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Terminal deoxynucleotidyl Transferase is required for an optimal response to the polysaccharide α-1,3 Dextran[1]

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Abstract

An understanding of antibody responses to polysaccharides associated with pathogenic microorganisms is of importance for improving vaccine design, especially in neonates that respond poorly to these types of antigens. In this study, we have investigated the role of the lymphoid specific enzyme TdT in generating B cell clones responsive to α-1,3 Dextran (DEX). TdT is a DNA polymerase that plays a major role in generating diversity of lymphocyte antigen receptors during V(D)J recombination. In this study we show that the DEX-specific antibody response is lower and the dominant DEX-specific J558 idiotype (Id) is not detected in TdT−/− mice when compared to wild type BALB/c (WT) mice. Nucleotide sequencing of heavy chain CDR3s of DEX-specific plasmablasts, sorted post-immunization, showed that TdT−/− mice generate a lower frequency of the predominant adult molecularly-determined clone J558. Complementation of TdT expression in TdT−/− mice by early forced expression of the short splice variant of TdT restored WT proportions of J558 Id+ clones and also abrogated the development of the minor M104E Id+ clones. J558 Id V(D)J rearrangements are detected as early as 7 days after birth in IgM negative B cell precursors in the liver and spleen of WT and TdT transgenic mice but not in TdT−/− mice. These data show that TdT is essential for the generation of the predominant higher affinity DEX-responsive J558 clone.

Keywords

TdT; CDR3; antibodies; repertoire development; J558 idiotype

Introduction

Polysaccharides serve as important virulence factors for many pathogenic microorganisms (1) and induction of polysaccharide-specific antibodies is a major factor in successful vaccination against pathogens such as *Haemophilus influenzae* type b, *Streptococcus pneumoniae* and *Neisseria meningitidis* (2–4). An understanding of the cellular and

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molecular events involved in the generation of B cell clones that give rise to protective polysaccharide-specific antibodies will provide clues as how the immune system can be induced to make such antibodies.

Many antibody responses to polysaccharides in mice are T-cell independent and characterized by the rapid production of IgM and IgG3 (5), oligoclonality and low affinity (6–8). Polysaccharides are generally poor inducers of memory, although features of memory antibody responses to polysaccharides have been recently demonstrated ((9); Foote J. and J.F. Kearney, manuscript submitted).

Polysaccharides induce poor antibody responses in neonatal humans and mice (10–13) and several mechanisms have been proposed to account for this relative unresponsiveness compared to adults (reviewed in (14, 15)). One possible mechanism is that the neonate, in contrast to the adult, does not contain B cells with the appropriate polysaccharide-reactive immunoglobulin (Ig) receptors (8, 13). The neonatal B cell repertoire differs significantly from that of the adult with respect to Ig V_H, V_L, D_H and J_H gene usage (16–20). One notable difference between neonatal versus adult Ig repertoire is that heavy chain CDR3 lengths are shorter in the neonate due to the lack of, or lower Terminal deoxynucleotidyl Transferase (TdT) activity in mice (20, 21) and humans (22) respectively. In addition, in-frame rearrangements predominate, as a result of enhanced homology-mediated recombination, leading to increased representation of certain CDR3 sequences (23–25).

TdT is a lymphoid-specific DNA polymerase that plays a major role in the generation of B and T cell antigen receptor diversity (26–28). TdT is conserved among vertebrate species (29, 30) and of the TdT alternative splice variants, the short form of TdT (TdTS) has been shown to exert its diversifying activity by adding non-templated nucleotides (N-addition) at the V(D)J junctions of rearranging B and T cell receptors (27, 28, 31–33).

The presence or absence of TdT functional activity has been shown to play a significant role in mouse antibody responses to T-independent antigens. The germline-encoded T15 antibody specific for phosphorylcholine (PC), expressed on the surface of *Streptococcus pneumoniae*, is generated early in life in the absence of TdT (23) and protects against infection with this pathogen (34, 35). Forced expression of TdT during this period leads to the loss of the canonical T15 antibody in adulthood and hence loss of protection (36). In contrast, the activity of TdT is required for the generation of the M603 idiotype+ (Id+) B cell clone, responsive to PC expressed on *Proteus morganii* (37). Both of these studies provide examples of the significant role that TdT plays in modulating the B cell repertoire.

In this study we investigated the role of TdT during the generation of B cell clones involved in the antibody response to the polysaccharide α-1,3 Dextran (DEX) (38–40). DEX is a branched polymer containing α-1,3 glucose epitopes which are also expressed in glucans associated with a variety of organisms such as, *Enterobacter cloacae*, *Histoplasma capsulatum* yeast cell wall (41) and *Aspergillus fumigatus* (Dizon B.L. and J.F. Kearney, unpublished observations). The antibody response of adult BALB/c mice to DEX is oligoclonal and consists almost entirely of antibodies bearing the λ light chain (39) and the majority of anti-DEX antibodies possess idiotypic determinants cross-reactive with the

BALB/c plasmacytoma proteins J558 and M104E (39, 40, 42). Amino acid sequence analysis of DEX binding hybridoma proteins showed V_H region homology, with most diversity existing in the putative D_H region of the heavy chain CDR3. It has been shown previously that this region contributes to the individual idiotype identity expressed by distinct B cell clones (43). Now, by comparing the anti-DEX antibody response of TdT-deficient and TdT transgenic mice, we show that TdT activity is required for the generation of the optimal anti-DEX antibody response in adult BALB/c mice and the accompanying dominance of the J558 idiotype.

Materials and Methods

Mice

BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). TdT−/− were originally obtained from Dr. Diane Mathis (27) and crossed for 10 generations or more to the BALB/c background. TdTS transgenic mice were developed in our laboratory (36) and were crossed to TdT−/− mice in this study (TdTS x TdT−/−). All mice were bred and housed within the specific pathogen-free facility at The University of Alabama at Birmingham and used at 8 to 12 weeks of age according to protocols approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Immunization and serum analysis

Mice were immunized i.v. with 10^8 DEX-expressing heat-killed *Enterobacter cloacae* (strain MK7). ELISA was used to quantify serum antibodies by methods previously described (38). Plates were coated with either DEX at 2 μg /mL (Dr. Slodki, USDA, Peoria, IL) or the anti-J558 idiotype antibody EB3-7 at 1 μg /mL (44). Bound serum immunoglobulins were developed with alkaline-phosphatase conjugated anti-IgM for DEX-coated plates or anti-λ for EB3-7 coated plates (Southern Biotechnology Associates, Birmingham, AL).

Flow Cytometry and Cell Sorting

Single cell suspensions of spleen cells or peritoneal cavity cells were treated with Ab 93 (45) to block Fc receptors then surface stained with CD19 PE, DEX antigen labeled with Alexa 488 and anti- λ (JC5-1) for staining DEX-binding B cells. The “dump” channel included anti-CD3, anti-CD4, anti-CD8, anti-CD11c and anti-Gr-1 biotin labeled antibodies, which were then developed with SA-PerCP. DEX+ λ+ plasmablasts (B220 +/− Syndecan-1+) were sorted 7 days post-immunization with *Enterobacter cloacae* and genomic DNA was extracted. Cells were sorted into RPMI media supplemented with 10% fetal calf serum and the collection tubes were maintained at 4°C throughout the sort. The sorted cells were then centrifuged and digestion buffer (50 mM Tris-HCl, 100 mM EDTA, 100mM NaCl, 1% SDS, pH 8.0) + Proteinase K (0.5mg/mL) was added to the cell pellet and incubated overnight at 50°C. DNA was precipitated using isopropanol and the DNA pellet was washed with 70% ethanol, air-dried and resuspended in molecular grade water. All samples were analyzed using a FACSCalibur flow cytometer or FACSAria cell sorter (BD Biosciences, San Jose, CA). The data were analyzed using FLOWJO software (Tree Star, Inc.). All

antibodies were purchased from BD Biosciences except for Ab 93 and JC5-1 that were developed in our laboratory.

Polymerase Chain Reaction

V(D)J rearrangements were amplified by PCR from genomic DNA extracted from sorted plasmablasts using primers specific for gene segments J558.3 V_H 5'-AGCTGCAACAATCTGGACCT-3' and J_H1 5'-CCCCAGACATCGAAGTACCA-3'. PCR conditions were 95° for 3 min then 95° for 1 min, 58° for 1 min and 72° for 1 min for 35 cycles then 72° for 7 min. RT-PCR for detection of TdTS and Actin was done as described (32).

Cloning and Sequencing

PCR products were subcloned into TOPO-TA vector and transformed into TOP10 competent bacteria (Invitrogen, Carlsbad, CA). Templiphi Amplification Kits were used to isolate plasmid DNA from colonies (Amersham Biosciences, Piscataway, NJ). Plasmid DNA was sequenced at the Center for AIDS Research sequencing facility at the University of Alabama at Birmingham.

Sequence analysis

Any nucleotides that could not have been derived from a coding sequence or P addition were considered as non-germline nucleotides. In the case of D_H-J_H homology joining, where nucleotides could be assigned to either D_H or J_H, nucleotides were assigned to D_H except when P-addition to J_H gene was identified, in which case the nucleotides were assigned to J_H. HCDR3 was identified as the region between the 3' V_H encoded conserved cysteine (*TGT*) at Kabat position 92 (IMGT 104) and the 5' J_H -encoded conserved tryptophan (*TGG*) at Kabat position 103 (IMGT 118). Duplicate sequences (identical V_H, D_H, and J_H segments as well as junctional sequences) derived from individual PCR reactions were only counted once. Nucleotide sequences and deduced amino acid translations reported in this manuscript are available at GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) under the accession numbers GQ478711-GQ478848.

Statistics—Data comparing three or more groups were analyzed by a one-way ANOVA test, followed by Tukey's post test, for data with normal distribution and Kruskal-Wallis test, followed by Dunn's post test, for data that did not distribute normally. Data comparing two groups were analyzed by a two-tailed unpaired *t* test. To determine statistical significance for data comparing proportions, we calculated the 95% confidence interval of the mean for each group. Statistical significance was determined by a p value of <0.05.

Results

Ontogeny of B cell precursors responding to DEX

In previous ontogenetic studies, we showed that DEX-responsive B cells were first detected in BALB/c mice between 5–11 days of age and gradually increased to adult frequencies, which peaked at ≈30 days of age. By using idiotypic markers, it was determined that the M104E Id+ clone predominated during the emergence of the anti-DEX repertoire but was

eventually largely replaced by the J558 Id+ clone which normally dominates the adult anti-DEX repertoire (46) (Figures 1A, B). The cross-reactive idiotype (IdX) was consistently expressed by the majority of DEX-specific clones including J558 and M104E. The J558 and M104E B cell clones were originally defined by the amino acid sequences from corresponding plasmacytomas and have identical heavy and light chain amino acid sequences, except for two amino acids constituting the heavy chain CDR3 region (Figure 1C) (39, 42, 47, 48). Nucleotide sequence alignment of the two clones show that the difference lies in five nucleotides where TTACGAC (encoding amino acids YD) in the M104E heavy chain CDR3 is substituted by CCGCTAC or four nucleotides where TACGAC is replaced by AGGTAC (encoding amino acids RY) in the J558 heavy chain CDR3 (49). The differences in the heavy chain CDR3 of these clones are mostly encoded by D_H gene segment DSP2.2 usage by M104E, DSP2.10 usage (AGGTAC) or J_H1 usage (CCGCTAC) by J558. Both J558 clones show evidence of junctional diversity (P and N-nucleotides) in contrast to the M104E clone. In summary, the presence of clones with the ability to respond to DEX as well as the pattern of idiotype expression on anti-DEX antibodies is age-dependent, with the major responding clones differing only in their heavy chain CDR3 regions.

The antibody response to DEX is lower in TdT−/− mice due to loss of J558 Id expressing antibody that is partly restored by transgenic TdTS expression

Since the amino acid content of the heavy chain CDR3 is the major contributor to differences between J558 Id and M104E Id, we examined the antibody response to DEX in wild type (WT) versus TdT−/− BALB/c mice. TdT−/− mice showed a marked reduction in the total IgM anti-DEX response compared to WT mice, at day 7 post-immunization (Figure 2A) and J558 Id+ antibody was barely detectable (Figure 2B).

To determine whether J558 Id+ antibodies could be restored by the expression of TdTS, we next immunized transgenic TdTS x TdT−/− mice. Although the total DEX-specific antibody response elicited in TdTS x TdT−/− mice was lower than in WT mice (Figure 3A), J558 Id+ anti-DEX antibody levels were restored to that of the WT (Figure 3B). We suspect that the lower anti-DEX antibody level may be partly due to N-additions in the λ1 light chain of these TdTS transgenic mice, thus contributing to the partial loss of J558 Id expression. In addition, early forced expression of TdTS may have prevented the generation of the CDR3 that gives rise to the M104E Id, which is expressed by almost 25% of anti-DEX spleen B cell precursors detected in the adult by the splenic focus assay (Figure 1B). Taken together, these results suggest that the activity of TdTS is involved in the formation of the heavy chain CDR3 expressed by J558 Id+ anti-DEX antibodies.

TdT−/− and WT mice have similar numbers of DEX-specific B cells

We next asked whether the frequency of DEX-specific B cells in adult mice is dependent on the expression of TdTS. If this were true then we would expect TdT−/− animals to have a lower number of DEX-specific B cells than WT mice. Flow cytometric analysis showed that, within the limits of detection of this assay, the absolute cell number of λ light chain expressing B cells that bind to DEX was similar in the spleens and peritoneal cavity cells of TdT−/− (mean ± SEM: 670 ± 317 and 364 ± 211 cells respectively) compared to WT mice

(475 ± 124 and 171 ± 45 cells respectively) (Figures 4A, B). As a negative control we showed that B cells from the C57BL/6 strain, which is a DEX non-responder strain, did not stain with DEX and anti-λ light chain antibody (Figure 4A). These results show that the lack of TdTS does not appear to affect the frequency of DEX-binding B cells in the spleen and peritoneal cavity.

TdT−/− mice express a neonatal-like anti-DEX repertoire

To compare molecular associations with idiotype expression in DEX-responding B cells of WT, TdT−/− and TdTS x TdT−/− mice, we amplified and sequenced heavy chain V(D)J rearrangements from genomic DNA of DEX+ λ+ plasmablasts sorted from the spleens of *Enterobacter cloacae*-immunized animals. As expected, WT mice expressed mostly the J558 V(D)J rearrangement, giving rise to the amino acids arginine and tyrosine (RY) in HCDR3. In agreement with serum antibody idiotype expression, TdT−/− animals expressed a much lower proportion of this V(D)J rearrangement in DEX+ λ+ plasmablasts (Figure 5A). The frequency of RY-expressing DEX+ λ+ plasmablasts was completely restored in TdTS x TdT−/− animals. The frequency of M104E CDR3 sequences (YD in HCDR3) detected in TdT−/− DEX+ λ+ plasmablasts was similar to that of the WT, but barely detectable in TdTS x TdT−/− DEX+ λ+ plasmablasts (Figure 5A).

WT sequences showed a greater number of non-J558 non-M104E, but J558.3-J_H1 expressing, DEX-responsive rearrangements than sequences from TdT−/− mice. The frequency of non-J558, non-M104E sequences in TdTS x TdT−/− is also reduced, which, in addition to the absence of M104E clone, could partially explain the lower total DEX antibody response of these animals. These data show that the altered DEX-responsive repertoire in TdT−/− mice, as shown by the decreased expression of J558 and decreased frequencies of non-J558, non-M104E sequences, is an important factor in the overall decreased antibody response to DEX.

Although TdTS x TdT−/− DEX+ λ+ plasmablasts showed a restoration of the WT frequency of the dominant J558 sequence, examination of the deduced amino acid sequences of the V(D)J rearrangements amplified from DEX+ λ+ plasmablasts shows that diversity at the heavy chain CDR3 region was lower than the WT (Figure 5B). Comparison of nucleotide sequences from WT and TdTS x TdT−/− heavy chain CDR3 regions showed that although some of the WT sequences require one or two N-additions for their formation, over-expression of TdTS in TdTS x TdT−/− mice led to the abolition of these sequences and the emergence of other sequences with longer N-additions. As expected, the diversity in TdT−/− sequences was also lower compared to WT sequences with the M104E sequence being the most frequently used (Figure 5C).

J558 HCDR3 expression correlates with TdTS expression early in ontogeny of DEX-specific repertoire

To further confirm the requirement for TdTS in the generation of the J558 sequence and to rule out any role for antigen-receptor mediated selection, we examined the expression of TdTS in WT BALB/c day 7 neonates at the time when DEX-responsive B cells are first beginning to appear and correlated it with J558 sequence generation. TdTS mRNA message

levels were detected in B220+ IgM- B pro-B cells in the liver, spleen and bone marrow (Figure 6A). We then amplified antibody heavy chain V(D)J rearrangements that utilize V_H J558.3 and J_H1, the gene segments used in both M104E, J558 and other reported DEX-specific sequences, from these pro-B cell populations. With the exception of the bone marrow compartment, the expression of the J558 sequence correlated with the expression of TdTS as shown in Figure 6B. V(D)J rearrangements that give rise to DEX-responsive clones were barely detected in TdT−/− neonates, however they were readily detectable in neonates from TdTS x TdT−/− mice with J558 being the dominant sequence amplified at 7 days of age. In these B220+ IgM- cells no other (non-J558) sequences that give rise to known DEX-responsive B cells were detected at this time point. These data show that the expression of TdTS coincides with the expression of the J558 V(D)J rearrangement when DEX-responding clones start to emerge. They also show that selection for J558 could be taking place independently of antigen-receptor mediated signals.

Discussion

Immune protection against encapsulated bacteria, such as *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilis influenzae* is provided by antibodies against the bacterial capsular polysaccharides. We investigated the molecular mechanisms involved in generating an oligoclonal response to a typical type-2 T-cell independent antigen (DEX) and demonstrated that TdTS is important for the optimal antibody responses to DEX. We show that TdTS is required to generate a diverse DEX-responsive repertoire including optimal numbers of the J558 DEX-responsive clone involved in the adult response to DEX.

Earlier studies suggested that TdT is not required for the generation of efficient polyclonal T-cell and antibody responses to selected complex protein antigens (28, 50). However, there is mounting evidence that the advantage offered by the diversifying activity of TdT is subtle and is better measured at the level of clonal populations. Heterosubtypic immunity to Influenza A virus infection, which affords protection against a virus subtype differing from that used for immunization, is impaired in TdT−/− mice (51). This observation may be explained in part by the finding that TdT is required for the generation of private influenza virus-specific CD8+ repertoires that contribute to enhanced diversity in TCRs across the population of a species as a whole (52). Yewdell et al have also shown that TdT−/− mice mount a total CD8+ response to influenza and vaccinia viruses that is lower in magnitude and breadth compared to WT and that responses to immunodominant viral determinants are reshuffled (53). From these studies, as well as our findings, it is clear that TdT plays a significant role in the expression of a fully immuno-competent B and T cell repertoire in a given host population.

Alternatively it has been shown that the absence of TdT expression in neonatal life, and the window of limited diversity that ensues is required to generate certain antibody clones with protective, germline-encoded specificity. This was clearly demonstrated in TdTS transgenic mice, where forced TdT expression exerted its diversifying activity in fetal and neonatal repertoires, with the result that the mice were incapable of establishing the dominant T15 Id + anti-PC antibody that is protective against *Streptococcus pneumoniae* (36). TdT−/− mice have also been shown to generate a more robust T15 antibody response than WT animals

(37, 54). However, there are clearly other clones that require the activity of TdT for their generation, such as the M603 anti-PC antibody (37). In this study it was shown that TdT is responsible for the Asn/Asp substitution at the V-D junction of the T15 Id to generate the M603 Id+ clones. In our present study, we reveal another example of the requirement for TdT to generate the J558 Id instead of the germline M104E Id, both of which are responsive to the polysaccharide DEX.

The finding that TdT is required for the expression of the J558 Id may partially explain the observation in our earlier study that: 1) the frequency of anti-DEX spleen precursors, as detected by the splenic focus assay, gradually increases to adult levels subsequent to the onset of TdTS expression; 2) The idiotype pattern of the DEX-responding precursors gradually changes from a high M104E/J558 ratio to a much lower one. The difference between the two idiotypes lies in the heavy chain CDR3 region, where TdT exerts its diversifying activities. Our data confirm that the M104E sequence is germline as evidenced by its absence in TdTS x TdT−/− mice, where TdT is expressed constitutively in fetal and neonatal B cell precursors. The J558 Id+ heavy chain CDR3 could be encoded by DSP2.10 (AGGTAC) or by the nucleotides (CCGCTAC) which could be encoded by the first four nucleotides of the J_H1 segment, a P-nucleotide, an N-nucleotide and a D_H that cannot be assigned to a known D_H segment. We recover predominantly J558 Id+ sequences that express the latter CDR3 from our DEX+ λ+ plasmablasts after immunization with *Enterobacter cloacae*. This CDR3 region is GC rich, which is also suggestive that TdT is involved in its formation. However the generation of the J558 Id CDR3 is not absolutely dependent on TdT since we recover some sequences from TdT−/− mice that express this idiotype. These could be generated by the activity of other DNA polymerases, such as DNA Polymerase λ (Pol λ), that has been shown to be implicated in modifying gene segment ends during heavy chain V(D)J rearrangement (55). Examination of antibody responses by Pol λ −/− x TdT−/− would test this possibility. However, the two genes are on the same chromosome and it would be very difficult to obtain double knockout animals. The alternative would be to test Pol λ−/− neonates before TdTS is expressed, however B cells responsive to DEX in BALB/c mice do not emerge until 5 days of age, at which time point TdT is already beginning to be expressed (20, 21). Other members of the DNA Pol X family, which play a significant role in DNA repair, may also be playing a considerable role in V(D)J junctional diversity in the absence of TdT to contribute to these non-germline nucleotides. It is important to note that in Fig 5A we show that the overall frequency of DEX-specific plasmablasts of TdT−\− mice post immunization is lower than that of the WT. This may account for our observation that J558 Id-expressing antibodies in the serum of immunized TdT−\− mice are very low (3–58 μg/mL) or not detected by ELISA. It is possible that the higher affinity of the rare J558 clones that we find in TdT−\− mice could lead to their preferential enrichment in the pool of DEX-specific cells in our FACS sort. The frequency of DEX+ plasmablasts in Fig 5A suggests that the baseline numbers of J558 Id+ B cells in TdT−\− are much lower than wildtype. We have attempted to determine these baseline numbers by direct FACS staining of J558 Id+ B cells using EB3-7 (anti-J558 Id), however the background staining was too high to make any meaningful interpretation of the data. Alternatively, our method of sequence analysis, which excludes repeat sequences, may have led to an underrepresentation of the M104E germline sequences obtained from TdT−\−

plasmablasts. Regardless of the mechanism by which the J558 sequence is generated in the absence of TdTS, it is not as efficient in generating this clone as in WT mice.

The possibility exists that the absence of TdT alters the repertoire of other B cells that would have otherwise contributed to the selection of the J558 clone. There is evidence supporting a role for B cell idiotypic interactions early in life that modulate the development of B cell clones responsive to DEX and to other T-independent antigens (56, 57). Neonatal injection of the anti-PC antibodies, such as the T15 antibody that is presumably more abundant in TdT−/− mice, leads to a reduction in J558 idiotype expression when these mice were immunized with DEX as adults (58). Therefore, the mechanism by which J558 Id expression requires TdTS may be indirect and involves the selection of J558-expressing clones as a result of B cell interactions with other B cells or other immune cells.

However, the most likely explanation of our findings is that the J558 clone, along with HDEX9 (Ka= 2.0×10^4) and HDEX37 (Ka= 5.3×10^5) (all of which use RY amino acids at their CDR3 regions) show the highest affinity to DEX (59, 60). HDEX9 and HDEX37 sequences were not detected in our PCR assays because they use different V_H and J_H segments respectively. The difference in antibody affinity between J558 and the germline M104E to DEX is almost 100-fold (Ka= 1.4×10^5 versus 1.3×10^3) (57). This illustrates the importance of TdT in the generation of clones of higher affinity and which are likely involved in the higher antibody response in WT versus TdT−/− mice. The higher affinity of the J558 clone may also be responsible for its selective expansion in the splenic marginal zone and the peritoneal B1b cell compartment under the influence of environmental or self antigens (J. Foote and J.F. Kearney, manuscript submitted).

The diversity of the anti-DEX antibody response contributed by TdT is also evident in the increased number of non-J558, non-M104E sequences recovered from immunized WT versus TdT−/− animals. The activity of TdT promotes a more diverse usage of D_H segments and less homology-mediated joining that is characteristic of fetal and neonatal sequences. This is still evident despite the fact that we only amplified J558.3V_H- J_H1 rearrangements with the important limitation that TdT−\− mice may be forced to generate DEX-specific rearrangements that do not employ J558.3V_H- J_H1 due to their limited ability to generate the J558 rearrangement. However the response in BALB/c mice is known to be pauciclonal and the 2 major anti-DEX clones (J558 and M104E) use these specific gene segments. Overexpression of TdTS in TdTS x TdT−/− mice did not restore this element of antibody diversity. However, TdTS x TdT−/− mice are capable of harboring N-additions in their light chains, which in turn might compromise the ability of clones to bind to DEX, thus decreasing the frequency of overall DEX binding plasmablasts that were sorted. Therefore, sequences recovered from these plasmablasts might not show the same level of diversity as those from WT mice. An examination of the nucleotide sequences of V(D)J rearrangements amplified post-immunization from DEX-specific plasmablasts of these mice reveals that it is also possible that supra-physiological levels of TdTS activity might obliterate the generation of some of the DEX-specific sequences normally recovered from WT animals.

Examination of neonatal pro-B cells in the liver and spleen revealed a correlation between TdTS and J558 expression. This correlation did not hold true for neonatal bone marrow

cells, perhaps because the bone marrow has not been colonized with precursors capable of generating J558 at that point. Our assay did not detect other non-J558 sequences that would give rise to DEX-specific B cells in all neonatal pro-B cells examined, perhaps because the ontogenetic development of these clones is different and they may be generated at a later time point than 7 days after birth when the samples were taken. However, J558 contributes to about 70% of the adult response and detection of other DEX-responsive non-J558 sequences may require a very large sample size.

Most of the neonatal sequences we examined from spleen and liver pro-B cells could potentially give rise to J558 Id-expressing antibody. This suggests that selection, independent of BCR-mediated signaling, is taking place in these pro-B cells. J558-expressing heavy chain could be associating with the surrogate light chain in such a manner that leads to efficient pre-BCR assembly followed by rapid proliferation. Wasserman et al showed by transfecting different heavy chains in pro-B cell lines, that bias in V_H usage in the B cell repertoire can depend on the nature of the interactions between the heavy chain V(D)J rearrangement and the surrogate light chain which leads to different outcomes in fetal versus adult pro-B cells (61).

This report sheds light on the role of TdT on the host antibody response to a polysaccharide antigen. Antibodies vary in their protective abilities and our findings highlight another factor that may be considered to explain the molecular mechanism of these differences. A number of DNA polymerases have been shown to play a role in modulating heavy and light chain junctional diversity (55, 62). It would be of interest to examine the role of these factors in modulating the antibody response to polysaccharides. These findings have further implications for the observed differences in the ability of neonatal versus adult B cell repertoires to respond to polysaccharide antigens. The results lend support to the hypothesis that in some instances the poor neonatal response to polysaccharides may be due to the lack of B cell clones expressing the appropriate B cell receptor.

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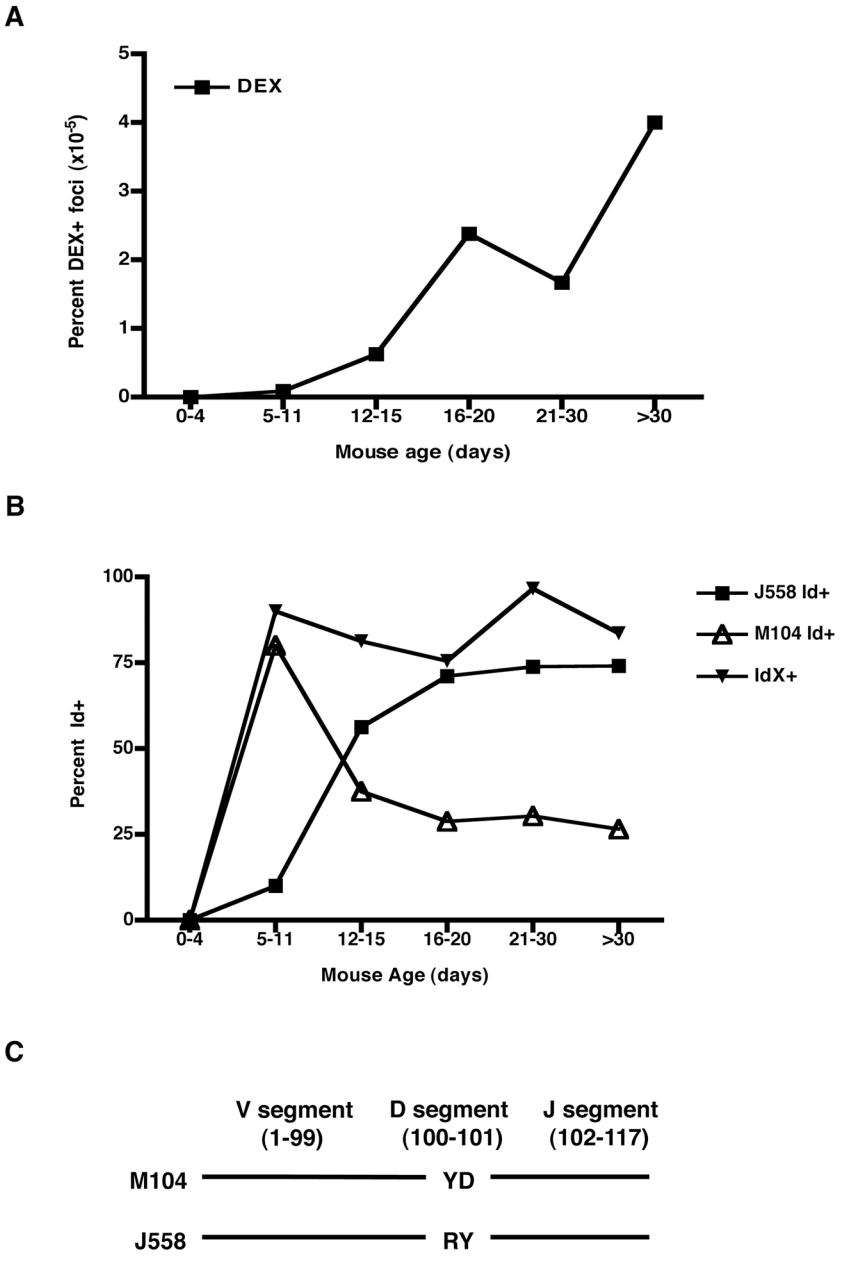


Figure 1. Ontogeny of DEX-responding B cell clones

Figure adapted from (46). (A) Percent of DEX-responsive spleen precursors in BALB/c mice from donors of the indicated ages was determined using the splenic focus assays. (B) The idiotype expression profile in each assay was determined by ELISA using anti-Id antibodies to detect J558 Id, M104E and IdX. IdX, the cross-reactive idiotype, is expressed by most DEX responding precursors. (C) Amino acid sequence alignment of the heavy chain of the two dominant anti-DEX clones M104E and J558 shows that the two clones are identical except for the CDR3 region.

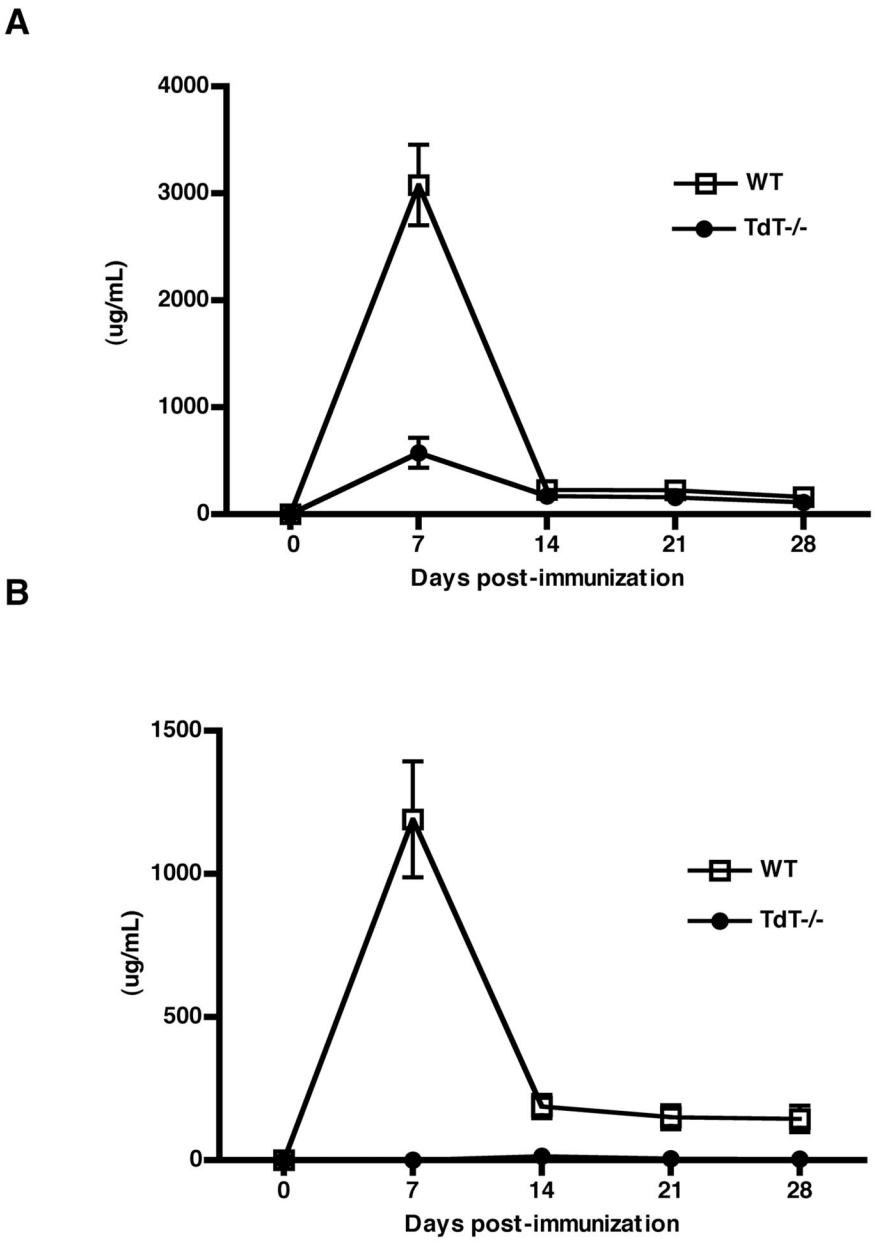


Figure 2. TdT−/− mice elicit a lower antibody response to DEX and fail to generate the J558 Id+ clone

Adult WT and TdT−/− mice were immunized i.v. with DEX-expressing *Enterobacter cloacae* and serum was collected at the indicated time points. ELISA was used to determine (A) DEX-specific IgM antibodies and (B) J558-specific antibodies (using EB3-7 anti-J558 Id).

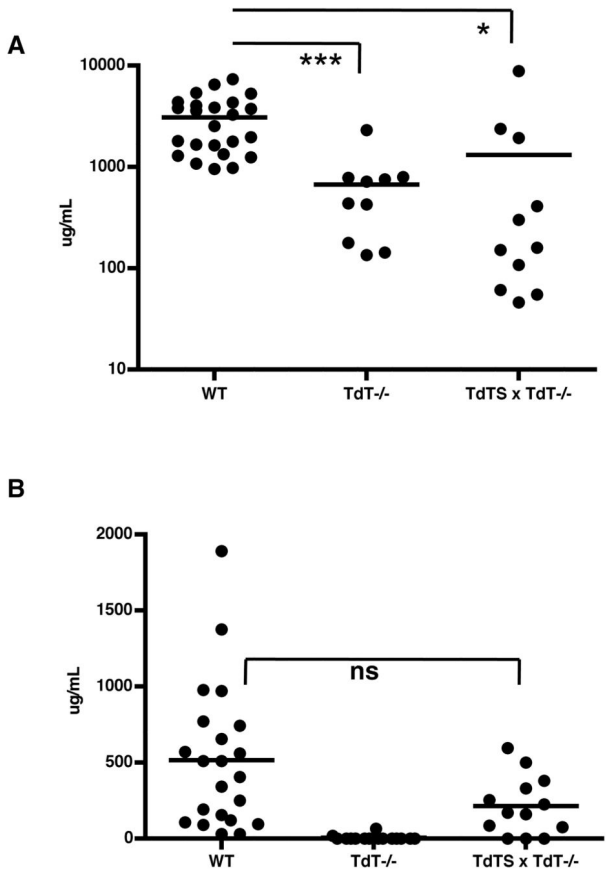


Figure 3. TdTS x TdT−/− restore J558 Id+ antibody levels to DEX

Adult WT, TdT−/− and TdTS x TdT−/− mice were immunized i.v. with DEX-expressing *Enterobacter cloacae* and serum collected day 7 post-immunization. ELISA was used to determine (A) DEX-specific IgM antibodies and (B) J558-specific antibodies. *** $p < 0.0001$, * $p < 0.05$ by One Way ANOVA post-hoc test, ns; not significant by Kruskal Wallis post-hoc analysis.

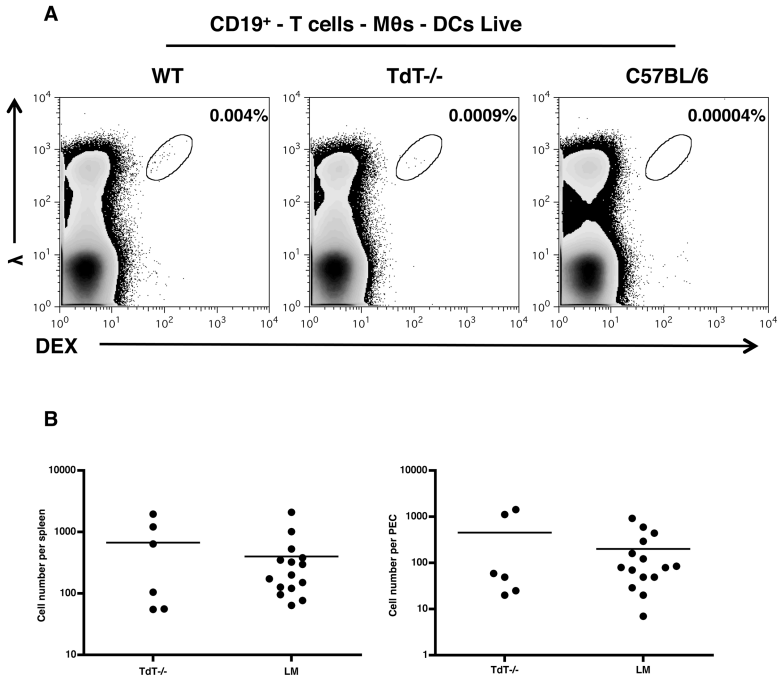
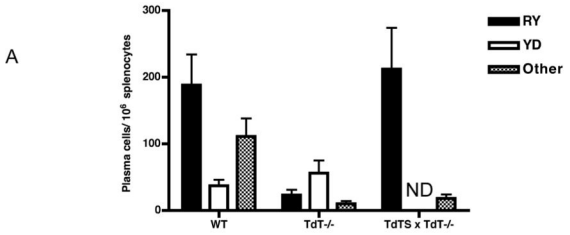


Figure 4. Adult WT and TdT−/− pre-immune mice have similar numbers of DEX-binding B cells

(A) A representative FACS density plot showing the percentage of CD19+ B cells in the spleen that are DEX+ JC5-1+ (λ+) in adult WT BALB/c and TdT−/− mice. C57BL/6 mice were used as negative control. (B) A graphical representation of the absolute cell numbers of DEX+ λ+ B cells in the spleens and peritoneal cavity cells (PEC) of WT and TdT−/− mice. The numbers were back-calculated from the FACS plots represented in (A). LM: littermate control mice.



B

WT

J558.3 V_H	N/P	D_H	N/P	J_H1	D_H gene	AA	
CDR3							
TGT GCA AGA				C TAC TGG TAC TTC GAT GTC TGG			
--- --- ---		GAC	CG	- --- --- --- --- --- --- ---	Dsp2.4	RY	(X 28)
--- --- ---	G	AT AGG TAC		--- --- --- --- --- --- ---	Dsp2.10	RY	
--- --- ---		GAT TAC GAC		--- --- --- --- --- --- ---	Dsp2.2	YD	(X 6)
--- --- --	GG	AC TGG GAC		--- --- --- --- --- --- ---	DQ52	WD	
--- --- ---	G	AC TGG GAC		--- --- --- --- --- --- ---	DQ52	WD	(X 2)
--- --- ---	GAT GA	T AAC T		-- --- --- --- --- --- ---	Dsp2.8	DN	(X 2)
--- --- ---	G	AT GGT AAC T		--- --- --- --- --- --- ---	Dsp2.8	GN	(X 3)
--- --- ---	GAT A	GT AAC T		-- --- --- --- --- --- ---	Dsp2.7	SN	(X 2)
--- --- --	G	GAT TAC G	C	C --- --- --- --- --- ---	Dsp2.2	YA	
--- --- ---	GA	T AAC TAC		--- --- --- --- --- --- ---	DSP2.8	NY	
--- --- --		A GAT A	C	- --- --- --- --- --- --- ---	DST4	TY	

TdT-/-

--- --- ---		GAT TAC GAC		--- --- --- --- --- --- ---	Dsp2.2	YD	(X 33)
--- --- ---		GAT TAC G	C	- --- --- --- --- --- --- ---	Dsp2.2	YA	(X 2)
--- --- ---		GAC	CG	- --- --- --- --- --- --- ---	Dsp2.4	RY	(X 17)
--- --- ---	GAT TCC G			-- --- --- --- --- --- ---		SD	
--- --- ---	GAC C	GG GAC		--- --- --- --- --- --- ---	DQ52	RD	

TdTS x TdT-/-

--- --- ---		GAC	CG	- --- --- --- --- --- --- ---	Dsp2.4	RY	(X 16)
--- --- ---	G	GAT AGG TAC		--- --- --- --- --- --- ---	Dsp2.10	RY	(X 3)
--- --- ---	G	AT GGT AAC T		-- --- --- --- --- --- ---	Dsp2.8	GN	(X 6)
--- --- ---	GA	T GGG A		-- --- --- --- --- --- ---	DQ52	GN	
--- --- ---	G	AC TGG GAC		--- --- --- --- --- --- ---	DQ52	WD	(X 3)
--- --- ---	GAC CGC TG			- --- --- --- --- --- ---		RC	

Figure 5. WT mice response to DEX shows higher J558 Id expression and greater diversity of anti-DEX antibodies than TdT−/− and TdTS x TdT−/−

DEX+ λ+ plasmablasts were sorted 7 days post-immunization with *Enterobacter cloacae* and V(D)J rearrangements were amplified from genomic DNA from WT, TdT−/− and TdTS x TdT−/− mice. (A) Frequency of DEX+ λ+ plasma cells utilizing J558 (RY), M104E (YD) or other sequences. ND; not detected. The frequency of plasmablasts expressing J558 or M104E was back calculated from the frequency of total DEX+ λ+ plasmablasts (as determined by FACS analysis, 7 days post-immunization) and the percentage of J558+ or M104E+ sequences obtained out of the total V(D)J sequences amplified from bulk DEX-specific plasmablasts. (B) Representative nucleotide and deduced amino acid sequences of heavy chain CDR3 regions amplified from WT, TdT−/− and TdTS x TdT−/−. In the case of sequence overlap nucleotides were arbitrarily assigned to the D_H gene segment except when P-nucleotides to J_H were identified, in which case they were assigned to J_H gene segment. P-nucleotides are underlined. AA: the two amino acids that differentiate each DEX-specific heavy chain sequence from the others. Data is representative of four independent FACS sorts for WT, five for TdT−/− and three for TdTS x TdT−/−.

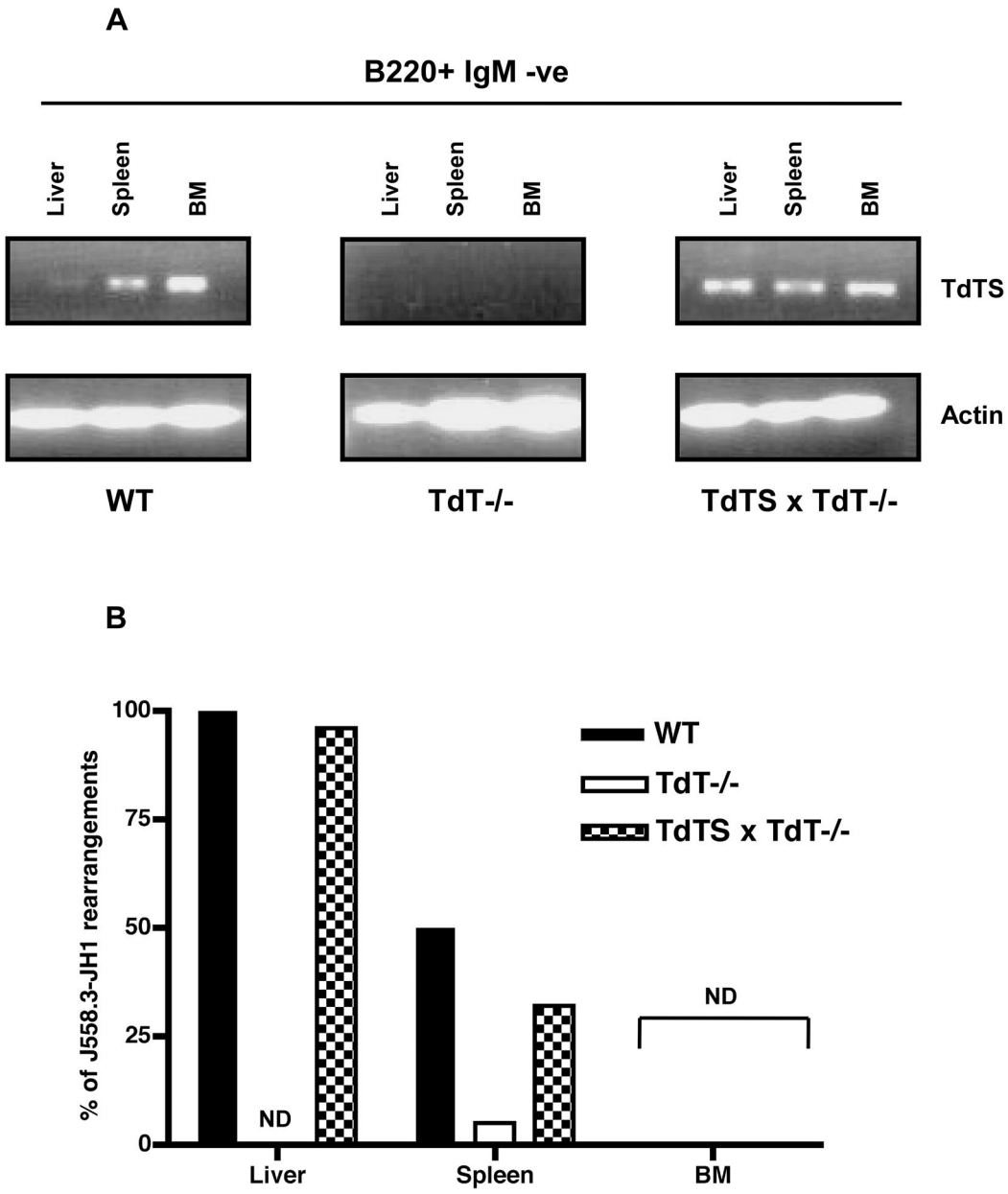


Figure 6. TdTS expression correlates with J558 expression when J558 Id starts to emerge

B220+ IgM- B cell progenitors were sorted from the spleens, liver and bone marrow of WT, TdT−/− and TdTS x TdT−/− mice at 7 days of age. (A) RT-PCR showing the expression of TdTS and Actin in the liver, spleen and bone marrow. (B) Percent of J558 sequence expression from J558.3-J_H1 V(D)J rearrangements amplified from sorted neonatal B cell progenitors in the liver and spleen. The figure is representative of two independent FACS sorts (one sort for TdTS x TdT−/−) from an entire litter of 7-day-old pups. ND: not detected. N= 17 for WT liver, 11 for WT spleen, 25 for TdT−/− liver, 18 for TdT−/− spleen, 9 for TdTS x TdT−/− liver and 18 for TdTS x TdT−/− spleen. The J558 sequence was not detected in bone marrow B cell progenitors at this time point.