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The role of evolutionary conserved germline D_H sequence in B-1 cell development and natural antibody production

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Abstract

Due to N addition and variation in the site of V–D–J joining, the third complementarity-determining region of the heavy chain (CDR-H3) is the most diverse component of the initial immunoglobulin antigen-binding site repertoire. A large component of the peritoneal cavity B-1 cell component is the product of fetal and perinatal B cell production. The CDR-H3 repertoire is thus depleted of N addition, which increases dependency on germ-line sequence. Cross-species comparisons have shown that D_H gene sequence demonstrates conservation of amino acid preferences by reading frame. Preference for reading frame 1, which is enriched for tyrosine and glycine, is created both by rearrangement patterns and by pre-BCR and BCR selection. In previous studies, we have assessed the role of conserved D_H sequence by examining peritoneal cavity B-1 cell numbers and antibody production in BALB/c mice with altered D_H loci. Here, we review our finding that changes in the constraints normally imposed by germ line–encoded amino acids within the CDR-H3 repertoire profoundly affect B-1 cell development, especially B-1a cells, and thus natural antibody immunity. Our studies suggest that both natural and somatic selection operate to create a restricted B-1 cell CDR-H3 repertoire.

Introduction

The third complementarity determining region-3 (CDR-H3) of the immunoglobulin heavy chain lies at the center of the antigen-binding site, where it often plays a critical role in epitope recognition and the subsequent immune response to the antigen.^{1,2} During B cell development, CDR-H3 is formed *de novo* by VDJ rearrangement, making it the focus for germ line–encoded combinatorial variation. Exonucleolytic nibbling and the addition of N nucleotides also make CDR-H3 the focus for somatic variation of the nascent IgM repertoire. In spite of the tremendous diversity introduced by these mechanisms, CDR-H3

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repertoires expressed by specific B cell subsets often exhibit characteristic categorical constraints, including biases in V_H, D_H, and J_H gene segment usage; in D_H reading-frame utilization; in the number of amino acids within the CDR-H3 loop (i.e., length), and in the physicochemical properties (e.g., hydrophobicity or charge) of those amino acids.^{3,4} These biases in the primary B cell repertoire appear to be a manifestation of preferences for specific, individual ranges of potential antigen-binding structures and complementarity surfaces that characterize the different B cell subsets. Here, we review our previous findings regarding the changes that occur in B-1 cell development, especially among B-1a cells, and in natural antibody immunity when the constraints that are normally imposed by the germ line sequence of the diversity (D_H) gene segments upon this range of antigen-binding sites are shifted as a result of altering the sequence of the D_H locus.⁵⁻⁹

Alteration of evolutionarily conserved D_H coding sequence affects B cell development

In order to gain insight into the mechanisms that regulate the composition of the antibody repertoire, to determine when during the B cell development constraints on CDR- H3 composition might be imposed, and to assess the role of germ line control of the repertoire in antibody production; we began a detailed examination of CDR-H3 repertoire development among key B cell subsets. We isolated B cell populations from the bone marrow, spleen, and peritoneal cavity (PerC) of 8- to 12-week-old BALB/c mice. In the bone marrow, we focused on the progenitor, immature, and mature B lineage subsets.³ In the spleen, we focused on the transitional (T1 and T2), marginal zone (MZ), and follicular (FO) B cell subsets.¹⁰ In the PerC, we focused on the B-1a, B-1b, and B-2 cell subsets.⁸ For each subset population; we cloned, sequenced, and deconstructed the CDR-H3 component of V_H7183DJ_HC_μ transcripts obtained from several mice.

We found that the distribution of gene segment usage, lengths, global amino acid content, and average hydrophobicity was highly similar among the different mice and exhibited a specific, controlled distribution of amino acids that was already apparent at the earliest stage of B cell development evaluated, Hardy fraction B.¹¹ This subset includes B lineage cells that have undergone DJ rearrangement as well as cells that have just completed VDJ rearrangement. Thus, these cells express little or no Ig_μ protein. We observed a consistent enrichment for tyrosine and glycine in CDR-H3 coupled with an underrepresentation of both positively charged and hydrophobic amino acids when compared to general *Mus musculus* codon usage.¹²

In previous cross-species comparisons of germ line Ig sequence, we had observed that the sequence of diversity gene segments exhibited conservation of amino acid preferences by reading frame.¹² In all the jawed vertebrates studied, by and large, RF1 tended to encode neutral amino acids as defined by the Kyte-Doolittle hydrophobicity scale, especially tyrosine and glycine. RFs 2 and 3 by deletion and RFs 2 and 3 by inversion (iRFs) tended to encode hydrophobic amino acids. RF3 and iRF3 also tended to include termination codons. Finally, iRF1 tended to encode charged amino acids. In the various jawed vertebrates studied, B cells used overlapping sets of mechanisms to encourage use of RF1 and avoid the use of the three iRFs.¹² Coupled with the finding that RF1-encoded tyrosines were overrepresented in VDJ joins among developing B cells, these observations led us to the

hypothesis that natural selection of the D_H germ line repertoire might play a key role in controlling the global composition of CDR-H3 and thus of the range of antigen-binding sites available to the functional repertoire. In our studies, we also observed subtle to major adjustments in amino acid usage as the B cells passed through sequential developmental checkpoints.³ Thus, we postulated that the constraints on CDR-H3 repertoire content that we had observed in peripheral B cell subsets were the product of both natural selection of germ line sequence and clonal somatic selection that operates in the individual in response to local antigenic exposure. However, the relative roles of these two processes in regulating the repertoire and in influencing antibody production in response to antigen challenge were unclear.

In order to test the role of natural selection of the D_H sequence on regulating the composition of CDR-H3, we created a panel of mice wherein we altered the composition of the D_H locus. We used cre-loxP-based gene targeting to delete 12 of the 13 D_H gene segments in the D_H locus of a BALB/c ES cell. The first IgH mutant retained a single wild-type *DFL16.1* segment (*D-DFL*), which in its physiologic state preferentially uses tyrosine-enriched RF1.⁵ Manipulation of the targeting construct also allowed us to alter the sequence of the remaining D_H. For the second D_H allele, two frameshift mutations in the coding sequence created a DFL16.1-derived gene segment (*D-DμFS*) that encouraged use of valine-encoding RF2 in place of RF1.^{7,13} Finally, the central portion of DFL161 was replaced with the inverted sequence of the *DSP2.2* gene segment (*D-iD*), introducing a preference for iRF1 sequence encoding highly charged amino acids, including arginine.⁶ In these mice, developing B cells have full access to all V_H and J_H gene segments, to all light chain rearrangements, to unrestricted class switching, to N addition, and to somatic hypermutation. The only change that has been made is an altered preference for charged or hydrophobic amino acids at the center of the antigen-binding site in the places normally occupied by tyrosine or glycine. Thus, unlike a classic, single-antibody transgene mutant mouse, altering the sequence of the D_H permits progenitor B cells to progress through all of the normal checkpoints of B cell development while expressing polyclonal mature antibody repertoires that differ only in the contribution of the altered D_H.

We found that alterations in the D_H loci did not preclude B cell production. We then sought to study the effect of altering the pattern of CDR-H3 hydrophobicity on B cell development. We compared the absolute numbers of B cells in D_H-altered mice with wild-type controls, focusing on key developmental subsets in the bone marrow, spleen, and peritoneal cavity. In this review, we will focus on our studies on the PerC B cell compartment, which constitutes the largest fraction of B-1 cells in normal mice (for further review, see Ref. 14). In order to place these results in a proper context, this discussion begins with a brief overview of our previous studies regarding the impact of genetic alterations in the CDR-H3 repertoire in the bone marrow and splenic B cell populations.

In the bone marrow of our D_H mutant mice, we observed an increase in the number of pro-B cells (Hardy fraction B), equivalence in the number of early pre-B cells (fraction C), and a varying decrease in both the numbers of late pre-B cells (fraction D) and the numbers of immature B cells (fraction E) in order from the lesser to the greater reduction of *D-DFL*, *D-iD*, and *D-DμFS*. Figure 1 illustrates the percent divergence in absolute numbers of B

cells in these key subsets between individual D_H -altered and wild-type littermate controls. We interpreted the increase in the pro-B cell population as reflective of the loss of the majority of the D_H locus rather than the change in the sequence of CDR-H3. The decrease in the late pre-B and immature B cell subsets appeared to reflect the effects of both the change in the number of D_H gene segments and the changes in the sequence of the remaining D_H , suggesting that pre-BCR selection was being influenced by the sequence of the D_H . A testing of this hypothesis is underway in our laboratory.

Once released from the bone marrow, the absolute numbers of cells within the individual B cell subsets differed by IgH allele. In mice limited to the use of a single, normal *DFL16.1* gene segment (*D-DFL*), the absolute numbers of transitional, MZ, and follicular cells in the spleen, and the numbers of mature (fraction F) cells recirculating in the bone marrow proved statistically indistinguishable from wild-type littermates. In contrast, in the mice that were forced to increase CDR-H3 usage of either hydrophobic (*D-D μ FS*) or positively charged (*D-iD*) amino acids, the numbers of conventional splenic follicular and bone marrow mature B cells were nearly halved. In our studies, normalization of MZ B cell numbers was observed in *D-D μ FS* mice as opposed to the mice forced to use arginine-enriched *D-iD*, in which MZ B-cell numbers increased by one-third. These data suggested to us that the MZ compartment might be tolerant, or even preferentially accepting, of antibodies with antigen-binding sites containing charged CDR-H3s. In later studies, we confirmed this prediction by examining the composition of CDR H3s cloned from wild-type MZ B cells. We found that the repertoire is normally enriched for positively charged amino acids.¹⁰

Finally, we studied the effect of altering the pattern of hydrophobicity of CDR-H3 among PerC B-1a, B-1b and B-2 cell subsets. In both the *D-DFL* and *D-D μ FS*, the numbers of all three B cell subsets were similar to the wild type (Fig. 1).⁷ On the other hand, mice forced to produce a repertoire enriched for highly charged CDR-H3s (*D-iD* mice) demonstrated a decrease in these populations.⁶ Unlike the follicle, where both hydrophobic and charged sequences were being suppressed, our findings suggested that the PerC B cell compartment might be permissive of immunoglobulin with hydrophobic CDR-H3s while still suppressing charged CDR-H3s.^{3,10} To test this hypothesis, we examined the composition of CDR-H3 cloned from wild-type PerC B cell subsets more closely. The latter section of this review will present a new analysis of our previous published data⁸ and discuss our findings in greater detail.

The role of evolutionarily conserved D_H coding sequence in natural antibody immunity

One of the most studied examples of the association between evolutionary conserved germ line VDJ gene rearrangement and antigen specificity among B-1a cells is the natural antibody repertoire against phosphorylcholine (PC). PC is present on the cell wall of a number of common pathogens, including *Streptococcus pneumoniae*.^{15,16}

Although many antibodies can bind PC, those containing the germ line canonical T15 idiotype dominate the preimmune anti-PC repertoire. It is believed that B-1a cells bearing the T15-Id are selected and expanded in response to endogenous oxidation-specific epitopes on low-density lipoprotein (OxLDL) and apoptotic cells.^{17,18} The ability of non-mutated

T15 antibodies to provide optimal protection against lethal infection with *S. pneumoniae* in mice suggested a link between endogenous self-reactivity and protective activity against exogenous pathogens.^{19,20}

The T15-Id is conserved across multiple mouse strains and is tightly associated with the use of a specific V_H (V_HS107.1) and a specific V_L (V_K22).²¹ Disruption of the V_HS107.1 gene results in an inability to mount a protective immune response to *S. pneumoniae*.²² In common with many other B-1a immunoglobulins; canonical anti-PC T15 CDR-H3s lack N-region addition and use their D_H, in this case DFL16.1, in only one of six potential reading frames, RF1.²³ Taken together, these findings raised the possibility that the complete evolutionary conserved sequence of DFL16.1 in general, and of RF1 in particular, is critical for creating a category of antibodies with naturally selected germ line CDR-H3 sequence that would provide protection against an ubiquitous epitope on the surface of common pathogens.

To gain insight into the relative roles of natural (germ line) versus somatic selection of CDR-H3 sequence in the generation and function of the anti-PC NAb repertoire, and to directly challenge the hypothesis of the role of evolutionarily conserved D_H sequences in regulating antibody-dependent protection against a common pathogen, we turned to our panel of D_H-altered BALB/c mice.⁹ Each strain of these D_H-altered mice presents a different variation in the sequence of the *DFL16.1* segment, which is the gene normally used to encode the T15-Id Nab. This pattern of *DFL16.1* variation made these mice ideal for testing the role of D_H conservation in the anti-PC/T15 system,

The details of the variation in sequence become very important in analyzing the data that we obtained. In the context of T15 prototypic sequences, mice using the *D-DFL* IgH allele use only *DFL16.1*, which happens to be the D_H component of the canonical T15-Id⁺ anti-PC repertoire. In the perinatal period where N addition is rare, mice containing a *D-DFL* IgH allele would be predicted to produce antibodies with a classical T15 CDR-H3-type sequence with high efficiency. The second D_H-altered mouse (*D-DμFS*) contains slightly altered *DFL16.1* sequence. There is a preference for RF2, but RF1 sequence can still be accessed. However, the second frameshift mutation introduced a termination codon near the end of the RF1 sequence. Thus, creation of a CDR-H3 with a canonical T15 sequence would require extensive nucleotide nibbling at the 3' end of the D_H segment accompanied by N nucleotide addition at the D→J junction that could replace the termination codon with a codon for the missing terminal RF1 sequence. Thus in this D_H-altered strain production of the classical T15 CDR-H3 amino acid sequence would require TdT-catalyzed N addition to mend *DFL16.1*. T15 antibodies typically arise in the perinatal period, which lacks N addition. Creation of a T15 sequence in a *D-DμFS* mouse would thus require the contribution of a postnatal-developing B-1a cell that had experienced TdT activity. The third D_H-altered mouse (*D-iD*) completely lacks the core *DFL16.1* RF1 sequence. Creation of a classical T15 CDR-H3 amino acid sequence would entail a complete rebuilding of DFL16.1 amino acid sequence that would require both extensive exonucleolytic nibbling of the germline D_H and extensive N addition. Given the likely low frequency of such a product, population of the B-1a compartment with such cells would further require somatic selection and massive clonal expansion.

To test the effect of the change in CDR-H3 content on natural antibody (Nab) production, we quantified the serum titers of anti-PC IgM Nabs in the different D_H-altered strains to test the requirement for the *DFL16.1* RF1 sequence in the generation of Nabs specific for PC. In unmanipulated mice, we found that there was a direct relationship between serum anti-PC titer and the likelihood of using *DFL16.1* in its germ line form (Fig. 2A). This suggested that the availability of B cells incorporating germ line-encoded *DFL16.1* RF1 sequence into their Ig H chains might be directly influencing the physiological production of natural anti-PC antibodies. We also found a correlation between the availability of *DFL16.1* RF1 and the levels of T15-Id⁺ Nabs (Fig. 2A). While there was an equivalent number of B-1a cells bearing anti-PC BCR in the PerC, the frequency of T15-Id⁺ B-1a cells directly correlated with the likelihood of access to *DFL16.1* RF1 sequence.⁹ These data suggested that the potential to secrete T15-Id⁺ antibodies, but not total anti-PC, might be, as predicted, dependent on the availability of naturally selected *DFL16.1* sequence.

To test this prediction, we sorted, sequenced, and analyzed the CDR-H3 interval using anti-PC T15-Id⁺ B-1a cells from the PerC of wild-type and homozygous D_H-altered mice. Classic T15 CDR-H3 amino acid sequence was only observed in B-1a cells with access to at least the core of the germ line sequence of *DFL16.1*. Moreover, the presentation of this sequence among antibodies expressing PC and T15-binding BCR proved dependent on its availability for usage (Fig. 2B). Among the sequences isolated from AB1.2/PC-DEX⁺ B-1a cells from the *D-D μ FS* mouse, which has a damaged *DFL16.1* sequence, we found an example of a clone that had recreated the classic T15 anti-PC amino acid sequence by means of N addition. No such clones were found in the sequences obtained from the *D-iD* mouse, which completely lacks *DFL16.1*. Together, these findings suggest that while somatic, clonal selection pressure is being exerted to create the classic T15 anti-PC CDR-H3 sequence, the mechanism of N addition was able to help correct a D_H that contained core RF1 sequence, but failed to recreate a T15 sequence where the D_H completely lacked this core sequence. Production of anti-PC with canonical T15 CDRH3 thus appeared highly dependent on natural selection of germ line immunoglobulin sequence.

Immunization with heat-killed bacteria and even exposure to live bacteria in a life-threatening situation proved insufficient to elicit classic T15-Id anti-PC antibodies in the absence of evolutionary conserved D_H sequences.⁹ That is, the available B-1a population proved incapable of providing that first line of defense against infection that is normally contributed by the natural antibody repertoire.

Of note, this same T15-Id, induced by endogenous OxLDL, has been suggested to play a physiological role in atherosclerosis by preventing potentially atherogenic uptake of this lipid by macrophages.^{18,24} Interestingly, anti-OxLDL reactivity was preserved in all strains of D_H-altered mice, including *D-iD* mice, which completely lack the evolutionary conserved *DFL16.1* segment (Fig. 2A). Moreover, these Nabs elicited in the absence of evolutionary conserved D_H sequence are capable of preventing the uptake of OxLDL by macrophages (data not shown and Ref. 9). It would appear that antibodies encoded by alternative germ line sequences are being induced to replace the wild-type anti-OxLDL antibody repertoire. These data support the view that access to conserved germ line D_H

sequence is not required to generate a fully functional physiological repertoire against self-antigens of this type.

These studies support the notion that D_H gene segments have a dual role in repertoire generation. In addition to serving as a nidus for two rounds of N addition; preservation of amino acid content signatures by reading frame coupled with reading frame control also enable the D_H to shape, or delimit, the range of diversity that is generated in the antigen-binding site repertoire. This concept brings a novel perspective on the mechanisms that natural selection may employ to shape the natural immunoglobulin repertoire in order to provide anticipatory resistance to disease. By altering the germ line D_H repertoire, we were able to sever the link between natural self-reactivity and anticipatory protection against a bacterial pathogen.⁹

The preservation of D_H coding sequence seems to be directly related to the hydrophobic-enriched CDR-H3 in the B-1 cell subset

As discussed above in this manuscript, we have previously identified somatically imposed sequence signatures in the range of the CDR-H3 repertoires expressed in BALB/c wild-type mice by MZ and FO B cells in the spleen¹⁰ and by the peritoneal cavity (PerC) B-1a, B-1b, and B-2 B cell subsets.⁸ In the latter, we found that, whereas each PerC B cell subset retains a number of CDR-H3 sequence signatures that mark their respective fetal and/or adult origins,²⁵ the final result includes signature features that are unique to the PerC compartment. The most striking feature found was the permissiveness for hydrophobic CDR-H3s in the PerC B cell populations.

As noted previously, in spite of access to six different D_H reading frames, each with a different amino acid sequence, the majority of B cells in all jawed vertebrates studied to date prefer to express Igs that use D_H RF1 in their CDR-H3s. Because B-1a cells seem to display higher degree of hydrophobicity, we reasoned that if the use of D_H coding sequence was primarily responsible for the enhanced use of hydrophobic-enriched CDR-H3 in the PerC B cells, by means of alternative RF usage, for instance RF2, there would be a positive correlation between the length of the sequence contributed by the D_H and the hydrophobicity index.

To assess the actual contribution of D_H sequence to CDR-H3 loop hydrophobicity in the peritoneal cavity (PerC) B cell populations, we revisited our previously published sequences from immature and mature bone marrow B cells (Hardy fractions E and F, respectively),³ and PerC B cells (B-1a, B-1b, and B-2) of BALB/c wild-type mice.⁸ We plotted the D_H length of each retrieved coding sequence against its respective average hydrophobicity (Fig. 3). In both immature (BM E) and mature (BM F) bone marrow B cells, we found no significant correlation between the length of the sequence contributed by the D_H and hydrophobicity ($P > 0.05$ in both BM E and BM F) (Fig. 3A). In both B-1a and B-1b PerC B cell populations, however, longer lengths of remaining D_H sequence correlated with greater hydrophobicity ($P = 0.001$ and $P = 0.018$, respectively). The same trend was seen in the PerC B-2 population, but it did not reach statistical significance ($P = 0.086$) (Fig. 3A).

To assess the relative contribution of N addition versus D_H sequence to CDR-H3 loop hydrophobicity, a similar analysis was applied to each B cell population, but in this case by plotting the length of the sequence encoded by N nucleotides against the hydrophobicity index. No direct effect on the hydrophobicity index was observed as the contribution of N nucleotides became more prominent (Fig. 3B). These findings provided support for the hypothesis that the increase in hydrophobic CDR-H3s in PerC B cells reflected increased preservation of germ line RF2 sequence from the D_H gene segment.

Antibodies with hydrophobic patches have been suggested to play key roles in both self- and non-self– (pathogenic) antigen recognition. For example, two broadly neutralizing antibodies that recognize the membrane-proximal external region (MPER) of the HIV-1 gp41 transmembrane glycoprotein, the monoclonal antibodies 2F5 and 4E10, contain hydrophobic patches in CDR-H3 that are required for stable interactions between antibody and antigen that are needed for neutralization.^{26,27} With respect to autoantigens, self-reactive antibodies have been implicated both in physiological and pathological processes.²⁸ Interestingly, they appear to be enriched for hydrophobic residues in CDR-H3;²⁹ this is in agreement with our findings on B-1a repertoire, as B-1a B cells are the major producers of natural antibodies. The antigens targeted by autoantibodies with hydrophobic CDR-H3 remain largely unknown. Identification of these antigens is an important area of research.³⁰ As discussed above, antibodies that retain the hydrophobic patches can be difficult to elicit, likely due to the scarcity of antibodies with this feature in the major B cell compartments. Our findings indicate that the peritoneal compartment may be a potentially targetable source for these types of rare hydrophobic antibodies.

Concluding remarks

Both natural and somatic selection appear to be used to create a restricted, unique, and functional B-1 cell CDR-H3 repertoire. Our findings strongly suggest that D_H gene sequence play a major role in regulating the antigen-binding site features of the natural antibodies. Harnessing the power of this naturally selected repertoire to medical interventions such as vaccination and immunotherapy may yield new forms of treatment for common diseases. Conversely, the absence of specific immunoglobulin germ line sequence may help explain why some forms of vaccination have been ineffective and why our species is sensitive to some pathogens, but not others. In parallel, another set of data we obtained suggested to us that the B-1 repertoire might be either permissive or even selective for hydrophobic sequences, in a D_H-dependent manner. Future studies will investigate, in depth, whether B cells enriched for hydrophobic CDR-H3s are being actively selected into or expanded in the PerC B cell compartment, and whether they may be detrimental to B cell responses in conventional tissues. It is tempting to speculate that these hydrophobic CDR-H3s exhibit unique antigen-binding patterns that could serve as a reservoir for the diversity needed to respond to unusual antigens or vaccines, such as HIV virus epitopes.²⁷

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References

1. Kabat, EA.; Wu, TT.; Perry, HM.; Gottesman, KS.; Foeller, C. Sequences of proteins of immunological interest. U.S.Department of Health and Human Services; Bethesda, Maryland: 1991.
2. Xu JL, Davis MM. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity*. 2000; 13:37–45. [PubMed: 10933393]
3. Ivanov, Schelonka RL, Zhuang Y, Gartland GL, Zemlin M, Schroeder HW Jr. Development of the expressed Ig CDR-H3 repertoire is marked by focusing of constraints in length, amino acid use, and charge that are first established in early B cell progenitors. *J Immunol*. 2005; 174:7773–80. [PubMed: 15944280]
4. Zemlin M, Klinger M, Link J, Zemlin C, Bauer K, Engler JA, et al. Expressed murine and human CDR-H3 intervals of equal length exhibit distinct repertoires that differ in their amino acid composition and predicted range of structures. *J Mol Biol*. 2003; 334:733–49. [PubMed: 14636599]
5. Schelonka RL, Ivanov, Jung DH, Ippolito GC, Nitschke L, Zhuang Y, et al. A single DH gene segment creates its own unique CDR-H3 repertoire and is sufficient for B cell development and immune function. *J Immunol*. 2005; 175:6624–32. [PubMed: 16272317]
6. Ippolito GC, Schelonka RL, Zemlin M, Ivanov, Kobayashi R, Zemlin C, et al. Forced usage of positively charged amino acids in immunoglobulin CDR-H3 impairs B cell development and antibody production. *J Exp Med*. 2006; 203:1567–78. [PubMed: 16754718]
7. Schelonka RL, Zemlin M, Kobayashi R, Ippolito GC, Zhuang Y, Gartland GL, et al. Preferential use of DH reading frame 2 alters B cell development and antigen-specific antibody production. *J Immunol*. 2008; 181:8409–15. [PubMed: 19050258]
8. Vale AM, Tanner JM, Schelonka RL, Zhuang Y, Zemlin M, Gartland GL, 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; 185:6085–95. [PubMed: 20956345]
9. Vale AM, Kapoor P, Skibinski GA, Elgavish A, Mahmoud TI, Zemlin C, et al. The link between antibodies to OxLDL and natural protection against pneumococci depends on DH gene conservation. *J Exp Med*. 2013; 210:875–890. [PubMed: 23589567]
10. Schelonka RL, Tanner J, Zhuang Y, Gartland GL, Zemlin M, Schroeder HW Jr. Categorical selection of the antibody repertoire in splenic B cells. *Eur J Immunol*. 2007; 37:1010–21. [PubMed: 17345580]
11. Hardy RR, Hayakawa K. B cell development pathways. *Annu Rev Immunol*. 2001; 19:595–621. [PubMed: 11244048]
12. Ivanov, II.; Link, JM.; Ippolito, GC.; Schroeder, HW, Jr. Constraints on hydrophobicity and sequence composition of HCDR3 are conserved across evolution.. In: Zanetti, M.; Capra, JD., editors. *The Antibodies*. Taylor and Francis Group; London: 2002. p. 43-67.
13. Zemlin M, Schelonka RL, Ippolito GC, Zemlin C, Zhuang Y, Gartland GL, et al. Regulation of repertoire development through genetic control of DH reading frame preference. *J Immunol*. 2008; 181:8416–24. [PubMed: 19050259]
14. Schroeder HW Jr, Zemlin M, Khass 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; 30:327–44. [PubMed: 20666706]
15. Potter M, Leon MA. Three IgA myeloma immunoglobulins from the BALB/ mouse: precipitation with pneumococcal C polysaccharide. *Science*. 1968; 162:369–71. [PubMed: 4175691]
16. Briles DE, Nahm M, Schroer K, Davie J, Baker P, Kearney J, et al. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 streptococcus pneumoniae. *J Exp Med*. 1981; 153:694–705. [PubMed: 7252411]
17. Binder CJ, Silverman GJ. Natural antibodies and the autoimmunity of atherosclerosis. *Springer Semin Immunopathol*. 2005; 26:385–404. [PubMed: 15609021]
18. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, et al. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J Clin Invest*. 2009; 119:1335–49. [PubMed: 19363291]

19. McDaniel LS, Benjamin WH Jr, Forman C, Briles DE. Blood clearance by anti-phosphocholine antibodies as a mechanism of protection in experimental pneumococcal bacteremia. *J Immunol.* 1984; 133:3308–12. [PubMed: 6491288]
20. Briles DE, Forman C, Hudak S, Claflin JL. Anti-phosphorylcholine antibodies of the T15 idiotypic are optimally protective against *Streptococcus pneumoniae*. *J Exp Med.* 1982; 156:1177–85. [PubMed: 7153709]
21. Crews S, Griffin J, Huang H, Calame K, Hood L. A single VH gene segment encodes the immune response to phosphorylcholine: somatic mutation is correlated with the class of the antibody. *Cell.* 1981; 25:59–66. [PubMed: 6791833]
22. Mi QS, Zhou L, Schulze DH, Fischer RT, Lustig A, Rezanka LJ, et al. Highly reduced protection against *Streptococcus pneumoniae* after deletion of a single heavy chain gene in mouse. *Proc Natl Acad Sci U S A.* 2000; 97:6031–6. [PubMed: 10811914]
23. Feeney AJ. Predominance of the prototypic T15 anti-phosphorylcholine junctional sequence in neonatal pre-B cells. *J Immunol.* 1991; 147:4343–50. [PubMed: 1753104]
24. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, et al. Natural antibodies with the T15 idiotypic may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest.* 2000; 105:1731–40. [PubMed: 10862788]
25. Montecino-Rodriguez E, Dorshkind K. B-1 B cell development in the fetus and adult. *Immunity.* 2012; 36:13–21. [PubMed: 22284417]
26. Ofek G, McKee K, Yang Y, Yang ZY, Skinner J, Guenaga FJ, et al. Relationship between antibody 2F5 neutralization of HIV-1 and hydrophobicity of its heavy chain third complementarity-determining region. *J Virol.* 2010; 84:2955–62. [PubMed: 20042512]
27. Kwong PD, Mascola JR, Nabel GJ. Rational Design of Vaccines to Elicit Broadly Neutralizing Antibodies to HIV-1. *Cold Spring Harb Perspect Med.* 2011; 1:a007278. [PubMed: 22229123]
28. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol.* 2011; 11:34–46. [PubMed: 21151033]
29. Butler JE, Lemke CD, Weber P, Sinkora M, Lager KM. Antibody repertoire development in fetal and neonatal piglets: XIX. Undiversified B cells with hydrophobic HCDR3s preferentially proliferate in the porcine reproductive and respiratory syndrome. *J Immunol.* 2007; 178:6320–31. [PubMed: 17475861]
30. Wu X, Liu B, Van der Merwe PL, Kalis NN, Berney SM, Young DC. Myosin-reactive autoantibodies in rheumatic carditis and normal fetus. *Clin Immunol Immunopathol.* 1998; 87:184–92. [PubMed: 9614934]
31. Vale AM, Nobrega A, Schroeder HW. J. Linkage of Natural Antibody Immunologic Homeostasis to Anticipatory Protection against a Common Pathogen Requires Natural Selection of Immunoglobulin Diversity Gene Segment Sequence. *Journal of Immunology & Clinical Research.* 2013; 1:2005.

Figure 1.

Schematic model of bone marrow B cell differentiation and the peripheral distribution of the major mature B cell subsets. Shown is the divergence in the absolute numbers of B cells from homozygous $D-DFL$, $D-D\mu FS$, and $D-iD$ mice relative to their wild-type littermate controls. Percent loss or gain relative to wild-type are shown in bone marrow, splenic and peritoneal cavity (PerC) B cell subsets. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; and **** = $P < 0.0001$. Also shown is the distinct perinatal B cell progenitor that give rise to the B-1 cell subsets. The data presented are modified from Ref. 7.

Figure 2.

Loss of evolutionarily conserved D_H sequence affects natural antibody immunity. (A) The median serum concentration of natural IgM anti-PC, T15-Id⁺, and anti-OxLDL, by mouse genotype are plotted as a function of the relative availability of DFL16.1 in RF1 compared to wild type, which ranges as follows: $D-iD \ll D-D\mu FS < WT \ll D-DFL$. The levels of IgM antibodies for each mouse strain are shown as an equivalent titer ($\mu\text{g/ml}$) to the BALB/c IgM anti-PC-producing hybridoma BH8. (B) Flow cytometry plots show representative gates used to sort peritoneal B-1a cells that stain highly for PC-DEX and AB1.2. We performed CDR-H3 analysis of transcripts of sorted PC-DEX^{hi} AB1.2⁺ B-1a cells from each mouse strain. Clonal diversity pie charts depict the proportion of individual CDR-H3 sequences. The T15 prototypes are shown in gray. The bold italic letters represent N-region addition. Modified from Refs. 9 and 31.

Figure 3.

Relative contributions of wild-type D_H coding sequences to the CDR-H3 loop hydrophobicity. Shown is a new analysis of our previous published data.⁸ (A) Sequences of CDR-H3 transcripts from immature and mature bone marrow B lineages, and PerC B cell subpopulations were retrieved from our previous studies,^{3,8} and the length of the D_H nucleotide coding sequence was plotted against its respective average of CDR-H3 hydrophobicity. (B) Length of sequence encoded by N nucleotides was plotted against the respective average hydrophobicity.