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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

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Abstract

When compared to adult bone marrow, the composition of the perinatal liver CDR-H3 repertoire is marked by a paucity of N nucleotides and by enrichment for use of J_H proximal DQ52; and D_H-proximal V_H and J_H gene segments. To test the extent to which these differences reflect limited perinatal TdT activity versus differences in the fetal/adult environment, we used the Hardy scheme to sort Fraction B-F B lineage cells from TdT deficient BALB/c adult bone marrow. V_H7183-containing VDJCμ transcripts from these cells were amplified, cloned, sequenced, and compared to transcripts from wild-type perinatal liver and adult bone marrow. The pattern of $V_H DJ_H$ usage in TdT deficient bone marrow largely matched that of TdT sufficient adult cells. What minor differences were detected in the pro-B cell stage tended to diminish with B cell maturation, suggesting strong environmental or antigen-driven pressure to achieve a specific range of $V_H DJ_H$ usage regardless of the extent of N addition. However, although the patterns of $V_H DJ_H$ usage in the TdT deficient B lineage cells paralleled that of wild-type adult cells, the length distribution, global amino acid composition, and charge distribution of the CDR-H3 repertoire proved to be a close, although not exact, homologue of the CDR-H3 repertoire first expressed by late pre B cells in the TdT insufficient perinatal liver. Thus, while differing in V_H content, TdT deficient mice appear to represent a good, although not perfect, model for testing the role of perinatal CDR-H3 limitations on late B cell development and antibody responses.

Keywords

Terminal deoxynucleotidyl Transferase; Repertoire Development; Antibodies/Immunoglobulin; Mice

Introduction

For immunoglobulin (Ig), the B cell antigen receptor (BCR), diversity is the property of the variable (V) domains of the heavy (H) and light (L) chains, which are manufactured and

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then sequentially tested and selected during B cell development (1–5). Diversity is asymmetrically distributed within each V domain (6, 7). In the primary sequence, three intervals of hypervariability, termed complementarity determining regions (CDRs), are separated from each other by four relatively conserved framework regions (FRs). The heavy chain CDR3 (CDR-H3), which is encoded by the 3' end of the V_H, the 5' end of the J_H, and the entire D_H, is the direct product of V(D)J joining and can be supplemented by non-germline encoded nucleotides (N nucleotides) introduced randomly at the sites of joining by terminal deoxynucleotidyl transferase (TdT). Its location at the center of the antigen-binding site means that CDR-H3 often plays a critical role in antibody specificity (6–8). The inclusion of N nucleotides in CDR-H3 allows B cells to escape potential germline constraints on the sequence of their antigen receptor repertoires (1, 2, 9–11).

In previous studies we have shown that the essential outlines of the adult TdT sufficient CDR-H3 repertoire, including patterns of gene segment utilization, amino acid composition, charge, predicted base and loop structure and length are established early in B cell development, prior to the expression of H chain protein (12). B lineage cells sequentially express a pre-B cell receptor, rearrange a light chain gene, express surface IgM and, as they are released into the periphery, begin to co-express surface IgD. During this developmental process, the CDR-H3 repertoire is sequentially focused to fit into what appears to be a preferred range in terms of the distributions of length, amino acid composition, and average hydrophobicity. This developmental process is heavily influenced by the amino acid composition of the reading frame of the included D_H (12).

The composition of the CDR-H3 repertoire varies during ontogeny. For example, the perinatal liver CDR-H3 repertoire, which has its own distinct pattern of VDJ gene segment usage and lacks N nucleotides, differs significantly from the CDR-H3 repertoire expressed in adult bone marrow (13–15). It has been proposed that the differences in these repertoires contribute heavily to the differences between the neonatal and the adult response to antigens, i.e. the antigenic hierarchy (16) that underlies both vaccination schedules and the altered susceptibility of young children to infection.

By comparing the CDR-H3 repertoire of V_H7183-containing transcripts from TdT deficient mice to that of physiologically TdT insufficient perinatal liver and to that of TdT sufficient adult bone marrow, we sought to test the extent to which differences between the perinatal repertoire and the adult repertoire reflect the effects of N nucleotide inclusion. Although the patterns of VDJ usage in early pre-B cell progenitors from the bone marrow of TdT deficient mice differed from those observed in the physiologically TdT insufficient perinatal liver, we found that the CDR-H3 repertoire expressed by the mature bone marrow TdT deficient B cells is similar, but not exact homologue of the CDR-H3 repertoire expressed by mature B cells in the TdT insufficient perinatal liver.

Materials and methods

Mice

TdT deficient animals generated by Gillfilan and colleagues (Gilfillan et al., 1993) on a mixed 129/C57BL/6 background were the kind gift of Dr. John Kearney of the University of Alabama at Birmingham. The animals were backcrossed for 10 generations onto BALB/cJ (Jackson lab, Stock No 000651) and bred in the UAB vivarium. The mice were maintained in a specific pathogen free barrier facility. All experiments with live mice were approved by and performed in compliance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee regulations.

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Flow cytometric analysis and fluorescence activated cell sorting

Flow cytometric analysis and fluorescence activated cell sorting from mononuclear cells from the liver was performed as previously described for mononuclear cells from the bone marrow of 8 week old BALB/c mice (12, 17, 18). A MoFlo instrument (Cytomation, Ft. Collins, CO) was used for cell sorting. Developing B lineage cells in the liver were identified on the basis of the surface expression of CD19, CD43, IgM, BP-1, and/or IgD.

RNA preparation, RT-PCR and sequencing

Total RNA isolation, V_H7183 specific VDJCμ RT-PCR amplification, cloning, sequencing, and sequence analysis was performed as previously described (12, 17, 18). The sequences reported in this paper have been placed in the GenBank database (www.ncbi.nlm.nih.gov/Genbank/) under the accession numbers **HM154548-HM154936**. A listing of the 470 unique, in-frame V_H7183DJCμ sequences used for analysis in this work is provided in Supplemental Table (S1).

Structural Analysis

We used the "H3" rules, as published by Shirai (19, 20), to predict structural features of the CDR-H3 base and loop, as previously described (21). Briefly, the structure of the CDR-H3 base (termed kinked, extra-kinked, or extended) can be predicted in sequences that contain a minimum of five amino acid residues, including IMGT positions 105–118 (Kabat positions 93–103). In approximately 25–30% of the sequences with a kinked or extra-kinked CDR-H3 base, the H3-rules can predict whether an intact hydrogen bond ladder may be formed within the loop of the CDR-H3 region or whether the hydrogen bond ladder is likely to be broken. For example, proline residues tend to inhibit formation of a stable hydrogen bond ladder, the presence of a V_H-encoded arginine at the amino terminus of CDR-H3 in conjunction with a J_H-encoded aspartic acid at the C terminus permits formation of a salt bridge that stabilizes the base, and glycine residues permit greater flexibility (19).

Statistical analysis

Differences between populations were assessed, where appropriate, by the Student's t test, two tailed; Fisher's exact test, two tailed; χ^2; or Levene's test for the homogeneity of variance. Analysis was performed with JMP version 7.0 (SAS Institute). Means are accompanied by the standard error of the mean.

Results

Isolation of B lineage cells and RT-PCR cloning of Ig transcripts from TdT deficient adult bone marrow

From the bone marrow of 8 week old TdT deficient congenic BALB/c mice, we isolated B lineage cells belonging to Hardy fractions B through F (22). We used 3' Cμ and 5' V_H7183-specific primers to RT-PCR amplify and then clone V_H7183DJCμ transcripts. Subsequently, we obtained 469 unique, in-frame, open reading frame V_H7183DJCμ sequences. Of these, 68 were cloned from fraction B (pro-B cells), 162 from Fraction C (early pre-B cells); 84 from Fraction D (late pre-B), 83 from Fraction E (immature B cells), and 73 from Fraction F (mature B cells) [Supplemental Table (S1)]. We compared these to 53, 55, 92, 146, and 135 unique, in-frame, open reading sequences from Fractions B through F from four samples of BALB/c perinatal liver (15); and to 194, 373, 279, 255, and 254 previously published Fractions B through F unique, in-frame, open reading frame sequences from the bone marrows of eleven different 8 week old adult mice (12, 17, 18, 23). Of these eleven adult mice, four were TdT sufficient littermates of the TdT deficient mice (12, 17, 18, 23). To more easily visualize the divergence between the repertoires expressed in TdT deficient

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adult bone marrow and those expressed in physiologically TdT insufficient perinatal liver and TdT sufficient adult bone marrow, we also graphed the differences in the prevalence (expressed as a percentage of the total) of each individual feature.

Usage of V_H81X (V_H7183.1) and V_H7183.10 in early B cell progenitors was affected by the presence of TdT

V_H81X (V_H7183.1) has been shown to be a prominent contributor to the initial H chain repertoire (15, 24–27). In previous studies, we confirmed that V_H81X is preferentially used in TdT sufficient early B cell progenitors and demonstrated that the same held true in mice with altered, but still TdT sufficient, CDR-H3 repertoires (12, 17, 18, 23). In all of these TdT sufficient mice, use of V_H81x then progressively declines with development (32% of B through 2% of F, $p < 0.0001$ in wild type mice) with a different gene segment in this family, V_H7183.10, serving as the most highly utilized V_H from Fraction C onwards.

In the absence of TdT in adult bone marrow, both V_H81X (V_H7183.1) and V_H7183.10 utilization in Fraction B was reduced (Figure 1). Intriguingly the use of the next upstream V_H, particularly V_H7183.2, increased to the point that, when added together, the prevalence of these two most J_H-proximal V_H gene segments was identical to that of TdT sufficient adult mice. The prevalence of V_H81X also rose in Fractions C through E and was greater than wild-type adult BM in each of these stages (Fraction C p 0.001, Fraction D p 0.01, and Fraction E p 0.05; respectively). Conversely, use of V_H7183.2 declined to match the wild-type controls. Although statistical significance was not achieved, we observed a statistical trend for increased use of V_H81X in the TdT deficient mice when compared to TdT sufficient controls. The prevalence of V_H7183.10, which is the most commonly used V_H7183 gene segment in adult bone marrow (12), steadily increased from Fraction C through Fraction F. At Fraction F, the prevalence of V_H7183.10 in the TdT deficient mice was statistically indistinguishable from the TDT sufficient mice.

Use of V_H81X in adult TdT deficient fraction B was also significantly lower than that of physiologically TdT insufficient perintatal liver fraction B ($p<0.0001$), but significantly increased in fraction C ($p<0.0005$; Figure 1). Similarly, the relative use of V_H7183.10 was lower in TdT deficient fractions C and D (p 0.0001) than in the corresponding samples from fetal liver. Among Fractions E and F the use of both V_H81X and V_H7183.10 in the adult bone marrow TdT deficient cells proved virtually identical to that observed in the perinatal liver (Figure1).

The use of V_H7183 gene segments distal to V_H7183.10 matched the use in TdT sufficient adult bone marrow

The linear position relative to the D_H/J_H locus of the other V_H7183 gene segments also appears to correlate with differences in the frequency of their use in the fetal versus adult (15). To examine this effect in the context of adult TdT deficient bone marrow, we grouped the V_H gene segments upstream of V_H7183.10 into one block (block 18-8); and the V_H gene segments downstream of V_H7183.10, and upstream of V_H81X and V_H7183.2, into a second block (block 6-3) of gene segments. We previously showed that use of D_H/J_H distal block 18-8 in the perinatal liver consistently lagged behind that of D_H/J_H proximal block 6-3 (15). The pattern of the D_H distal V_H gene block (18–8) in the TdT deficient mice proved identical to adult wild-type mice, and thus divergent from the perinatal TdT insufficient mice (Figure 2). The pattern of the D_H proximal V_H gene block 6-3 proved more torturous, sharing identity with adult bone marrow in Fraction B and diverging from both adult bone marrow and perinatal liver in fraction D. Interestingly, however, the three groups of mice converged to the same pattern in early pre-B cell fraction C, and then in immature B cell fraction E and mature B cell fraction F. (Figure 2).

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Increased identification of D_H DSP family members in CDR-H3s from TdT deficient B cells

In previous deconstructions of V_H7183DJCμ sequences from adult TdT sufficient wild-type bone marrow, we were unable to identify the donor D_H in up to one-sixth of the CDR-H3 sequences (12, 17, 18, 23). By contrast we were able to identify the donor D_H in 99% of the transcripts we obtained from TdT deficient fraction F (Figure 3). This was associated with a striking increase in the donor assignment of the various members of the DSP gene segment family. The contribution of the other D_H families, DFL, DQ, and DST, was statistically indistinguishable from adult TdT sufficient wild-type bone marrow.

Although there was a statistical trend for decreased use of J_H1 and J_H3 among the CDR-H3 sequences from the TdT deficient mice, with a potential compensatory increase in the use of J_H4, none of the differences achieved statistical significance (Figure 3).

Increased use of DQ52, increased use of J_H2 and decreased use of J_H3 and J_H4 are hallmarks of the perinatal liver V_H7183DJCμ repertoire (15). D_H and J_H usage among the sequences obtained from TdT deficient adult bone marrow matched the pattern observed in TdT sufficient adult bone marrow (Figure 3).

Increased use of reading frame 1 in CDR-H3s from TdT deficient B cells

We observed a statistical trend ($p=0.09$) for increased use of reading frame 1 among those sequences from TdT deficient adult bone marrow that used members of the DFL or DSP families, with 78% using RF1 in fraction B versus 89% in fraction F (Figure 3). The use of RF1 in the TdT deficient bone marrow repertoire was greater than adult bone marrow and less than perinatal liver in Fractions B, C and D. The converse was true for RF3. While this pattern of an increased use of RF1 and decreased use of RF3 was maintained between TdT sufficient and TdT deficient adult bone marrow continued in Fraction F, the differences in reading frame usage between TdT deficient adult bone marrow and TdT insufficient neonatal liver essentially resolved, creating a very similar pattern. Unlike RF1 and RF3, the use of RF2 in the TdT deficient adult bone marrow matched that of TdT insufficient perinatal liver at all five stages of bone marrow B cell development; with both using RF2 much less frequently than TdT sufficient adult bone marrow.

The frequency of CDR-H3s containing D-J and V-D overlaps increased with development in TdT deficient adult bone marrow

One mechanism known to influence D_H reading frame usage is a tendency for D→J rearrangement to occur at sites of sequence microhomology (13, 28). Rearrangement at these regions of microhomology has the effect of restricting the diversity of the repertoire by enriching for shared terminal amino acid sequence. The extent of this microhomology varies, with J_H1 and J_H2 gene segments sharing up to six nucleotides of homology with the 3' termini of twelve of the thirteen D_H gene segments and J_H4 sharing five nucleotides of homology, all in reading frame 1. J_H3, on the other hand, exhibits only one nucleotide of terminal homology in more than one D_H reading frame.

To test whether the increase in the use of RF1 reflected an increased incidence of rearrangement at the site of D-J microhomology in the absence of N nucleotides, we evaluated the frequency of rearrangements involving D-J microhomology (Figure 4). Among sequences obtained from TdT sufficient adult bone marrow, rearrangements occurring at sites of D-J microhomology represented only 2–5% of the transcripts. In contrast, among the sequences from TdT deficient adult bone marrow, approximately forty percent from fraction B had evidence of D-J homology rearrangements. The prevalence of these types of rearrangements exhibited a steady increase with development, representing approximately eighty percent of fraction F sequences. Among those sequences using

members of the DSP or DFL families that lacked evidence of rearrangement at sites of D-J microhomology, the prevalence of RF1 was 70% of the total regardless of developmental stage. Among those sequences containing evidence of rearrangement at sites of D-J microhomology, use of RF1 reached up to 100% in several fractions (data not shown). The non-RF1 exceptions in this latter category tended to use J_H3 and thus contained only one shared nucleotide.

The frequency of V→D overlap was also greater among the sequences from TdT deficient adult bone marrow. The prevalence of such sequences matched that observed in physiologically TdT insufficient perinatal liver for fractions B through E (Figure 4). However, in Fraction F, the prevalence of sequences sharing D-J or V-D overlaps proved lower in TdT insufficient perinatal liver than in TdT deficient adult bone marrow. The divergence in the prevalence of the D-J overlap bore no relation to the use of individual J_H gene segments.

The distribution of CDR-H3 lengths was focused with development

In TdT sufficient adult bone marrow the average CDR-H3 length increases with B cell development. This increase was also observed in TdT deficient bone marrow (10.0 ± 0.3 amino acids, fraction B, versus 10.4 ± 0.2, fraction F; respectively) (Figure 5A), although this slight increase did not achieve statistical significance (p=0.37). However, the two codons difference in the average CDR-H3 length in TdT deficient fraction F CDR-H3s versus TdT sufficient adult bone marrow fraction F (12.5 ± 0.2) was highly significant (p<0.0001).

While the average length of adult bone marrow CDR-H3 is significantly longer than perinatal liver at all stages in WT mice (15), the CDR-H3s from TdT deficient adult bone marrow Fraction B were almost one codon longer than the CDR-H3s from TdT insufficient perinatal liver Fraction B (p=0.05). The average length of the sequences from the TdT deficient mice approached that of TdT insufficient perinatal liver in Fraction C, and then converged in Fraction D. Equivalence was then maintained from Fraction E through Fraction F (Figure 5A). A major reason for the lower average length of CDR-H3 in both TdT deficient adult bone marrow and TdT insufficient perinatal liver when compared to TdT sufficient adult bone marrow is the complete absence of CDR-H3s containing more than 16 codons [Figure 6; (15)].

In TdT sufficient bone marrow the distribution of CDR-H3 lengths also becomes more focused with development (12). This primarily reflects a progressive decrease in the representation of shorter CDR-H3s. This same pattern was also observed in TdT deficient bone marrow (Figure 6), where a progressive decrease in the representation of sequences containing CDR-H3s of containing fewer than eight codons is readily apparent.

When the lengths of CDR-H3s from TdT deficient bone marrow and TdT insufficient perinatal liver were compared to each other, it became clear that the divergence in average length largely reflected the decreased number of very short CDR-H3s in the samples from adult bone marrow. However, as the developing B cells passed through their sequential checkpoints this divergence declined to the point that the CDR-H3 length distribution in Fraction F was nearly identical between the two sets of samples. (The single exception was an over-representation of CDR-H3s that use 9 codons in the TdT deficient adult cells.) (Figure 6**, right panel**).

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The amino acid composition of CDR-H3 was greatly restricted in the absence of TdT, and matches that of perinatal liver

To assess the effect of N addition on CDR-H3 amino acid content, we compared the utilization of individual amino acids in the CDR-H3 loop, which comprises amino acids 95 to 100 by the Kabat nomenclature (6). In Figure 7, the amino acids are arranged by relative hydrophobicity, as assessed by the Kyte-Doolittle scale (29) as normalized by Eisenberg (30).

When compared to the TdT sufficient adult bone marrow repertoire, the TdT deficient adult bone marrow repertoire was significantly enriched for use of tyrosine, histidine, and alanine. Use of aspartic acid and asparagine was also variably increased. Conversely, the TdT deficient adult bone marrow repertoire was depleted of arginine, threonine, and leucine; and virtually devoid of lysine, glutamine, glutamic acid, proline, methionine, cysteine, phenylalanine, valine, and isoleucine. There was relatively little change in this pattern with development.

The amino acid composition of the CDR-H3 repertoire in TdT deficient adult bone marrow proved strikingly similar to the TdT insufficient perinatal liver repertoire. The few differences that achieved statistical significance were primarily detected in Fraction B. Indeed, for B cells in Fraction F there were no statistically significant differences in global amino acid usage between the two repertoires.

The perinatal repertoire was enriched for use of DQ52 (15), which encodes tryptophan and glycine in RF1, threonine, leucine and glycine in RF2, and asparagine, aspartic acid and glycine in RF3. Close inspection revealed a statistical trend for an increase in the use of threonine, aspartic acid, asparagine, and tryptophan in the perinatal versus the adult N-less repertoires. However, the numbers of sequences were insufficient to achieve statistical significance. What differences were present appeared to be minimized in Fraction F (Figure 7).

Average CDR-H3 hydrophobicity declined with development

The calculation of average CDR-H3 hydrophobicity provides a measure of the relative assortment of amino acids by a particular physical property, water solubility. In TdT sufficient adult bone marrow, the average hydrophobicity of CDR-H3 becomes more, then less hydrophobic in the transition from Fraction B to C to D. It then stabilizes in a slightly hydrophobic range (−0.18 ± 0.2, Figure 5B) for Fractions E and F. The average hydrophobicity of CDR-H3s produced by TdT deficient bone marrow B lineage cells began at the same point in Fraction B (−0.15 ± 0.05), but then progressively declined from B to C to D, becoming increasingly charged. It then stabilized through Fraction F, achieving an average hydrophobicity of −0.24 ± 0.03. The difference between the TdT WT and KO adult repertoires just missed achieving statistical significance ($p=0.06$).

The average hydrophobicity of the CDR-H3 repertoire produced by TdT insufficient perinatal liver B lineage cells starts more heavily charged than either TdT sufficient or TdT deficient adult bone marrow. There is again a set of transitions from B to C to D, but in the opposite direction from TdT sufficient adult bone marrow. The charge differential in Fraction C achieved statistical significance when compared to both TdT sufficient and TdT deficient adult bone marrow ($p<0.001$). And then, in a pattern that matched that observed for the progression in the average length of CDR-H3 (Figure 5A), there was convergence among cells that have progressed to Fraction D. At the end of this selection process, the average hydrophobicity for both TdT deficient adult bone marrow and TdT insufficient neonatal liver Fraction F cells proved the same (Figure 5B).

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In TdT sufficient adult bone marrow, the distribution of average hydrophobicity in the CDR-H3 repertoire achieved a tighter focus as the cells progress through successive developmental checkpoints (12). This focusing was associated with a progressive loss of highly charged and highly hydrophobic CDR-H3s. This same general process was observed in TdT deficient adult bone marrow, with one major exception. At all stages of development, there was a relatively paucity of highly hydrophobic CDR-H3s (Figure 8, left panel). Still, the paucity of both highly hydrophobic and highly charged CDR-H3s was most apparent in Fraction F, with evidence by inspection of the same trimming and focusing pattern observed in TdT sufficient bone marrow (12).

As with the differences in average length, distribution of lengths, and average charge, the greatest divergence between the distribution of charge in CDR-H3s from TdT deficient bone marrow and those from TdT insufficient perinatal liver occurred in fractions B and C. The distribution pattern nearly normalized in fraction D, and then showed mild patterns of divergence in Fractions E and F, none of which achieved statistical significance (Figure 8).

CDR-H3s from perinatal liver and TdT deficient bone marrow demonstrated a similar range of base and loop structures

The base of the CDR-H3 loop most typically contains alanine and arginine contributed by the V_H at Kabat positions 93 and 94; and phenylalanine, aspartic acid, and tyrosine contributed by the J_H at Kabat positions 100K-102. To assess the effect of N addition on CDR-H3, we calculated the properties of the CDR-H3 base and CDR-H3 loops according to Shirai's H3 rules (19) (Figure 9). In the CDR-H3s from both TdT deficient adult bone marrow and TdT insufficient perinatal liver, all of the sequences with a predictable loop structure contained intact hydrogen bond ladders, whereas in the sequences from wild type adult bone marrow, more than half of the CDR-H3 loops were deformed (Figure 9, right panel).

Although N nucleotides rarely contribute to the CDR-H3 base, extended CDR-H3-bases were significantly more frequent among bone marrow Fraction B sequences from TdT KO mice than from WT mice ($p = 0.007$) (Figure 9, left panel). No significant differences were observed in this regard among Fraction C through F sequences.

Discussion

The two most prominent differences between the perinatal liver and the adult bone marrow H chain repertoire in BALB/c mice are a divergence in V_H, D_H and J_H gene segment usage, and the paucity or abundance of N nucleotides (13–15). We sought to test what role, if any, the paucity of N addition during the perinatal period might play in the control of V_H, D_H, or J_H gene segment usage, CDR-H3 amino acid content and CDR-H3 structure. Our studies focused exclusively on gene segment utilization within the 7183 family because this family is preferentially utilized in perinatal tissues. Although these studies do not address a potential role for N addition in overall V_H family usage, they allowed us to directly compare the two repertoires in the context of the same V_H family, thus avoiding the potentially confounding issue of differences introduced by alterations in V_H family usage.

The absence of TdT in the adult reduced the initial use of V_H81X, the most commonly used V_H7183 gene segment in fetal liver progenitor B cells, and VH7183.10, the most commonly used V_H7183 gene segment in the mature B cell population. The mechanisms for these differences are unclear, but one potential possibility is that TdT influences the interaction between the RAG rearrangement complex and the rearranging gene segments, perhaps due to steric hindrance. However, as the developing B cells passed through sequential checkpoints of development and became more and more dependent on the antigen-binding

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properties of the V domain, we found that the usage of these gene segments in the TdT-deficient state converged and then matched their usage in the normal TdT-sufficient state.

For the rest of the V_H, D_H, and J_H gene segments, there were few, if any, statistically significant differences between TdT deficient and TdT sufficient adult bone marrow other than increased recognition of the use of DSP gene segment sequences. Indeed, it is possible that most 'unassignable' CDR-H3s in the TdT sufficient adult repertoire are derived from DSP-containing DJ rearrangements. What differences were present at early stages also tended to diminish with maturation of the B cells.

Between TdT deficient adult bone marrow and physiologically TdT insufficient perinatal liver, the divergence between V_H, D_H, and J_H gene segment sequence and reading frame preference proved greatest in Fraction B and the least in Fraction F. However, the signature differences in D_H and J_H gene usage that mark the perinatal repertoire, i.e. preferential use of DQ52 and J_H2, were not recapitulated in the absence of TdT and N nucleotides. Thus the activation and rearrangement of D_H and J_H do not appear to be as sensitive as V_H81X and $V_H7183.10$ to the presence or absence of TdT. And, these differences appeared to be less amenable to the antigen receptor-influenced selective pressures that act to converge V_H usage among the mature B cell fraction. This suggests that increased usage of DQ52 and J_H2 reflects either the effects of the fetal environment or differences in gene activation that are unique to the fetal B lineage cell. Determination of the contribution of these two possibilities likely will require transplantation studies.

Shirai's H3 rules (19) exclusively predicted intact hydrogen bond ladders for the CDR-H3 loops of adult TdT deficient and perinatal liver antibodies, whereas the adult TdT sufficient repertoire contained more than 50% deformed hairpins. Thus compared to adult WT, the antigen binding grooves of TdT deficient and perinatal TdT insufficient mice are deeper due to shorter CDR-H3 loops and much less diverse due to the predominance of intact hydrogen bond ladders. It can be assumed that these structural properties may contribute to the functional features of the fetal antibody repertoire, which is characterized by low affinity, poly-reactivity and mild auto-reactivity. The minor differences in CDR-H3 structures between sequences from TdT deficient and adult WT mice most likely reflect differences in J_H gene usage and were only present in Fraction B.

Similarly greatest divergence in amino acid composition, charge, and length between TdT deficient adult bone marrow B lineage cells and TdT sufficient adult bone marrow or TdT insufficient perinatal liver also occurred in Fractions B and C, with B greater than C. These fractions are the most likely to reflect differences in the stromal environment or in endogenous factors related to the derivation of the cells themselves. The perinatal liver, for example, clearly presents a different range of adjacent external cells and soluble molecules, and the progenitor cells that give rise to the B-1 lineage that predominates in the fetus are supported by TSLP (thymic stromal lymphopoietin) (31) whereas the progenitor cells that give rise to the B-2 lineage that predominates in the adult depend strictly on IL-7 (32). However, as the cells transition from Fraction C to Fraction D, a developmental checkpoint that depends on the successful association between the nascent H chain and surrogate light chain, the composition of the CDR-H3 repertoire in the TdT deficient bone marrow became very similar to the composition of the CDR-H3 repertoire in the TdT insufficient perinatal liver. Using Occam's razor, this would suggest that the surrogate light chain is acting to select a specific range of CDR-H3 lengths and charge irrespective of external environment or cell derivation. In particular, the surrogate light chain appears to discriminate against very short or highly hydrophobic CDR-H3s.

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The two most apparent differences between the H chain repertoire expressed in TdT insufficient perinatal liver and the repertoire expressed in TdT deficient adult bone marrow reflect differences in VDJ usage. First, adult cells can access a full range of V_H families, and, second, fetal B cell progenitors enrich for the use of DQ52 and for D_H proximal J_H2. Studies of the anatomy of hot spots in protein interfaces (34) have shown that arginine, tyrosine, and tryptophan contribute disproportionately to binding energies. In this light, our data would suggest that with regards to CDR-H3 the use of tyrosine and arginine are similar and the primary difference between TdT insufficient perinatal liver and TdT deficient adult bone marrow reflects the loss of DQ52-encoded tryptophan in CDR-H3. This latter population appears to be small, but could still be significant in certain cases.

In the final analysis, while we found small, but subtle, differences in the composition of the CDR-H3 repertoire between TdT deficient adult bone marrow and TdT insufficient perinatal liver in Fraction F, the overall view is one of extensive similarity on a global level between the CDR-H3s of TdT deficient adult bone marrow and TdT insufficient perinatal liver. Since the fetus is relatively protected from external environmental antigens, these observations would support a role for self-antigen as the major selective pressure, an interpretation supported by the extensive studies of Nussenweig and colleagues (33). It should be noted, however, that since V_H use differs so drastically between fetus and adult, it is possible that focusing on the 7183 family repertoire might miss environmental influences from external or commensal antigens that affect selection of V_H families other than 7183. Studies of the repertoire expressed by germ-free mice are currently being conducted in our laboratory.

These observations would suggest that study of immune responses in TdT deficient animals are likely to allow us to continue to gain some insight into the biologic significance of CDR-H3 control on the antigen hierarchy. Studies that have already been completed include an examination of the effect of N addition on immunity to infectious agents or polysaccharide antigens. Heterosubtypic immunity to influenza A virus infection is impaired in TdT−/−mice (35) and Mahmoud and Kearney have shown that TdT activity is required for the generation of optimal anti-DEX Ab response and the dominance of the J558 idiotype in adult BALB/c mice (36). These studies would suggest that restrictions in the addition of N nucleotides could function as a mechanism for the increased susceptibility of the young to some infectious diseases. Conversely, a number of studies have shown that autoimmune disease is inhibited by the absence of N nucleotides (37–41). In particular, the decrease in the use of arginine may diminish the likelihood of generating pathogenic anti-DNA antibodies (42).

Gene expression patterns in the embryo and fetus differ significantly from that of the adult. Thus it is possible that control of the CDR-H3 repertoire with the resultant limitations in the diversity of the immunoglobulins expressed by the developing child functions to buffer the effects of a changing universe of self-antigens, preventing the developing of autoimmunity in the very young. Still, the extensive similarities in CDR-H3 content between the mature B cell repertoire in TdT deficient adult bone marrow and TdT insufficient perinatal liver suggest that TdT deficient mice represent a good, although not perfect, model for the testing the role of the limited perinatal CDR-H3 repertoire on antibody responses to antigens. Such studies are currently being pursued on our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BM	bone marrow
CDR-H3	complementarity determining region 3 of the immunoglobulin heavy chain
PNL	perinatal liver
RT-PCR	polymerase chain reaction amplification of transcripts cloned into cDNA by reverse transcription of mRNA
TdT	Terminal deoxynucleotidyl Transferase
WT	wild-type

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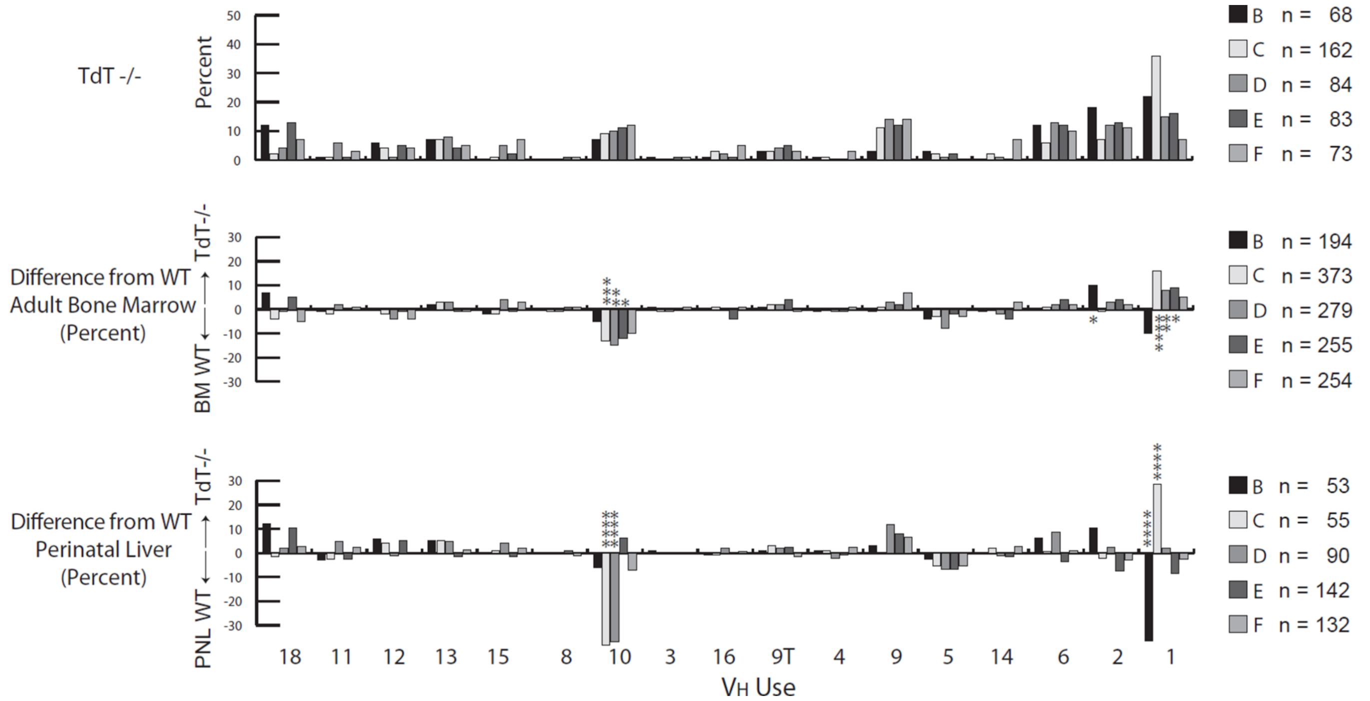

Figure 1. V_H usage in V_H7183DJCμ transcripts during B cell development in TdT-KO adult bone marrow: divergence between TdT-deficient versus WT adult bone marrow and perinatal liver

(Top) Percent of unique, in-frame sequences using the specified V_H gene segment in the adult bone marrow from Hardy fractions B (CD19+ CD43+ IgM− BP-1−), C (CD19+ CD43+ IgM− BP-1+), D (CD19+ CD43− IgM− IgD−), E (CD19+ CD43− IgM+ IgD−), and F (CD19+ CD43− IgM^{lo} IgD^{hi}) from TdT-deficient mice. (Middle) Divergence in the percentage of V_H gene segment use in TdT-deficient versus wild-type TdT sufficient adult bone marrow. (Bottom) Divergence in the percentage of V_H gene segment use in TdT-deficient adult bone marrow versus wild-type TdT insufficient perinatal liver. The number of sequences analyzed for each fraction in each mouse tissue is shown. All comparisons were made using χ^2-test or Fisher's exact test as appropriate. Significant differences among each fraction in the different mice are indicated by asterisks: *p 0.05, **p 0.01, *** p 0.001, **** p 0.0001.

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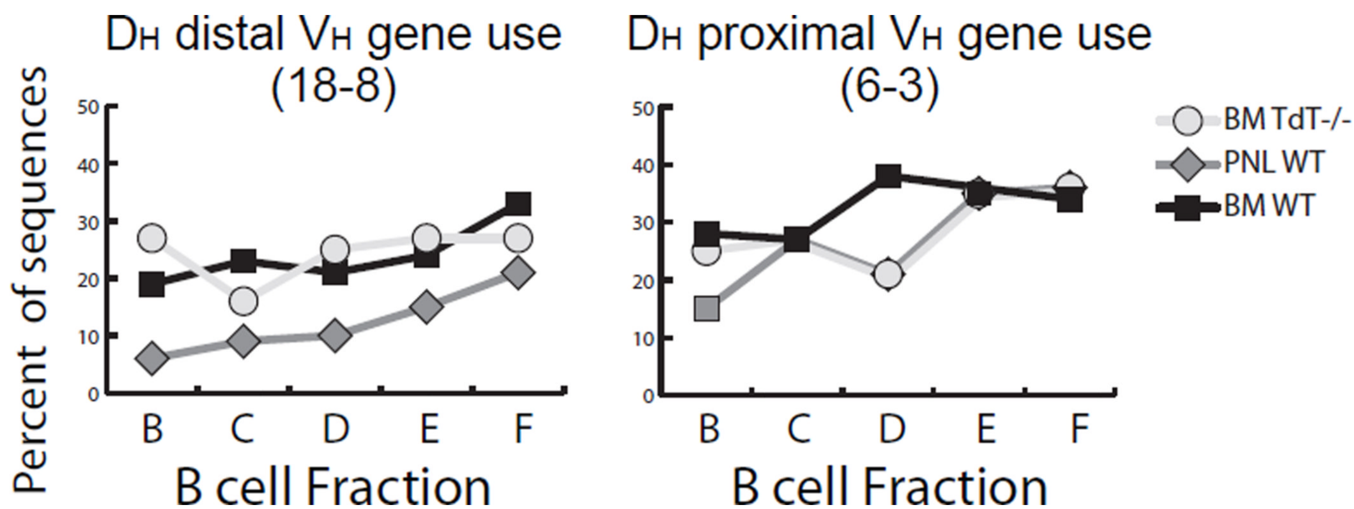

Figure 2. D_H distal and proximal V_H gene usage during B cell development in TdT-deficient adult bone marrow, WT adult bone marrow and perinatal liver

The V_H gene segments upstream of VH7183.10 were grouped into one block (block 8-18); and the V_H gene segments downstream of VH7183.10, but upstream of VH81X and VH7183.2, were grouped into a second block (block 3-6). V_H block usage is reported as the percent of the sequenced population of unique, in-frame, transcripts from TdT-deficient adult bone marrow, wild-type TdT sufficient adult bone marrow and wild-type TdT insufficient perinatal liver Hardy fractions B through F.

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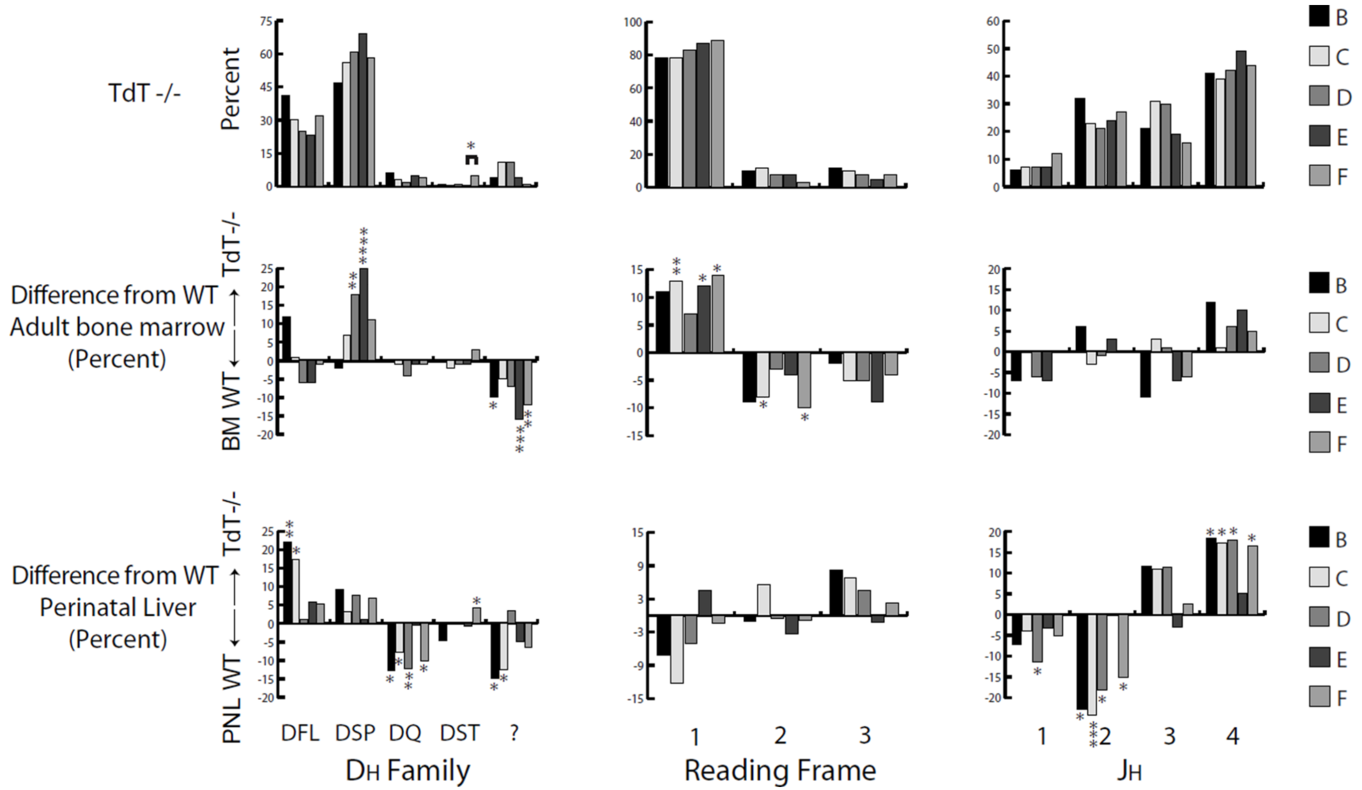

Figure 3. D_H family, D_H reading frame and J_H usage in V_H7183DJCμ transcripts during B cell development in TdT-deficient adult bone marrow: divergence between TdT-deficient versus WT adult bone marrow and perinatal liver

(Top) D_H family and J_H usage is reported as the percent of the sequenced population of unique, in-frame transcripts from TdT-deficient adult bone marrow Hardy fractions B through F. D_H reading frame usage is reported as the percent of the sequenced population of DFL- and DSP-containing transcripts from each B cell fraction. (Middle) Divergence in the percentage of D_H, DSP and DFL reading frame, and J_H usage in TdT-deficient versus wild-type TdT sufficient adult bone marrow. (Bottom) Divergence in the percentage of D_H family, DSP and DFL reading frame, and J_H usage in TdT-deficient adult bone marrow versus wild-type TdT insufficient perinatal liver. All comparisons were made using χ^2-test or Fisher's exact test as appropriate. Significant differences among each fraction in the different mice are indicated by asterisks: *p 0.05, **p 0.01, *** p 0.001, **** p 0.0001.

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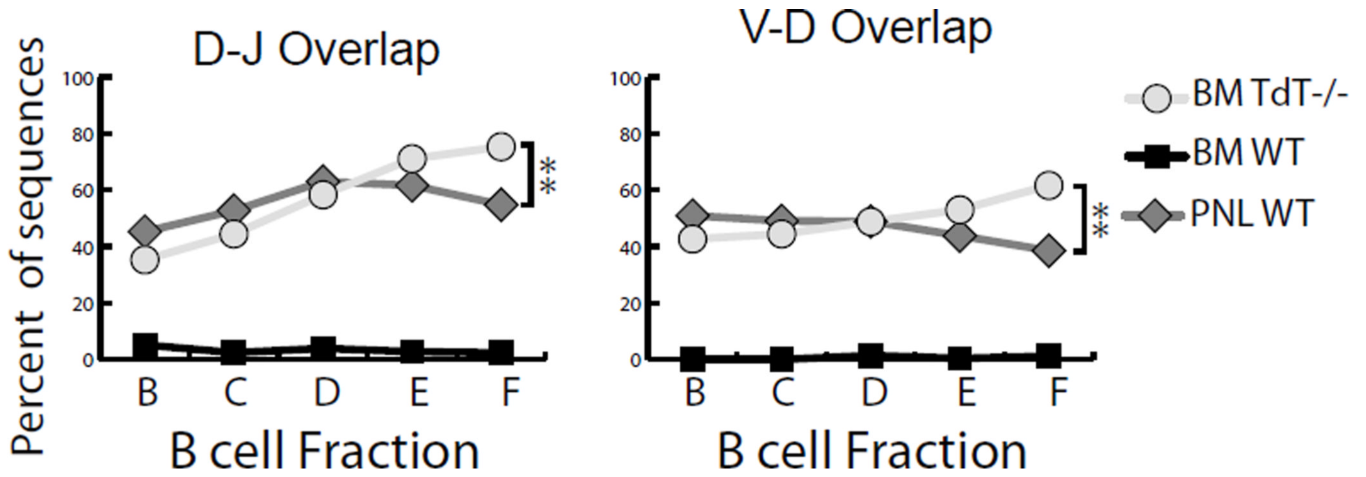

Figure 4. Frequency of D_H-J_H and V_H-D_H overlap during B cell development in TdT-deficient adult bone marrow, WT adult bone marrow and perinatal liver

D_H-J_H overlap (left) and V_H-D_H overlap (right) are reported as the percent of the sequenced population of unique, in-frame transcripts from TdT-deficient adult bone marrow, wild-type TdT sufficient adult bone marrow and TdT insufficient perinatal liver Hardy fraction B through F. All comparisons were made using χ^2-test or Fisher's exact test as appropriate. Significant differences among each fraction in the different mice are indicated by asterisks: *p 0.05, **p 0.01, *** p 0.001, **** p 0.0001.

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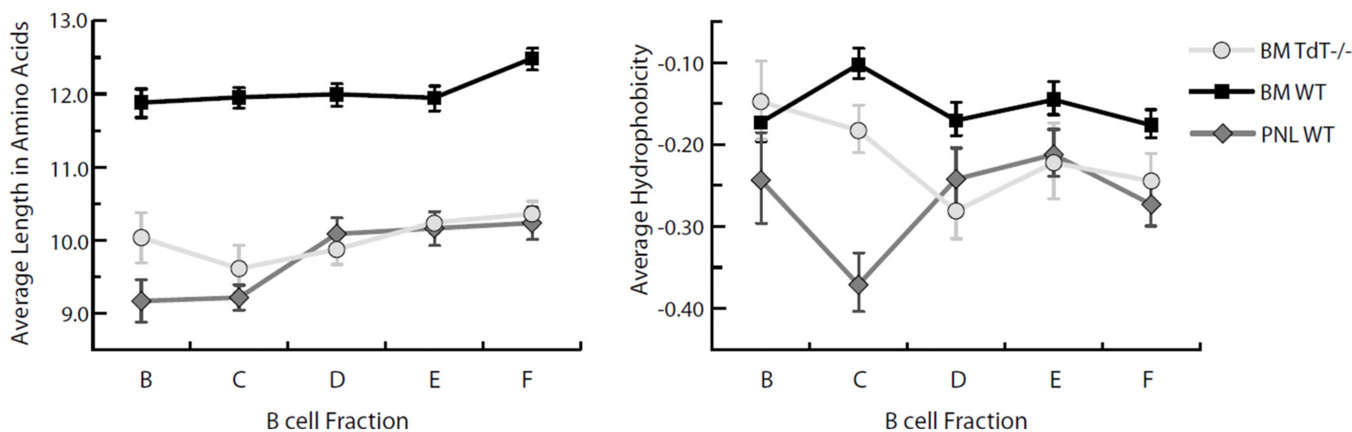

Figure 5. Average CDR-H3 length and average CDR-H3 loop hydrophobicity as a function of B cell development in TdT-deficient adult bone marrow compared to that in the adult bone marrow and perinatal liver of WT mice

(a) Average CDR-H3 length and (b) average CDR-H3 loop hydrophobicity encoded by the VH7183DJCμ transcripts from TdT insufficient perinatal liver, TdT deficient adult bone marrow, and TdT sufficient (wild-type) adult bone marrow. The standard error of the mean is shown.

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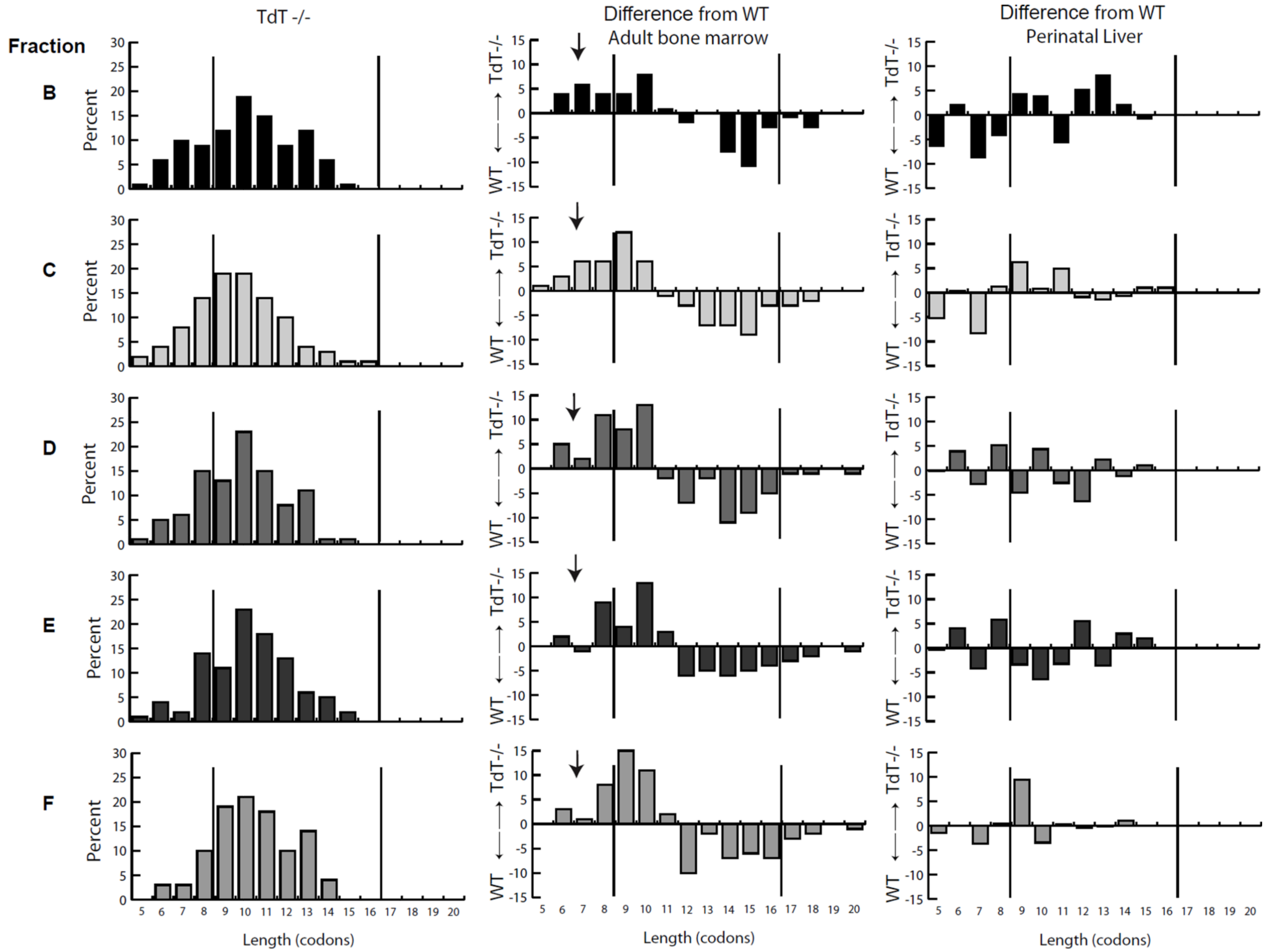

Figure 6. Distribution of CDR-H3 length of V_H7183DJCμ transcripts as a function of B cell development in TdT-deficient adult bone marrow: divergence between TdT-deficient versus WT adult bone marrow and perinatal liver

(Left) Distribution of CDR-H3 lengths of the sequenced population of unique, in-frame transcripts from TdT-deficient adult bone marrow Hardy fraction B through F. (Center) Divergence in the distribution of CDR-H3 lengths in TdT-deficient versus wild-type TdT sufficient adult bone marrow. (Right) Divergence in the distribution of CDR-H3 lengths in TdT-deficient adult bone marrow versus wild-type TdT insufficient perinatal liver. To facilitate visualization of the change in variance of the distribution, the vertical lines mark the preferred range of lengths in the bone marrow fraction F. Arrows point to features of particular interest, see text for detail.

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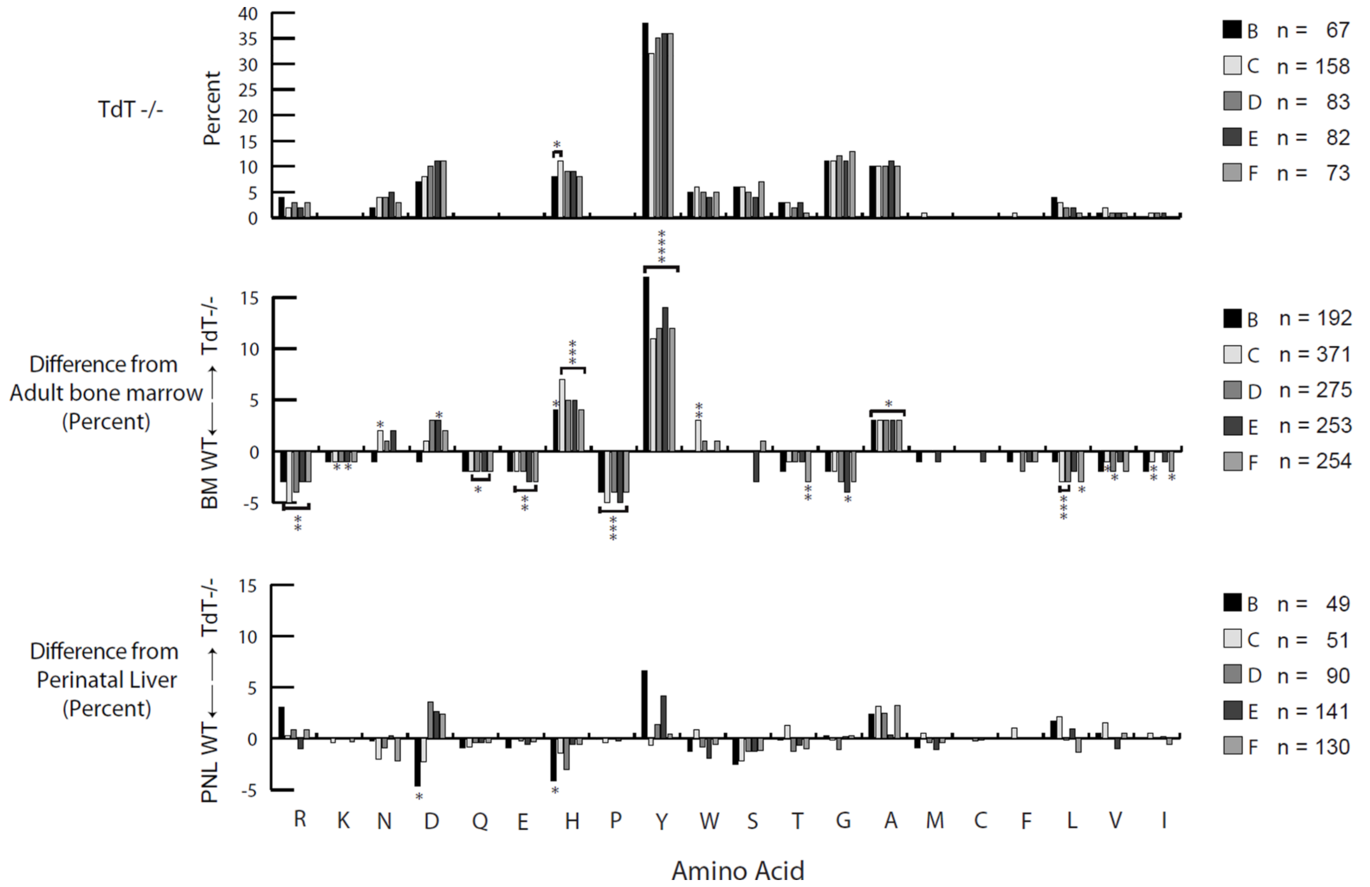

Figure 7. Distribution of amino acids in the CDR-H3 loops of the V_H7183DJCμ transcripts during B cell development in TdT-deficient adult bone marrow: divergence between TdT-deficient versus WT adult bone marrow and perinatal liver

(Top) Distribution of amino acid usage is shown as the percent of CDR-H3 loop sequences as a function of B cell development in TdT-deficient adult bone marrow. (Center) Divergence in the distribution of individual amino acid use in the CDR-H3 loop between TdT-deficient versus TdT sufficient (WT) adult bone marrow. (Right) Divergence in the distribution of individual amino acid use in the CDR-H3 loop between TdT-deficient adult bone marrow versus wild-type TdT insufficient perinatal liver. The amino acids are arranged by relative hydrophobicity, as assessed by a normalized Kyte-Doolittle scale (29, 30). . All comparisons were made using χ^2-test or Fisher's exact test as appropriate. Significant differences among each fraction in the different mouse are indicated by asterisks: *p 0.05, **p 0.01, *** p 0.001, **** p 0.0001.

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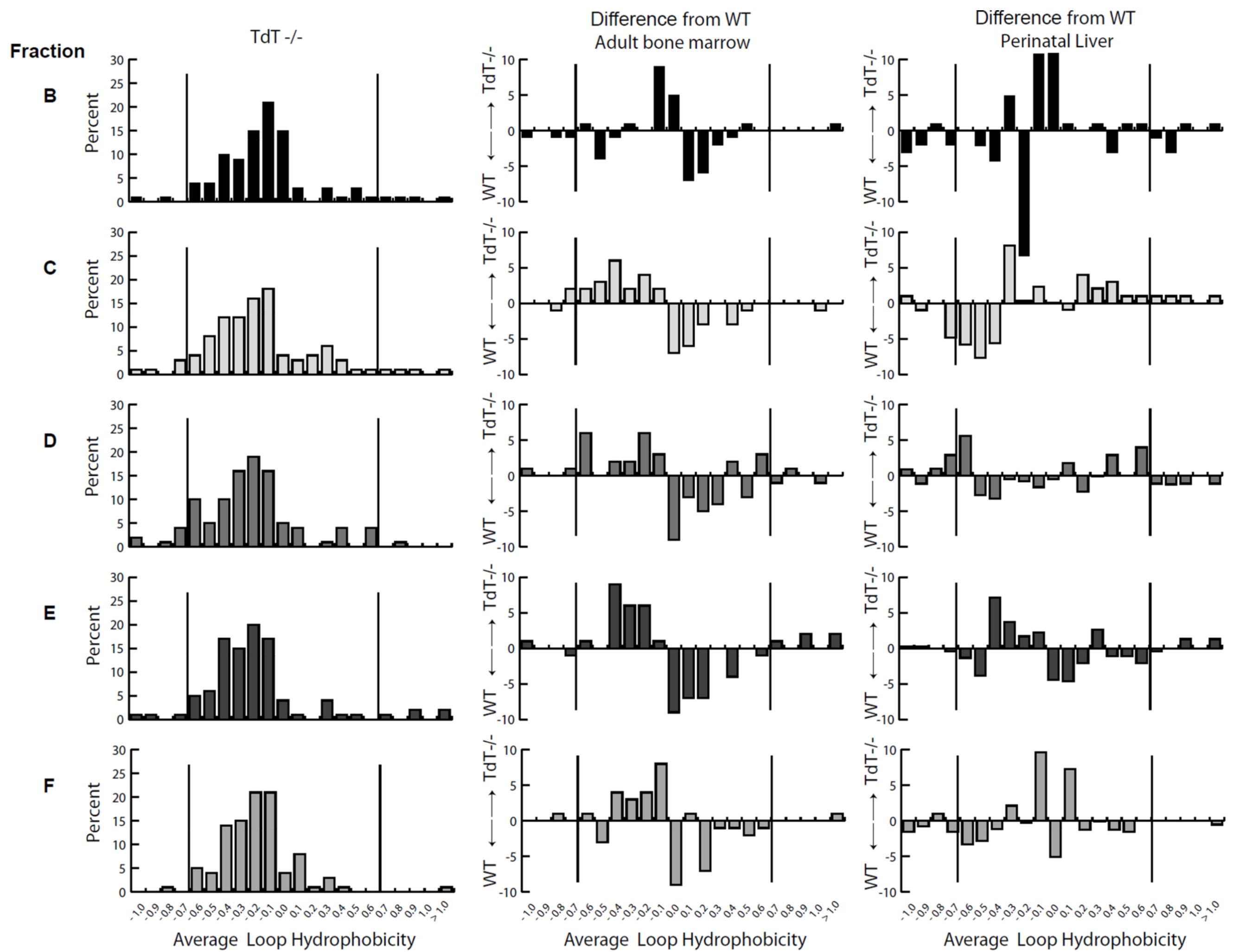

Figure 8. Distribution of CDR-H3 loop charge of V_H7183DJCμ transcripts as a function of B cell development in TdT-KO adult bone marrow: divergence between TdT-deficient versus WT adult bone marrow and perinatal liver

(Left) Distribution of CDR-H3 loop hydrophobicity of the sequenced population of unique, in-frame transcripts from TdT-KO adult bone marrow Hardy fraction B through F. (Center) Divergence in the distribution of CDR-H3 loop hydrophobicity in TdT-deficient versus TdT sufficient wild type (WT) adult bone marrow. (Right) Divergence in the distribution of CDR-H3 loop hydrophobicity in TdT-deficient adult bone marrow versus TdT insufficient perinatal liver. The normalized Kyte-Doolittle hydrophobicity scale (30) has been used to calculate average hydrophobicity. Although this scale ranges from −1.3 to + 1.7, only the range from −1.0 (charged) to +1.0 (hydrophobic) is shown. To facilitate visualization of the change in variance of the distribution, the vertical lines mark the preferred range of average hydrophobicity in the bone marrow fraction F.

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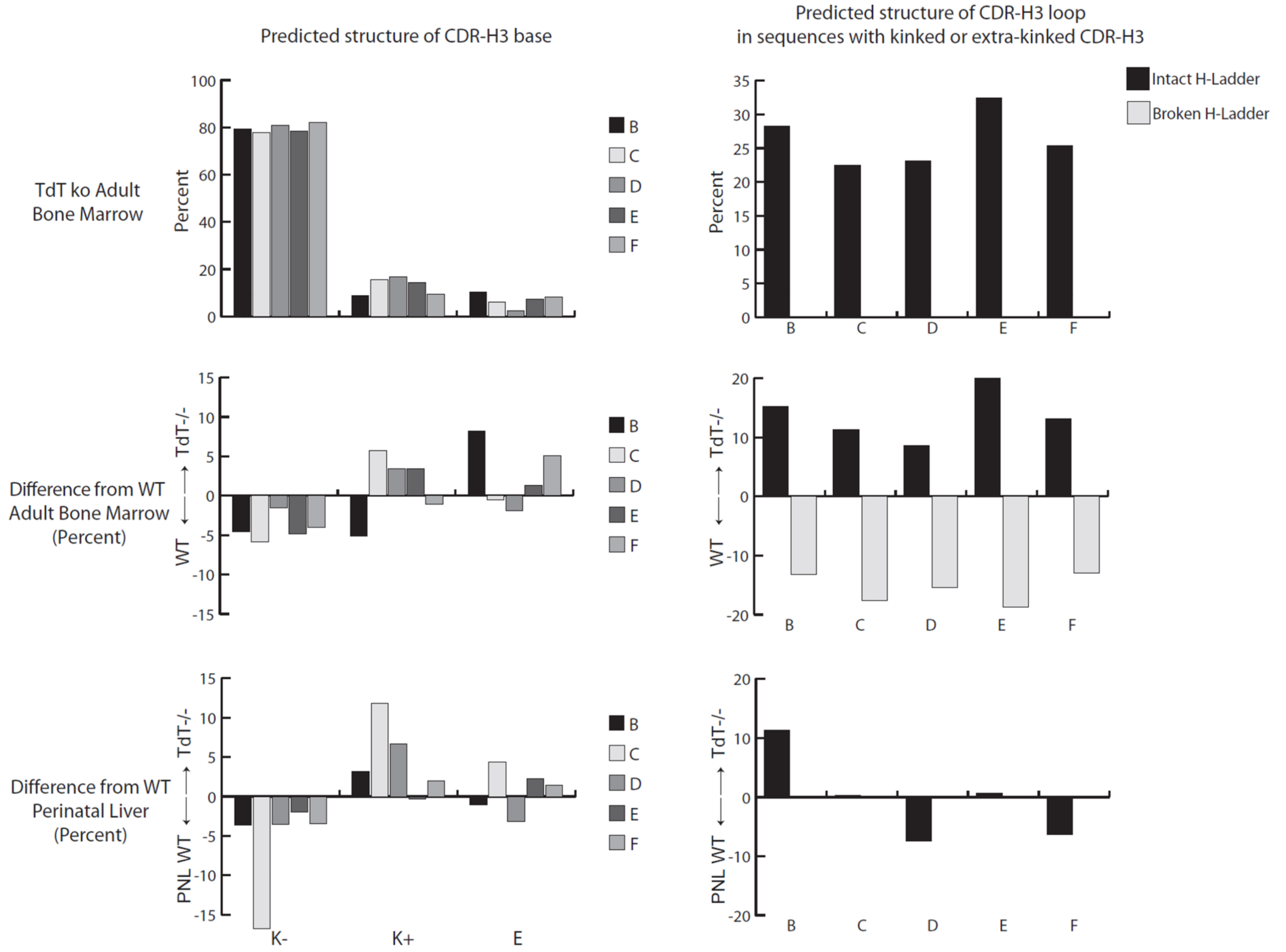

Figure 9. Distribution of the predicted structures of the base and the loop of CDR-H3 from V_H7183DJCμ transcripts as a function of B cell development in TdT-KO adult bone marrow: divergence between TdT-deficient versus WT adult bone marrow and perinatal liver

(Left column) Frequency of kinked (K–), extra- kinked (K+) and extended (E) CDR-H3 bases. (Right column) Frequency of broken and intact hydrogen bond ladders within the CDR-H3 loop for those H chains that contain kinked or extra-kinked bases. (Top) Transcripts from $TdT^{-/-}$ adult bone marrow Hardy fraction B through F. (Middle) Divergence in the transcripts from $TdT^{-/-}$ versus WT adult bone marrow. (Bottom) Divergence in the transcripts from TdT-KO adult bone marrow versus WT perinatal liver.