/White-derived inbred strain that develops early-onset lupus nephritis in both sexes (14). Although C57BL/6 mice do not normally develop autoimmune disease, their genetic background appears to facilitate expression of autoantibodies and development of autoimmune disease when susceptibility alleles are bred into their genome (14). Backcrossing the NZM2410 genome onto C57BL/6 led to the identification of three novel genomic intervals, *sle1* on chromosome 1, *sle2* on chromosome 4, and *sle3* on chromosome 7, which are associated with susceptibility to lupus (15). In the congenic strain B6.NZMc1, the *sle1* locus is associated with potentiating a strong, spontaneous humoral response to H2A/H2B/DNA subnucleosomes. In the B6.NZMc4 strain, *sle2* leads to B-cell hyperactivity, elevated levels of B1a cells in the spleen and peritoneal cavity, and increased total serum IgM; but no evidence of IgG anti-nuclear antigen (ANA) antibodies, T cell defects, or glomerulonephritis. In the congenic strain B6.NZMc7, *sle3* promotes an elevated CD4:CD8 ratio with an increase in activated CD4 T cells, decreased susceptibility to apoptosis, and a break in humoral tolerance. These mice produce low ANA titers. Triple congenic C57BL/6 *sle1,2,3* mice approach the autoimmune disease phenotype of the parental NZM2410 strain, including high ANA titers.

CDR-H3 content has been shown to be altered in *sle3* mice (16), and thus abnormal regulation of B cells bearing categories of CDR-H3 that are typically avoided or discarded in normal mice could play a major role in disease susceptibility. B cells producing autoreactive antibodies are present within the normal B cell repertoire but are continuously eliminated by different mechanisms, depending on the developmental stage. Therefore, we here tested whether the NZM2410-derived *sle1*, *sle2* or *sle3* loci could affect the developmental fate or the Igh CDR-H3 repertoire of B cells homozygous for the arginine enriched *D-iD* allele, and whether the combination of *sle* loci and arginine enriched D_H could affect the prevalence of dsDNA binding antibodies.

Material and methods

Mice

Wild type C57BL/6 mice were bred in the UAB vivarium. To enrich for arginine, we had previously altered a BALB/c D_H locus to contain a single D_H enriched for arginine in reading frame 1, the preferred reading frame for VDJ rearrangements. We termed this allele *D-iD* (3). We previously backcrossed the BALB/c *D-iD* allele onto C57BL/6 for 22 generations (17). C57BL/6 mice congenic for the *sle1*, *sle2* or *sle3* loci were the kind gift of Dr. Chandra Mohan (UT Southwestern Medical Center). All the strains were maintained in a specific pathogen free barrier facility. The total number of mice used for evaluating absolute numbers of different B cell populations was 10 wild type C57BL/6 (WT), 10 *sle1*, 10 *sle1/D-iD*, 8 *sle2*, 8 *D-iD/sle2*, 6 *sle3* and 8 *D-iD/sle3*. The absolute numbers of B cells at specific developmental stages were counted when the mice were 8 weeks of age. All experiments with live mice were approved by and performed in compliance with Institutional Animal Care and Use Committee regulations.

Flow cytometry and cell sorting

Mononuclear cells from bone marrow and spleen were prepared as previously reported (4, 18). B cell sub populations were separated based on expression of specific surface proteins. Early B cell subsets in the bone marrow were identified using the scheme of Melchers (19). Transitional B cell subsets were identified using the scheme of Allman (20). Marginal zone and follicular B cells were identified using the scheme of Loder (21). The following sets of monoclonal antibodies were used: Anti-B220 (PerCP) [BD Pharmingen, San Diego, CA], anti-BP-1 (PE) (a gift from John Kearney, UAB), and anti-IgM (Cy5) [Jackson ImmunoResearch], West Grove, PA], anti-cKit (allophycocyanin) [BD Pharmingen, San Diego, CA], CD19 (SPRD) [Southern Biotech, Birmingham, AL], anti IgD (biotin) [BD Pharmingen], anti CD21 (PE) [BD Pharmingen], anti-CD25 (FITC) [BD Pharmingen, San Diego, CA], anti CD21 (PE) [BD Pharmingen, San Diego, CA], anti AA4.1 (Pecy7) [ebioscience, San Diego, CA] and anti CD23 (FITC) [BD Pharmingen, San Diego, CA].

Sorting, RNA preparation, RT-PCR and sequencing

Total RNA isolation, V_H7183 specific VDJC_μ RT-PCR amplification, cloning, sequencing, and sequence analysis were performed as previously described (6, 12). A listing of 52, 39 and 26 unique, in-frame VDJC_μ sequences from mature recirculating B cells from C57BL/6//*D-iD*, *sle1/ D-iD* and *sle2/ D-iD* mice, and 39 sequences each from marginal zone B cells from *sle1/ D-iD* and *sle3/ D-iD* mice are included in the supplementary materials.

Anti-DNA ELISA

ELISAs were performed as previously reported. Plates were treated with DNA sodium salt from calf thymus (Sigma-Aldrich) after applying poly-L-lysine solution for 2 hours. Serially diluted sera samples (three 1:2 serial dilutions) were added. Diluted HRP-labeled secondary antibodies against mouse IgM and IgG (Southern (Birmingham, AL, USA) in 1.5% BSA-PBS were then applied. Development of the reaction was performed using 100 μL of 1X TMB ELISA substrate solution (eBioscience, San Diego, CA, USA). After incubation at room temperature in dark for 10 minutes, the reaction was stopped using 50 μL of 2NH₂SO₄. Analysis was performed using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany).

Statistical analysis

Differences between populations were assessed where appropriate by Student's t test, two tailed. Analysis was performed with JMP version 12 (SAS Institute, Inc., Cary, NC), or with GraphPad Prism 5.03 (GraphPad Software, Inc., La Jolla, CA). Means are accompanied by the standard error of the mean.

Results

Different effects of the *sle1*, *sle2* and *sle3* alleles on early B cell development

When compared to WT, no significant differences in B cell progenitor numbers were observed in C57BL/6 mice congenic for *sle1* as compared to C57BL/6 (Figure 1). Mice congenic for *sle2* had a 58% decrease in proB cell numbers, but preB and immature B cell

numbers were equivalent to wild type. Mice congenic for *sle3* demonstrated a 29% reduction in B cell progenitors examined, and this reduction was statistically significant for late preB and immature B cells ($p < 0.01$).

Varying effects of the *D-iD* allele on early B cell development in C57BL/6 wild type and the *sle* congenic mice

Previously, we showed that the introduction of the *D-iD* allele led to a statistically significant decrease in the numbers of preB and immature B cells relative to wild type C57BL/6 mice (17). Introduction of either the *sle1* or *sle2* alleles to the *D-iD* allele had no additional effect (Figure 1). Among the *sle3* mice, however, the detrimental effect of the introduction of the *D-iD* allele on C57BL/6 background was delayed to later developmental stages. Early preB cell numbers were unchanged. There was an additional 15% decrease in numbers of late preB cells in *sle3/ D-iD* compared to *sle3* alone, but this did not achieve statistical significance. The difference between *sle3* and *sle3/ D-iD* grew to 20% in the immature B cell compartment, which reached statistical significance ($p < 0.05$).

A relative decrease in transitional T1 cell numbers in *sle2/ D-iD* mice

The introduction of the *D-iD* allele alone on the C57BL/6 background decreased the absolute numbers of transitional T1, T2, and T3 B cells by 30–50% ($p < 0.01$, $p < 0.0001$, and $p < 0.0001$; respectively) (Figure 2). The introduction of *sle1* increased absolute T1 cell numbers by 14%, in agreement with a previous report (22). The introduction of *sle2* alone increased T1 cell numbers to 40% of WT in contrast to 30% decrease in case of C57BL/6/*D-iD* mice.

Addition of the *D-iD* allele to the *sle1* strain led to a decrease of the same magnitude among transitional B cell numbers observed in C57BL/6/*D-iD* mice (Figure 2). Addition of the *D-iD* allele to the *sle2* strain further impaired T1 cell numbers, but then ameliorated the effect in both T2 and T3 populations (Figure 2). The differences in the absolute numbers of T1 and T2 cells between the *sle2* and *sle2/ D-iD* strains were both statistically significant ($p < 0.01$ and $p < 0.01$, respectively).

Increased numbers of marginal zone B cells in *sle1/ D-iD* and *sle2/ D-iD* mice

Unlike BALB/c, where the introduction of the *D-iD* allele leads to an increase in marginal zone (MZ) B cell numbers (12), MZ numbers in C57BL/6 *D-iD* mice were equivalent to wild type C57BL/6 (Figure 3). Among the *sle* congenic strains, *sle1* alone had no effect on MZ B cell numbers, *sle2* led to an 41% increase ($p < 0.01$), and *sle3* led to non-statistically significant 20% decrease in MZ B cell numbers. The introduction of the *D-iD* allele to *sle1* congenic strain led to a highly significant 69% increase in MZ B cell numbers relative to wild type or *sle1* ($p < 0.0001$). No additional effect was observed in case of *sle2* with *D-iD* on MZ B cell numbers. The co-introduction of *D-iD* and *sle3* also led to an increase in MZ B cell numbers which was significantly different than *sle3* alone (49% difference, $p < 0.05$).

The *sle* alleles normalized the numbers of follicular B cells

Follicular B cells (FO) carrying a *D-iD* allele were eliminated whether the allele presented alone or when combined with any of the studied *sle* loci (Figure 3). The presence of *sle1*,

sle2 or even *sle3* alone without the *D-iD* allele promoted the survival of FO B cells bringing their numbers into WT limits. The addition of *D-iD* to the *sle1*, *sle2*, or *sle3* loci also led to a 35–60% reduction in the number of follicular B cells ($p<0.0001$, $p<0.0001$, and $p<0.01$, respectively).

The difference between the presence of any of the *sle* loci alone or when combined with *D-iD* was significant in case of *sle1* and *sle2*, $p<0.0001$ and $p=0.005$ in case of *sle3* (Figure 3).

***sle1* and *sle3* reduced the numbers of mature, recirculating B cells**

Unlike BALB/c, where the introduction of the *D-iD* allele leads to a halving of mature, recirculating B cells; in C57BL/6 the *D-iD* allele has no numerical effect (17). The addition of *sle1* or *sle3* alone led to a 57% or 42% reduction in mature B cell numbers, whereas *sle2* had no effect (Figure 3). The addition of the *D-iD* allele had no additional effect on mature B cell numbers in the presence of *sle1* or *sle2*. However, in the presence of *sle3* there was a 25% more decrease in mature B cell numbers ($p<0.01$).

The distribution of CDR-H3 loop length, charge and amino acid content can be influenced by *sle* alleles

In C57BL/6/ *D-iD* mice, the CDR-H3 repertoire is marked by longer than normal CDR-H3 loops in the mature recirculating B cells (Figure 4). The longer CDR-H3 lengths were preserved in mature recirculating B cells of *sle1*/ *D-iD* mice. However, in the *sle2*/ *D-iD* mice we observed a broadening of the repertoire to include shorter CDR-H3s. In the marginal zone, longer CDR-H3s were observed in *sle1*/ *D-iD* mice; moreover *sle3*/ *D-iD* B cells exhibited a return to the mean length, although the numbers of sequences were insufficient to achieve statistical significance.

The use of the *D-iD* allele enhances expression of more highly charged CDR-H3 loops (Figure 5) (3, 17). To test whether the *sle* alleles could influence the distribution of charge in the CDR-H3 repertoire, we sampled the repertoire of selected B cell subsets. Even though the mature, recirculating B cells in the *sle1*/ *D-iD* mice were more than halved in number (Figure 3), the CDR-H3 repertoire remained enriched for highly charged sequences. Conversely, we observed enrichment for more neutral or hydrophobic CDR-H3s in the *sle2*/ *D-iD* mice. Thus, these mice had broadened the repertoire and reduced the use of arginine. 15 of the 26 sequences from *Sle2*/ *D-iD* mice used the reading frame controlled by the *D-iD*, meanwhile 7 of the 26 sequences used an alternative reading frame ($p<0.05$). The difference in alternative reading frame use was significant at $p=0.048$, suggesting that the *sle2* allele may affect reading frame usage.

Among marginal zone B cells in the *sle1*/ *D-iD* mice, which exhibited an increase in B cell numbers when compared to wild type, we observed a broadening of the repertoire to include more charged and hydrophobic sequences (Figure 5). This did not occur in the *sle3*/ *D-iD* mice which had only charged sequences.

When the CDR-H3 repertoire was evaluated on a position-by-position basis from amino acid 99 to 103, where the cysteine was at position 96, major changes were observed. In the mature, recirculating B cell fraction from C57BL/6 *D-iD* mice, enrichment for arginine at

position 101 followed the pattern observed in BALB/c. In C57BL/6 *D-iD/sle1* mice, loss of B cell numbers in the mature, recirculating fraction was associated with selection for tyrosine and loss of arginine at position 101 when compared to C57BL/6 *D-iD*. In C57BL/6 *D-iD/sle2* mice, we observed enrichment for alternative reading frames in 7 of the 26 sequences (27%), and enrichment for hydrophobic valine and isoleucine in place of arginine at position 101 in the mature, recirculating fraction. In the marginal zone, C57BL/6 *D-iD/sle1* mice demonstrated increased selection for valine at position 101; whereas C57BL/6 *D-iD/sle3* mice, which had an increase in cell numbers, maintained the preference for arginine at position 101.

Enhanced production of IgM and IgG anti-ds-DNA antibodies when charged CDR-H3 are combined with *sle* loci

Introduction of the *D-iD* allele into BALB/c mice led to the appearance of dsDNA binding IgG antibodies in ~40% of the homozygous and heterozygous mice at 12 months of age; however, dsDNA binding IgM was suppressed (13). In C57BL/6, the introduction of the *D-iD* allele led to an increase in the average levels of dsDNA binding antibodies at 12 months of age for both IgM and IgG, but these differences did not achieve statistical significance (Figure 6).

Mice congenic for *sle1* did not express higher levels of dsDNA binding IgM or IgG when compared to WT (Figure 6). For IgM, mice doubly congenic for *D-iD* and *sle1* had higher levels of dsDNA binding antibodies than WT ($p<0.0001$), *sle1* alone ($p<0.01$) or *D-iD* alone ($p<0.0001$), respectively. For IgG, mice doubly congenic for *D-iD* and *sle1* had higher levels of dsDNA binding antibodies than WT ($p<0.01$) or *sle1* alone ($p<0.05$), but not *D-iD* alone.

Mice congenic for *sle3* expressed higher levels of dsDNA binding IgM than WT, but the differences between the means did not achieve statistical significance due to the variance. Levels of dsDNA binding IgG were unchanged (Figure 6).. For IgM, mice doubly congenic for *D-iD* and *sle3* had higher levels of dsDNA binding antibodies than WT ($p<0.01$), *sle3* alone ($p<0.05$) or *D-iD* alone ($p<0.01$), respectively. For IgG, mice doubly congenic for *D-iD* and *sle3* had higher levels of dsDNA binding antibodies than *sle3* alone ($p<0.05$), but not WT or *D-iD* alone.

Discussion

We monitored the passage of B cells bearing the arginine-promoting *D-iD* allele through sequential central (17) and peripheral developmental checkpoints (Figures 1–3). We assessed the absolute number of B cells at each developmental stage in wild-type C57BL/6, and then assessed the percent increase or decrease in absolute numbers in the C57BL/6/ *D-iD* strain. As was also the case in BALB/c, early B cell development was affected, with a significant decrease in preB and immature B cell numbers. However, in contrast to BALB/c, the numbers of mature, recirculating B cells were equivalent to wild type, indicating greater tolerance in this subset to the presence of highly charged, arginine-enriched CDR-H3s (17).

In this work, we expanded our developmental studies of the C57BL/6/ *D-iD* strain to the periphery (Figures 2–3). We found that all three transitional subsets (T1, T2 and T3) were also decreased in number. In the spleen, the follicular subset was decreased by 60%, matching or even slightly exceeding the decrease seen in BALB/c (3). However, unlike BALB/c where there was more than 30% increase in marginal zone B cell numbers, MZ B cells carrying the *D-iD* allele were normal in numbers. Thus, C57BL/6 B cells using the *D-iD* allele appeared to be tolerated, but not favored.

With this information in hand, we were then able to test whether the introduction of *sle1*, *sle2* or *sle3* influenced the development of B cells using the *D-iD* allele. We assessed the percent increase or decrease in absolute numbers in the C57BL/6 *sle1*, *sle2* or *sle3* strains congenic for either a WT or *D-iD* D_H alleles (Figures 1–3). We found that the introduction of the *D-iD* allele had a different effect in each of the *sle* congenic lines. In the bone marrow, the presence of the *sle2* allele preserved early preB cell numbers, but the detrimental effect of *D-iD* on bone marrow development re-appeared at the immature B cell stage. In the spleen, the presence of the *sle1* allele with *D-iD* led to a 69% increase in the numbers of marginal zone *D-iD* B cells. *Sle2* did not have a significant effect. And, when *sle3*/ *D-iD* was compared to *sle3* alone, there was a 49% increase in marginal zone B cell numbers and a 25% relative decrease in mature, recirculating B cell numbers. We have previously shown that the changes in B cell numbers that occur in the bone marrow of BALB/c mice due to the alteration of the CDR-H3 repertoire reflect varying rates of cell division and apoptosis (7). Elucidation of the mechanisms behind changes in B cell development in the *sle* strains will require similar kinetic studies.

We have shown that global, including position-specific, changes in the CDR-H3 repertoire influence epitope recognition and binding (7, 23), the ability to protect against infection (3, 24), and, most pertinent for this work, the likelihood of producing autoreactive antibodies (13). To test whether the introduction of *sle1*, *sle2* or *sle3* influenced the development of B cells using the *D-iD* allele, we performed sequence analysis of $V_H1783DJC\mu$ transcripts from selected B cell subsets, focusing on CDR-H3 loop hydrophobicity and length. Among mature, recirculating B cells, the introduction of the *D-iD* creates a more charged repertoire with longer CDR-H3s (17). The introduction of *sle1* reduces the absolute number of these B cells, and this reduction is unchanged with the addition of *D-iD* (Figure 3). These B cells maintained the same high charged CDR-H3 distribution (Figure 5) as well as longer average length (Figure 4). However, enrichment for tyrosine at position 101 was observed. In contrast, the introduction of *sle2* results in a normal number of mature B cells irrespective of the D_H allele (Figure 3) and to a partial normalization of CDR-H3 hydrophobicity (Figure 5) and length (Figure 4). In part, the change in CDR-H3 hydrophobicity can be explained by the use of alternative reading frames, which leads to the enhanced use of hydrophobic amino acids at position 101 (Figure 5). In the spleen, increased numbers of marginal zone B cells in *sle1*/ *D-iD* mice showed a minority population of more hydrophobic, valine-enriched CDR-H3s with a broader length distribution. In contrast, *sle3*/ *D-iD* marginal zone cells maintained a greater charge and the preference for arginine at position 101, but with a slightly shorter length distribution (Figures 4 and 5).

The *sle1* and *sle3* alleles both increased the numbers of marginal zone B cells in mice that have the *D-ID* allele (Figure 3). Since autoreactive B cells are often enriched in the marginal zone, we tested whether the double mutants would show an increase in dsDNA binding antibody production, and discovered unexpected strain-specific differences. Unlike in BALB/c, where dsDNA antibody production was increased but restricted to IgG antibodies (13), in 12 month old C57BL/6, IgM dsDNA binding antibodies were increased, and IgG dsDNA binding antibodies were increased when either *sle1* or *sle3* were introduced. In both cases, these findings indicate that categorical control of the CDR-H3 amino acid composition in the developing repertoire by natural selection can function as a factor to limit the development of autoreactive antibodies.

In the final analysis, our finding that alterations in the repertoire due to the presence of *sle* alleles indicates that the peripheral repertoire can be globally manipulated by non-immunoglobulin gene effects. This raises the possibility that pharmacologic interventions could alter the repertoire and thus the range of antigen binding sites, which could provide a means to elicit vaccine responses that are otherwise rare, to manipulate the response to infection, or to suppress the development of autoimmunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Yingxin Zhuang and Pratibha Kapoor for outstanding technical assistance. We thank Chandra Mohan for providing us with the *sle1*, *sle2* and *sle3* mice. This work was supported, in part, by NIH AI48115, AI117703, and DE022736. Flow cytometry was supported by NIH AR48311 and AI027767.

References

1. Sang A, Zheng YY, Morel L. Contributions of B cells to lupus pathogenesis. *Molecular immunology*. 2014; 62:329–338. [PubMed: 24332482]
2. Kirkham PM, Mortari F, Newton JA, Schroeder HW Jr. Immunoglobulin VH clan and family identity predicts variable domain structure and may influence antigen binding. *EMBO Journal*. 1992; 11:603–609. [PubMed: 1537339]
3. Schroeder HW Jr, Zemlin M, Khas M, Nguyen HH, Schelonka RL. Genetic control of DH reading frame and its effect on B-cell development and antigen-specific antibody production. *Crit Rev Immunol*. 2010; 30:327–344. [PubMed: 20666706]
4. Ivanov, et al. Development of the expressed immunoglobulin CDR-H3 repertoire is marked by focusing of constraints in length, amino acid utilization, and charge that are first established in early B cell progenitors. *J Immunol*. 2005; 174:7773–7780. [PubMed: 15944280]
5. Vale AM, et al. The peritoneal cavity B-2 antibody repertoire appears to reflect many of the same selective pressures that shape the B-1a and B-1b repertoires. *J Immunol*. 2010; 185:6085–6095. [PubMed: 20956345]
6. Schelonka RL, et al. Categorical selection of the antibody repertoire in splenic B cells. *Eur J Immunol*. 2007; 37:1010–1021. [PubMed: 17345580]
7. Khas M, et al. A new role for VpreB: an invariant surrogate antigen that selects Ig antigen binding sites. *Science Immunology*. 2016; 1
8. Verkoczy L, et al. Induction of HIV-1 broad neutralizing antibodies in 2F5 knock-in mice: selection against membrane proximal external region-associated autoreactivity limits T-dependent responses. *Journal of immunology*. 2013; 191:2538–2550.

9. Meffre E, et al. Immunoglobulin heavy chain expression shapes the B cell receptor repertoire in human B cell development. *The Journal of clinical investigation*. 2001; 108:879–886. [PubMed: 11560957]
10. Radic MZ, et al. Residues that mediate DNA binding of autoimmune antibodies. *J Immunol*. 1993; 150:4966–4977. [PubMed: 8496598]
11. Krishnan MR, Jou NT, Marion TN. Correlation between the amino acid position of arginine in VH-CDR3 and specificity for native DNA among autoimmune antibodies. *J Immunol*. 1996; 157:2430–2439. [PubMed: 8805642]
12. Ippolito GC, et al. Forced usage of positively charged amino acids in immunoglobulin CDR-H3 impairs B cell development and antibody production. *Journal of Experimental Medicine*. 2006; 203:1567–1578. [PubMed: 16754718]
13. Silva-Sanchez A, et al. Violation of an evolutionarily conserved immunoglobulin diversity gene sequence preference promotes production of dsDNA-specific IgG antibodies. *PLoS One*. 2015; 10:e0118171. [PubMed: 25706374]
14. Bolland S, Yim YS, Tus K, Wakeland EK, Ravetch JV. Genetic modifiers of systemic lupus erythematosus in FcγRIIB(−/−) mice. *Journal of Experimental Medicine*. 2002; 195:1167–1174. [PubMed: 11994421]
15. Wakeland EK, Morel L, Mohan C, Yui M. Genetic dissection of lupus nephritis in murine models of SLE. *Journal of clinical immunology*. 1997; 17:272–281. [PubMed: 9258766]
16. Wakui M, Kim J, Butfiloski EJ, Morel L, Sobel ES. Genetic dissection of lupus pathogenesis: Sle3/5 impacts IgH CDR3 sequences, somatic mutations, and receptor editing. *Journal of Immunology*. 2004; 173(12):7368–76.
17. Khass M, et al. Recirculating bone marrow B cells in C57BL/6 mice are more tolerant of highly hydrophobic and highly charged CDR-H3s than those in BALB/c mice. *Eur J Immunol*. 2013; 43:629–640. [PubMed: 23225217]
18. Zemlin, M., et al. 13th International Congress of Immunology. Kalil, J.Cunha-Neto, E., Rizzo, LV., editors. Medimond International Proceedings; Bologna: 2007. p. 507-511.
19. Rolink AG, et al. B cell development in mice with a defective lambda 5 gene. *Eur J Immunol*. 1993; 23:1284–1288. [PubMed: 7684685]
20. Allman D, et al. Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. *J Immunol*. 2001; 167:6834–6840. [PubMed: 11739500]
21. Loder F, et al. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *Journal of Experimental Medicine*. 1999; 190:75–89. [PubMed: 10429672]
22. Kumar KR, et al. Regulation of B cell tolerance by the lupus susceptibility gene Ly108. *Science*. 2006; 312:1665–1669. [PubMed: 16778059]
23. Wang YKP, Parks R, Silva-Sanchez A, Alam SM, Verkoczy L, Liao HX, Zhuang Y, Burrows PD, Levinson M, Elgavish A, Cui X, Haynes BF, Schroeder HW Jr. HIV-1 gp140 epitope recognition is influenced by immunoglobulin DH gene segment sequence. *Immunogenet*. 2016; 68:145–155.
24. Vale AM, et al. The link between antibodies to OxLDL and natural protection against pneumococci depends on DH gene conservation. *Journal of Experimental Medicine*. 2013; 210:875–890. [PubMed: 23589567]

Figure 1. Percent change in the absolute numbers of bone marrow B cell progenitors in C57BL/6 mice congenic for the *D-iD* allele and/or *sle1*, *sle2* or *sle3* alleles relative to their littermate controls

Melcher's scheme was used to identify cells as pro B, early preB, late preB and immature B cells. The standard error of the mean of each B lineage subpopulation for the littermate controls averaged approximately 10% of the absolute number of cells in each subpopulation (gray area). Data represent an analysis of at least five mice per group. Student's t test was used for statistical analysis. Significance is denoted as *p 0.05, **p 0.01, ***p 0.001, and ****p < 0.0001.

Figure 2. Percent change in the absolute numbers of transitional B cells in homozygous female 8 week C57BL/6 mice congenic for the *D-iD* allele and/or *sle1* or *sle2* alleles relative to their littermate controls

Absolute numbers of T1 (left), T2 (middle) and T3 (right column) cells were identified according to the Allman scheme. Cells obtained from C57BL/6/ *D-iD* (upper panel), *sle1* and *sle1/ D-iD* (middle panel) and *sle2* and *sle2/ D-iD* (lower panel) mice were compared to C57BL/6 littermate controls. Standard error of the mean of each B lineage subpopulation for the littermate controls averaged approximately 10% of the absolute number of cells in each subpopulation (gray area). Data represent an analysis of at least 5 mice per group. Student's t test was used for statistical analysis. Error bars depict the standard error of the mean. Significance values are marked as reported in Figure 1.

Figure 4. Distribution of the lengths of CDR-H3 loops from the selected B cell populations

We cloned, sequenced, identified and analyzed 39 and 26 non-redundant $V_H7183DJC\mu$ transcripts from mature recirculating B cells from *C57BL/6/sle1/ D-iD* and *C57BL/6/sle2/ D-iD* mice, respectively, compared to 52 sequences from *C57BL/6/ D-iD* mice. We also analyzed 39 $V_H7183DJC\mu$ transcripts from *C57BL/6/sle1/ D-iD* and 39 from *C57BL/6/sle3/ D-iD*, respectively. Distribution of CDR-H3 lengths in the obtained transcripts. The vertical lines represent the preferred length distribution in normal healthy B cells.

Figure 5. Distribution of the hydropathicity of CDR-H3 loops from the selected B cell populations

(Left) The average hydropobicity of the CDR-H3 loop from transcripts analyzed. The vertical line represents the preferred range of charge distribution in normal B cells. Arrows indicate the range of highly hydrophobic (right) or highly charged (left) sequences. (Right) CDR-H3 amino acid usage by percentage rank order in positions 99 through 103, where cysteine is found at position 96.

Figure 6. Serum levels of dsDNA binding IgM and IgG at 12 months of age in wild type C57BL/6 mice compared to mice congenic for *D-iD* and or the *sle1* (left) or *sle3* (right) intervals
Data represent an analysis of at least 15 mice per group. Anti ds-DNA was measured in ug/ml. Student's t test was used for statistical analysis. Significance is illustrated as *p 0.05, **p 0.01, ***p 0.001, and ****p < 0.0001.