Preimmune Control of the Variance of TCR CDR-B3: Insights Gained From Germline Replacement of a TCR Dβ Gene Segment With an Ig D_H Gene Segment

Mohamed Khass1,2†, Michael Levinson1†, Robert L. Schelonka3, Pratibha Kapoor1, Peter D. Burrows4 and Harry W. Schroeder Jr.5*

1 Division of Clinical Immunology and Rheumatology, Department of Medicine, The University of Alabama at Birmingham, Birmingham, AL, United States, 2 Division of Genetic Engineering and Biotechnology, National Research Center, Cairo, Egypt, 3 Division of Neonatology, Department of Pediatrics, Oregon Health Science Center, Portland, OR, United States, 4 Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, United States, 5 Division of Clinical Immunology and Rheumatology, Department of Medicine, Microbiology, and Genetics, The University of Alabama at Birmingham, Birmingham, AL, United States

We have previously shown that the sequence of the immunoglobulin diversity gene segment (D_H) helps dictate the structure and composition of complementarity determining region 3 of the immunoglobulin heavy chain (CDR-H3). In order to test the role of germline D sequence on the diversity of the preimmune TCRβ repertoire of T cells, we generated a mouse with a mutant TCRβ DJC locus wherein the Dβ2-Jβ2 gene segment cluster was deleted and the remaining diversity gene segment, Dβ1 (IMGT:TRDB1), was replaced with DSP2.3 (IMGT:IGHD2-02), a commonly used B cell immunoglobulin D_H gene segment. Crystallographic studies have shown that the length and thus structure of TCR CDR-B3 places amino acids at the tip of CDR-B3 in a position to directly interact with peptide bound to an MHC molecule. The length distribution of complementarity determining region 3 of the T cell receptor beta chain (CDR-B3) has been proposed to be restricted largely by MHC-specific selection, disfavoring CDR-B3 that are too long or too short. Here we show that the mechanism of control of CDR-B3 length depends on the Dβ sequence, which in turn dictates exonucleolytic nibbling. By contrast, the extent of N addition and the variance of created CDR3 lengths are regulated by the cell of origin, the thymocyte. We found that the sequence of the D and control of N addition collaborate to bias the distribution of CDR-B3 lengths in the pre-immune TCR repertoire and to focus the diversity provided by N addition and the sequence of the D on that portion of CDR-B3 that is most likely to interact with the peptide that is bound to the presenting MHC.

Keywords: germline, T cell receptor, gene segment, immunoglobulin, D gene segment

INTRODUCTION

V(D)J rearrangement has been calculated to yield potential repertoires of more than 10^{16} different T cell receptor (TCR) or immunoglobulin (Ig) antigen binding sites (1, 2). A fundamental issue is the extent to which diversity is random or directed. The former would imply that diversification of the repertoire is purely a matter of chance. The latter would suggest that diversification takes
place under germline control in order to optimize the creation of a functional repertoire and minimize autoreactive clones.

A major component of antigen receptor diversity comes from the inclusion of a diversity (D) gene segment into the rearrangement process. In B cells, D gene segments contribute to Ig heavy (H) chain diversity and in αβ T cells they contribute to TCRβ diversity. In both antigen receptors, amino acids encoded by the D are positioned at the center of complementarity determining region 3 (CDR-H3 for the immunoglobulin H chain and CDR-B3 for the T cell receptor beta chain), which is the direct product of V(D)J rearrangement (3). In both Ig and TCR, D gene segment-encoded amino acids within CDR3 commonly contribute directly to the recognition and binding of cognate antigens. The inclusion of a D gene segment also allows two rounds of junctional diversification during VDJ rearrangement. The somatic mechanisms of CDR3 junctional diversification include terminal exonucleolytic “nibbling,” P junction extension and N nucleotide addition.

Although potential CDR3 diversity is astronomical, we have previously shown that there are constraints on the structures of the immunoglobulin CDR-H3 repertoire that can be detected through analysis of as few as ten to twenty sequences, not thousands or millions. For example, in progenitor B cells, constraints on germline D$_H$ sequence can heavily influence the structure and composition of immunoglobulin H chain (CDR-H3) (4). Thus, constraints on D$_H$ germline content represent one mechanism by which the structural diversity of the repertoire can be directed.

In order to further test the role of germline D sequence on the shape of the preimmune CDR3 repertoire, we turned to the TCRβ locus and created a mouse with a mutant TCRβ DJC locus wherein the Dβ2-Jβ2 gene segment cluster had been deleted (Dβ2ko) and the remaining Dβ1 gene segment [ImMunoGeneTics (IMGT) database (5) (IMGT: TRBD1)] replaced with a commonly used D$_H$ gene segment DSP2.3 [IMGT: IGHD2-7(BALB/c)].

We found that the mechanism of control of CDR-B3 length, which is important for optimal MHC:peptide interactions, depends on the Dβ3 length, which is important for optimal MHC:peptide

**MATERIALS AND METHODS**

**Generation of Targeted ES Cells and the DβYTL Mouse**

Plasmids containing the germline C57BL/7 Dβ1 and Jβ1 loci were the kind gift of Dr. Barry Sleckman. The targeting construct was generated using a plNtk targeting vector containing a Sall–loxP–Neo′–loxP–XhoI-TK cassette (Supplementary Figure S1). A 4.4 kb KpnI–SacII 3′ homology arm containing the Jβ1 gene segments was subcloned into the XhoI site by blunt-end ligation.

A plasmid (BSSK5′M) containing Dβ1 was used as a substrate for PCR directed replacement of TCRβ Dβ1 by IgH DSP2.3. Overlapping 64 base pair primers containing the sequence of DSP2.3 in place of Dβ1 were generated. These were 5′ tgttaagcttgaa catttgc TCTACTATTGTTTACGAC caaggtg ataattatatgg 3′ and 5′ cttctcatggaatgacaccgggggtgggattagataaatgc 3′. The sequence of the D is in caps and the heptamers are separated by spaces from the rest of the sequence. Each of these was individually paired with a forward primer (5′ aataaccttgagggacagcctgaaaccggcccgggctgggattagataaatgc 3′) downstream of a HindIII site. The overlapping PCR products were then annealed and PCR amplification was performed with the upstream of Bsu36I and downstream of HindIII primers alone. The resulting PCR amplified product was cut with Bsu36I and HindIII, and back cloned into the BSSK5′M plasmid, thus replacing Dβ1 with DSP2.3.

A 2.6 kb NotI–ClaI 5′ homology arm was cut from the BSSK5′M plasmid and subcloned by blunt-end ligation into a SalI upstream of the first loxP site in the plNtk + 3′ homology arm plasmid. The resulting 14.9 kb targeting vector was linearized with PvuI and electroporated into 129 derived Dβ2−/− (Dβ2ko) mouse ES cells (6, 7). Briefly, 1 × 10$^6$ ES cells were electroporated with 25 µg of linearized vector DNA in a 0.4 cm cuvette at 240 V and 500 µF (Bio-Rad Gene Pulsar, Bio-Rad Laboratories, Hercules, CA). Individual ES cell clones were selected with 200 µg/mL G418 (positive selection) and 2 µM Ganciclovir (negative selection) from 24 h after electroporation for a total of 2 weeks. The transfection efficiency was 3%.

ES cell clones with homologous recombination were identified by long PCR using LA Taq DNA polymerase (Takara Bio USA, Mountain View, CA, United States). The PCR program used was (1) denaturation at 94°C for 1 min, (2) 94°C for 20 sec, 68°C for 7 min for 31 cycles, (3) 68°C for 10 min, and (4) hold at 4°C. The primers used to identify the correct 3′ end of the recombinant were a 5′ primer from the mouse TCRDβ1 region (5′ gtaggtcattcattgtaggggaaggttaggtg 3′) and a 3′ primer from the Neo loxP region of the targeting vector (5′ gcagccaaagggcggaaatggctttgg 3′). The primers used to identify the correct 3′ end of the recombinant were a 5′ primer from Neo loxP region (5′ acgggggggtgtgggggtgttgattaaagtc 3′) and a 3′ primer from mouse TCRDβ1 region (5′ ccatggaatgctagggcagcaggtttg 3′).

The TCRβ DJC locus resulting from this manipulation contained the original germline Dβ1 recombination signal sequences that now flanked immunoglobulin D$_H$ DSP2.3 in place of Dβ1. It contained the Jβ1 and Cβ1 locus in its entirety as well as the Cβ2 constant domain but lacked Dβ2 and Jβ2 sequences. We termed this new D$_H$ substituted TCR locus DβYTL, which refers to the central amino acids in each of its three reading frames (i.e., tyrosine, threonine, and leucine). For the purposes of this manuscript, we renamed the original Dβ2−/− gene targeted locus Dβ2ko to emphasize the deletion of Dβ2.

Two original Dβ2ko ES cells and two independently derived DβYTL ES cell clones were independently microinjected into C57BL6/J blastocysts. The resulting chimeric mice were bred to wild type C57BL6/J mice. The agouti offspring were genotyped by tail DNA PCR analysis to assess germline transmission of the DβYTL or Dβ2ko TCR alleles. Homozygous DβYTL mice
were bred to transgenic mice expressing the Cre recombinase from the CMV promoter to delete the LoxP-flanked Neo’ gene during early embryogenesis (Cre mice were obtained from Jackson Laboratories). Deletion of the Neo gene in the offspring was confirmed by PCR using Cre3 (5’gaattactgacctgac3 ’) and Cre4 (5’catcgccacctctccacag3 ’) primers. The homozygous progeny harboring mutant Djβ2ko mice, single cell suspensions were prepared from the thymus of two mice each. Red blood cells were removed with RBC lysing solution (1 mM KHCO₃, 0.15 M NaH₂Cl, and 0.1 mM Na₂EDTA). Cells were washed twice and resuspended in a master-mix of staining buffer containing optimal concentrations of monoclonal antibody reagents. Samples were sorted with a FACS Aria (Becton Dickinson). Double negative thymic cells were stained with PE-Cy7-CD25 (BD Cat #552880), APC-CD44 (BD Cat #559250), biotinylated-CD28 (BD Cat #553296) (developed secondarily with streptavidin), and a lineage stain [PE-CD3 (BD Cat #555275), monoclonal PE-CD4 (BD Cat #553049), PE-CD8a (BD Cat #553033), PE-B220 (BD Cat #561878), PE-CD11b (BD Cat #553111), PE-NK1.1 (BD Cat #553165)] to remove mature T, B, and NK cells. The cells were stained with propidium iodide (PI) to identify/sort live cells. DN2 cells were defined as CD44⁺ and CD25⁺ (Supplementary Figure S2).

### RNA, RT-PCR, DNA Cloning, and Sequencing

Total RNA was prepared from 1 × 10⁴ to 2 × 10⁴ cells of each individual subset, sorted directly into RLT lysing buffer using a QIAGEN RNase kit. RNA was used to synthesize cDNA using the QIAGEN RT-PCR Kit and the manufacturer’s recommended protocol under the following conditions: 95°C denaturation for 2 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final 72°C extension for 10 min. The reaction buffer contained 100 mM Tris-HCl, pH 8.8, 15 mM MgCl₂, and 750 mM KCl. Primers used were TCRB13-1 (5’-tgctggcaaccttcgaatagga-3’) and TCRBC1 (5’-tgagaattgtgactccacca-3’). PCR products were cloned (TOPOTA Cloning Kit; Invitrogen) and sequenced using the primer TCRB13-1 on an ABI 3730 sequencer.

### Sequence Analysis of CDR-H3 and CDR-β3

Eight of the thirteen D₂H gene segments in BALB/c mice and six of the ten D₂H in C57BL/6 mice belong to the DSP family. Due to the extensive sequence similarity among these gene segments, it is often difficult to determine exactly which DSP germline gene segment contributed to an individual CDR-H3. Thus, we grouped all of the sequences of CDR-H3 that had identifiable DSP family sequence into wild type controls.

194 V₅H7183 CDR-H3 sequences from BALB/c Hardy Fraction B [CD19⁺CD43⁺Bp⁻1⁻IgM⁻] (8) and 72 V₅H7183CDR-H3 sequences from the C57BL/6 Fraction B equivalent [B220⁺cKit⁺CD25⁻, BP⁻1⁻] (9) proB cells were previously published and analyzed as a whole (4). Of these there were 96 BALB/c sequences and 24 C57BL/6 sequences that contained an identifiable member of the DSP gene segment family (Figure 1 and Supplementary Table S1).

### Statistical Analysis

Statistical analysis was performed with JMP version 14 (SAS Institute) or GraphPad Prism 8 version 8 (GraphPad Software, San Diego, CA, United States). Population means were analyzed using one-way analysis of variance (ANOVA) test. Variance was assessed with the O’Brien Test for Homogeneity of Variance. Categorical comparisons were performed with Fisher’s exact test.

### RESULTS

In order to test the effect of changing the sequence of a D gene segment on VDJβ rearrangement and N addition, we replaced the Dβ1 gene segment with DSP2.3, a commonly used Ig D₂H gene segment, to create a new TCRβ allele we termed DjβYT1, which refers to the central amino acids in each of its three reading frames (i.e., tyrosine, threonine, and leucine). To simplify the analysis, we introduced this gene substitution mutation into an ES cell that had previously undergone a gene targeted deletion of the Dβ2-Jβ2 gene segment cluster (Djβ2ko). We chose to utilize a sequence of a member of the DSP D₂H family (Table 1 and Supplementary Table S2) because together the members of this family make up a majority of functional D₂H sequences in both BALB/c and C57BL/6 mice, the DSP2.3 sequence is found in the germlines of both strains and, unlike the case with many other D₂H segments, none of the three D₂H DSP2.3 reading frames rearranged by deletion contain a termination codon (11).

### Cloning of Representative CDR3 Sequences From Pre Selection Thymocytes

To assess the effect of the surrounding locus on natural selection of CDR-B3 and identify how the elements that contribute to CDR3 are being processed during development, we compared...
CDR-B3 content in DβYTL DN2 thymocytes to a panel of bone marrow proB and thymocyte DN2 controls. Most of our previous studies of the CDR-H3 repertoire were performed in BALB/c mice, hence sequences from BALB/c bone marrow proB cells were used as a major control for how DSP gene segments are normally handled. However, since the DβYTL allele was studied in C57BL/6 mice, we also compared CDR-H3 sequences from wild type C57BL/6 bone marrow proB cells as an additional control. We controlled for the effect of deleting the Dβ2-Jβ2 gene segment cluster by analyzing CDR-B3 sequences from Dβ2ko DN2 thymocytes, where the wild type Dβ1-Jβ1 locus was intact and the Dβ2-Jβ2 locus had been deleted. Together with the CDR-H3 sequences, study of these CDR-B3 sequences allowed comparison of how DSP gene segments were handled in two different strains and in the context of the TCR locus, as well as between the single DSP gene segment and Dβ1, both in the presence and absence of the Dβ2-Jβ2 locus.

We had previously cloned and sequenced immunoglobulin HC transcripts from BALB/c and C57BL/6 Fraction B cells that used members of the VH7183 family (4, 9). From this library of sequences, we identified 96 DSP containing sequences from BALB/c and 24 from C57BL/6 (Figure 1). To sample how the elements that contribute to CDR-B3 content are being processed, we randomly chose to analyze CDR-B3 sequences from TCR Vβ13.1 containing transcripts. We PCR amplified TCRβ sequences containing Vβ13.1 containing transcripts. We PCR amplified TCRβ sequences containing Vβ13.1 containing transcripts. We PCR amplified TCRβ sequences containing Vβ13.1 containing transcripts. We PCR amplified TCRβ sequences containing Vβ13.1 containing transcripts. We PCR amplified TCRβ sequences containing Vβ13.1 containing transcripts. We PCR amplified TCRβ sequences containing Vβ13.1 containing transcripts. We PCR amplified TCRβ sequences containing Vβ13.1 containing transcripts.

In Figures 2–5, we display the data in the order from left to right or from top to bottom for sequences obtained from BALB/c wild type Fraction B proB cells, C57BL/6 wild type Fraction B proB cells, C57BL/6 DβYTL DN2 thymocytes, C57BL/6 Dβ2ko DN2 thymocytes, and C57BL/6 wild type DN2 thymocytes. This arrangement facilitates visual comparison of the effects of D sequence and cell lineage on VDJ recombination and N region addition. Statistical comparisons are only shown for DβYTL versus the other four mouse strains. Comparisons where the p value is not shown had a p > 0.05.

Loss of Terminal D Nucleotides Is D Sequence Specific

There was a greater loss of nucleotides at the 3' end of the V and a lesser loss of nucleotides at the 5' end of the J in DN2 thymocytes than in Fraction B proB cells, irrespective of the sequence of the D (Figure 2). Conversely, the loss of 5' D sequence was greater and the loss of 3' D sequence lesser in progenitor cells that contained a DSP sequence than in progenitor cells that contained a Dβ1 sequence irrespective of the host cell type. Thus, although the sequence of the D did not control V or J nucleotide loss; the sequence of the D did control terminal loss of D nucleotides, irrespective of cell type. This is further evidence that the sequence of the D controls how that sequence is modified during VDJ rearrangement, with each D creating its own D-specific repertoire (8).

P junction gain at the termini of the D's also appeared D gene segment-specific. There was a greater gain of P junctions at the 5' end of the Dβ sequence than the DSP sequence, and a greater gain of P junction sequence at the 3' end of DSP sequences.
TABLE 1 | Germline V, D, and J CDR3 contributing sequences pertinent to this study.

| Strain | IMGT | Common | Terminus |
|--------|------|--------|---------|
| BALB/c | IGHV5-01 | VHV18X | GAAAGACA |
|        | IGHV5-02 | VHD6.06 | ......G. |
|        | IGHV5-06 | VH3.33.39 | ...... |
|        | IGHV5-14 | 7183.14 | ...... |
|        | IGHV5-05 | VH50.1 | ...... |
|        | IGHV5-09 | 7183.9 | ...... |
|        | IGHV5-04 | VHS7.1 | ...... |
|        | IGHV5-16 | 98-3G | ......G. |
|        | IGHV5-03 | VH283 | G......T |
|        | IGHV5-10 | 7183.1O | ...... |
|        | IGHV5-08 | VH10.19 | A......G. |
|        | IGHV5-15 | 68-5N | ...... |
|        | IGHV5-17 | 69-1 | A......G. |
|        | IGHV5-13 | 7183.13 | ...... |
|        | IGHV5-12 | 57-1M | ......G. |
|        | IGHV5-11 | VH7183.11 | ......G |
|       |        |        |         |
| CS7BL/6 | IGHV5-17 | ...... | ...... |
|        | IGHV5-16 | ......G. | ......G. |
|        | IGHV5-15 | ...... | ...... |
|        | IGHV5-14 | ...... | ...... |
|        | IGHV5-12 | ...... | ...... |
|        | IGHV5-09 | ...... | ...... |
|        | IGHV5-06 | ...... | ...... |
|        | IGHV5-04 | ......G. | ......G. |
|        | IGHV5-02 | ...... | ...... |
|        | TRBV13-1 | ...... | ...... |
|        |        |        |         |
| BALB/c | D2-02*01 | DSP2.03 | TCTACTATGGTTACGAC |
|        | D2-04*01 | DSP2.02 | ......A...... |
|        | D2-02*01 | DSP2.04 | ...... |
|        | D2-01*01 | DSP2.05 | ......A......T. |
|        | D2-10*01 | DSP2.07 | C......A......T. |
|        | D2-11*01 | DSP2.08 | C......A......T. |
|        | D2-03*01 | DSP2.09A | ......T. |
|        | D2-14*01 | DSP2.11 | C......A......G. |
|        | D2-03*01 | DSP2.09A | ......T. |
|        | D2-04*01 | DSP2.02 | ......A...... |
|        | D2-05*01 | DSP2.x | C......A......T. |
|        | D2-06*01 | DSP2.x | C......A......T. |
|        | D2-07*01 | DSP2.03 | ...... |
|        | D2-08*01 | DSP2.05 | ......A......T. |
|        | TRBV13-1 | ...... | ...... |
|       |        |        |         |
| CS7BL/6 | IGHJ1*01 | JH1 | CTACTGGTACCTCGATGTC |
|        | IGHJ2*01 | JH2 | AC......T.CTA. |
|        | IGHJ3*01 | JH3 | CCTCG......T.CTA. |
|        | IGHJ4*01 | JH4 | AT......ATGCTAG......G.CTA. |

TABLE 1 | Continued

| Strain | IMGT | Common | Terminus |
|--------|------|--------|---------|
|        | TRBJ1-1*01 | Jβ1 | CAAACACAGAA.TCT..... |
|        | TRBJ2-2*01 | Jβ2 | CAAACTGA.T.GA.T.CACC |
|        | TRBJ3-3*01 | Jβ3 | T.CTG.A.A.TACCTCTAT |
|        | TRBJ4-4*01 | Jβ4 | TT.CCA......GAAGAT.A |
|        | TRBJ5-1*02 | Jβ5 | TAA.AACG......GCTCCGGG.T |
|        | TRBJ6-1*01 | Jβ6 | TT.C......TAAT.CGG.C.CTCTA. |

Sequences are shown in comparison to a representative gene segment. The strain of origin is indicated.

than at the 3′ end of the Dβ sequence, irrespective of cell type, although the differences at the 5′ end did not achieve statistical significance. There was a greater gain of P junction nucleotides at the 3′ terminus of V gene segments in Fraction B cells than in thymocytes irrespective of the sequence of the D. The differences in P junctions in J’s were not statistically significant.

V/D overlap was greater in thymocytes and D/J overlap greater in Fraction B proB cells, although again these differences did not achieve statistical significance and the absolute contribution of nucleotides was small.

The presence of two or more microhomologous nucleotides between rearranging gene segments has been shown to influence the site of RAG mediated recombination in progenitor B cells (12, 13). Neither the 5′ terminus of the DSP gene segments nor the Dβ1 segment share dinucleotide microhomology with the 3′ terminus of the Ig or TCR V’s. The 3′ terminus of all DSP gene segments shares at least a two nucleotide microhomology (AC) with the 5′ terminus of the Jβ1 or Jβ2 gene segment recombination.

The extent of N region addition is cell type specific

The extent of N region addition between V and D as well as between D and J was greater in Fraction B proB cells than in DN2 thymocytes, irrespective of the sequence of the D (Figure 2).

Reading Frame Usage Is Random in DN2 Thymocytes Irrespective of D Sequence

Partly due to the microhomology between the 3′ end of the D and the 5′ end of the J, developing B cells demonstrate a bias against rearrangement into reading frame 2 (RF2). In the absence of extensive DβYTL-Jβ microhomology, RF 2 usage increased to a third of the rearrangements (Figure 3). Enrichment for RF1 was
FIGURE 2 | Comparisons of VDJ processing in the pre-selected repertoire. Shown in the left column is the average loss of terminal nucleotides in the V, D, and J gene segments. Shown in the middle column is the average amount of P junction addition. Shown in the right column is: row 1 and 2, the average amount of N nucleotide addition between V and D and between D and J, respectively; row 3 and 4, the extent of V/D and D/J overlap in sequence among sequences lacking N addition. In each case, the data are ordered, from left to right: wild type BALB/c DSP containing sequences, wild type C57BL/6 DSP containing sequences, Dβ1YTL containing sequences, Dβ1 containing sequences from the Dβ2ko mice and from wild type C57BL/6 mice. From left to right, the first three columns represent Dβ sequences and columns four and five are Dβ sequences. From left to right, the first two columns of each graph represent data from Fraction B proB cells, followed by the three columns of each graph representing data from DN2 cells from thymocytes. Error bars display the standard error of the mean. Statistical analysis was performed only for DβYTL in comparison with the four controls, and comparisons lacking a “p” value all have p > 0.05.
greater in BALB/c than in C57BL/6 with a compensatory loss of RF3. The mechanism underlying this difference is unclear.

**On Average, CDR-H3 Contains More Random N Nucleotides and Exhibits Greater Variance in Sequence Than CDR-B3**

The average number of total N nucleotides (5’ plus 3’) was greater in progenitor B cells than in thymocytes (Figure 3). Although there was no statistically significant difference in total N nucleotides between DßYTL CDR-B3 than in Dß1-Jß1 sequences from Dß2ko, there was less N addition in wild type Dß1-Jß1 sequences. The trend to have less N addition was observed both at the V→DJ and D→J joins. How the presence of an additional DßJß locus might influence total N addition is unclear.

Differences in N addition between cell types were also found in the variance and distribution of N nucleotides (Figure 4). The most variable range of lengths was observed in CDR-H3 of BALB/c transcripts and the least in CDR-B3 from wild type thymocyte DN2-derived TCRβ transcripts. Thus, the cell type clearly influenced the extent of N addition. There is also the suggestion that the absence of Dß2-Jß2 not only affected the absolute number of N nucleotides added, but also influenced the variance of N nucleotide addition.

The greater variance of N addition in fraction B proB cells vs. DN2 thymocytes contributed to a marked difference in the variance of the number of amino acids, or the lengths of the sequences, in CDR-H3 versus CDR-B3 (Figure 4). The variance in lengths was greater in progenitor B cells than in thymocytes. In DßYTL, the variance in lengths was similar to that of Dß2ko and wild type CDR-B3. Due in part to the difference in both the quantity and variability of N addition, the variance in the distribution of CDR-B3 lengths was significantly lower than the variance in the distribution of CDR-H3 lengths.

When viewed in toto, the greater length of the Vß and Jß portions of CDR-B3 coupled with the lower amount of N addition results in the average CDR-B3 containing more germline-encoded sequence than CDR-H3 (Figure 5). Intriguingly, however, despite differences in N addition and terminal nucleotide loss or gain, the average length of immunoglobulin CDR-H3s containing a DSP gene segment proved similar to that of TCR CDR-B3 containing Dß1 and Jß1 gene segments. This balance was affected when the sequence of the DJH was lengthened. DSP2.3 is five nucleotides longer than Dß1, and on average the length of CDR-B3 sequences containing DSP2.3 was 3.5 nucleotides longer than those containing Dß1 gene segment.
sequence. Thus the length of CDR-B3 can be heavily influenced by the length and terminal sequence of the D.

**DISCUSSION**

Adaptive immunity in jawed vertebrates is designed to produce immunoglobulin (Ig) and T cell receptor (TCR) repertoires of astronomic diversity in developing B cells and thymocytes (1, 2). These two antigen receptors have overlapping but distinct roles. While both Ig and TCR act as the antigen receptor for their respective host cells, Igs also have effector functions that require high affinity binding of high specificity. Conversely, while the interaction between Ig and antigen is bimolecular, T cell receptor recognition of antigen requires a trimolecular interaction with both peptide antigen and a member of the major histocompatibility complex (MHC). Secreted Ig clears pathogens through binding to target antigens, which induces a cascade of humoral and cellular reactions. On the other hand, T cells via their TCR can induce killing of target cells infected with the pathogens. Based on these major differences in functions, it is not surprising that the measures used to control their repertoires vary, even though genes encoding both receptors undergo the same process of VDJ recombination and N addition.

We have previously focused our efforts on testing whether the functional efficiency of the process of immunoglobulin diversification can be enhanced by means of natural selection of germline sequence (4). In addition to immunoglobulin, the diversity provided by VDJ recombination and N addition enables a broad array of TCR antigen specificity. The antigen receptors that are generated by both the immunoglobulin and TCR loci have the capacity to be autoreactive, protective, superfluous, or ineffective. Thus a completely random process would likely result in costly inefficiency. We have previously shown that the
sequence of D<sub>H</sub> can heavily influence immunoglobulin diversity, and that the features of this influence can be detected in broad outline by analysis of as few as ten to twenty sequences. Moreover, the changes induced by altering D<sub>H</sub> sequence negatively impact B cell development, antibody production, protection against infection, responses to allergens, and susceptibility to autoreactive antibody production (4, 14–16).

In this work, we sought to test whether the process of VDJ recombination and N region addition would be altered in T cells should the sequence of D<sub>β</sub> be changed. To test for potential germline constraints on both Ig and TCR repertoires, we replaced DB1 with a commonly used D<sub>β</sub> in a mouse lacking the D<sub>β</sub>-J<sub>β</sub> gene segment cluster. We evaluated the criteria of selection of TCR which included terminal nucleotide loss, terminal nucleotide P junction gain, V/D and D/J overlap, N addition, reading frame use, the relative contribution of N addition versus germline encoded content in CDR3, and total CDR3 length.

We compared the repertoires expressed in proB cells from the bone marrow to DN2 cells from the thymus. In developing B cells, D<sub>H</sub>→J<sub>H</sub> rearrangement precedes V<sub>H</sub>→D<sub>H</sub>J<sub>H</sub> rearrangement. Hardy Fraction B progenitor B cells contain these initial VDJ joins and by definition do not express appreciable levels of μH chain protein. Thus, the VDJ repertoire they express is considered to be preimmune because it is unselected by the immune properties of μH chain protein, including preBCR formation and antigen binding. Similarly, in the thymus D<sub>β</sub>→J<sub>β</sub> rearrangement precedes V<sub>β</sub>→D<sub>β</sub>J<sub>β</sub> rearrangement and, during thymocyte development, VDJCB transcripts are typically first found in DN2 cells. The homology between proB and DN2 cells is not exact since the definition of DN2 cells does not depend on the presence or absence of TCRβ protein. While it is possible that expression of nascent TCRβ protein could affect the fate of the cell, either directly or through its interaction with preTCRs, selection of the TCR β chain is primarily associated with the developmental checkpoint between DN3a and DN3bc (17). Thus, as with proB cells, DN2 thymocytes are considered to primarily express a preimmune TCRβ repertoire.

We found that the sequence of the D maintains control over the outcome of the rearrangement of the D regardless of whether recombination is occurring in proB cells or thymocytes. While the sequence of the D has minimal effects on V or J gene segment loss or gain of terminal sequence, it self-directs the extent of terminal D nucleotide loss or P junction gain.

The extent and variance in N addition appears affected by cell type and by the loci surrounding the rearranging gene segments (18, 19). This study was not designed to fully evaluate the independent contributions of these factors. However, it did disclose a potential role for the D<sub>β</sub>-J<sub>β</sub> locus in regulating the extent and distribution of N nucleotide addition. The activity of the N region addition machinery is regulated during ontogeny by controlling TdT expression and access to exposed terminal DNA sequence (20–22). We would surmise that one or both of these mechanisms are the means by which cell type and the presence or absence of a rearranging locus could influence N region length (20).

Structural studies have shown that amino acids at the tip of the TCR CDR-B3 loop are highly likely to interact directly with peptide antigens presented on the surface of the MHC molecule. The length distribution of CDR-B3 has been proposed to be restricted largely by MHC-specific selection, disfavoring TCRs with CDR3s that are longer than 13 amino acids (23). Our data would suggest that initial restrictions are controlled by natural selection. Potential mechanisms include preservation of D region sequence and regulation of N addition (24).

On average, the 3′ termini of TCR V<sub>β</sub> and the 5′ termini of TCR J<sub>β</sub> are longer than their immunoglobulin V<sub>H</sub> and J<sub>H</sub> counterparts (e.g., Table 1 and Supplementary Table S2). Natural selection of the germline sequences of TCR V<sub>β</sub>, D<sub>β</sub>, and J<sub>β</sub> coupled with control of CDR-B3 length variance has the effect of focusing the diversity provided by N addition and the sequence of the D to that portion of CDR-B3 that is most likely to interact with the peptide that is bound to the presenting MHC.

This manuscript has focused on the effect of D sequence on the preimmune repertoire at the nucleotide level. The contribution
of the amino acids encoded by the D, which differ in peptide signature between T cells and B cells, to repertoire selection, T cell development, and antigen responses is reported in a companion manuscript. Given our past experience with the immunoglobulin locus, we were not surprised that T cell biology is also heavily affected by violation of normal germline constraints on the amino acids encoded by the sequence of the D. We speculate that the threat of unrestrained inefficiency or potential hazard in the creation of antigen receptor repertoires by an entirely stochastic process of DNA rearrangement appears to have been constrained during evolution by controlling the sequences of the rearranging gene segments to optimize the products of recombination while engendering diversity.

**DATA AVAILABILITY STATEMENT**

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a manuscript that does not adhere to our open data policies.

**ETHICS STATEMENT**

The animal study was reviewed and approved by UAB IACUC.

**AUTHOR CONTRIBUTIONS**

MK took the lead in analyzing and interpreting the data, and writing the manuscript. ML took the lead role in sequencing the original Dβ2ko construct and reviewing and discussing the results of our studies. HS developed the concept of the project, directed the writing of the manuscript. All authors contributed to the planning of the experiments, interpreting the data, and editing the manuscript. HS developed the concept of the project, directed the planning and execution of the studies, reviewed the data, and directed the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02079/full#supplementary-material

**REFERENCES**

1. Rajewsky K. Clonal selection and learning in the antibody system. Nature. (1996) 381:751–8.
2. Jørgensen JL, Reay PA, Ehrich EW, Davis MM. Molecular components of T-cell recognition. Annu Rev Immunol. (1992) 10:835–73. doi: 10.1146/annurev.iy.10.040192.004155
3. Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. Sequences of Proteins of Immunological Interest. Bethesda, MY: U.S.Department of Health and Human Services (1991). p. 1–2387.
4. Khass M, Vale AM, Burrows PD, Schroeder HW Jr. The sequences encoded by immunoglobulin diversity (DH) gene segments play key roles in controlling B-cell development, antigen-binding site diversity, and antibody production. Immuno Rev. (2018) 284:106–19. doi: 10.1111/imr.12669
5. Lefranc MP. IMGT, the international ImMunoGeneTics database: a high-quality information system for comparative immunogenetics and immunology. Dev Comp Immunol. (2002) 26:697–705. doi: 10.1016/s0145-3055(02)00026-5
6. Bassing CH, Alt FW, Hughes MM, D’Auteuil M, Wehrly TD, Woodman BB, et al. Recombination signal sequences restrict chomosomal V(D)J recombination beyond the 12/23 rule. Nature. (2000) 405:583–6.
7. Sleckman BP, Bassing CH, Hughes MM, Okada A, D’Auteuil M, Wehrly TD, et al. Mechanisms that directly ordered assembly of T cell receptor beta locus V, D, and J gene segments. Proc Natl Acad Sci USA. (2000) 97:7975–80. doi: 10.1073/pnas.130190397
8. Zemlin M, Schelonka RL, Ippolito GC, Zemlin C, Zhuang Y, Garland GL, et al. Regulation of repertoire development through genetic control of D H reading frame preference. J Immunol. (2008) 181:8416–24.
9. Khass M, Buckley K, Kapoor P, Schelonka RL, Watkins LS, Zhuang Y, 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) 43:629–40. doi: 10.1002/eji.201242936
10. Wong HS, Chang CM, Kao CC, Hsu YW, Liu X, Chang WC, et al. V-J combinations of T-cell receptor predict responses to erythropoietin in end-stage renal disease patients. J Biomed Sci. (2017) 24:43. doi: 10.1186/s12929-017-0349-5
11. 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. doi: 10.1615/critrevimmunol.v30.i4.20
12. Feeney AJ. Predominance of VH-D-JH junctions occurring at sites of short sequence homology results in limited junctional diversity in neonatal antibodies. J Immunol. (1992) 149:222–9.
13. Gu H, Kitamura D, Rajewsky K. DH reading frame bias: evolutionary selection, antigen selection or both? Evolutionary selection. Immunol Today. (1991) 12:420–1.
14. Silva-Sanchez A, Liu CR, Vale AM, Khass M, Kapoor P, Elgavish 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. doi: 10.1371/journal.pone.0118171

15. Trad A, Tanasa RI, Lange H, Zemlin M, Schroeder HW Jr., Lemke H. Clonal progression during the T cell-dependent b cell antibody response depends on the immunoglobulin DH gene segment repertoire. Front Immunol. (2014) 5:385. doi: 10.3389/fimmu.2014.00385

16. 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 D(H) gene conservation. J Exp Med. (2013) 210:875–90. doi: 10.1084/jem.20121861

17. Teague TK, Tan C, Marino JH, Davis BK, Taylor AA, Huey RW, et al. CD28 expression redefines thymocyte development during the pre-T to DP transition. Int Immunol. (2010) 22:387–97. doi: 10.1093/intimm/dxq020

18. Vale AM, Nobrega A, Schroeder HW Jr. The role of evolutionarily conserved germ-line DH sequence in B-1 cell development and natural antibody production. Ann N Y Acad Sci. (2015) 1362:48–56. doi: 10.1111/nyas.12808

19. Vale AM, Tanner JM, Schelonka RL, Zhuang Y, Zemlin M, Garland 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.

20. Bogue M, Gilfillan S, Benoist C, Mathis D. Regulation of N-region diversity in antigen receptors through thymocyte differentiation and thymus ontogeny. Proc Natl Acad Sci USA. (1992) 89:11011–5.

21. Motea EA, Berdys AJ. Terminal deoxynucleotidyl transferase: the story of a misguided DNA polymerase. Biochim Biophys Acta. (2010) 1804:1151–66. doi: 10.1016/j.bbapap.2009.06.030

22. Schelonka RL, Ivanov II, Vale AM, Szymanska E, Zemlin M, Garland GL, et al. The CDR-H3 repertoire from TdT-deficient adult bone marrow is a close, but not exact, homologue of the CDR-H3 repertoire from perinatal liver. J Immunol. (2010) 185:6075–84.

23. Lu J, Van Laathem F, Bhattacharya A, Craveiro M, Saba I, Chu J, et al. Molecular constraints on CDR3 for thymic selection of MHC-restricted TCRs from a random pre-selection repertoire. Nat Commun. (2019) 10:1019. doi: 10.1038/s41467-019-08906-7

24. Zemlin M, Schelonka RL, Bauer K, Schroeder HW Jr. Regulation and chance in the ontogeny of B and T cell antigen receptor repertoires. Immunol Res. (2002) 26:265–78.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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