Out-of-frame T cell receptor beta transcripts are eliminated by multiple pathways In Vivo

Grace K. Mahowald  
*Washington University School of Medicine in St. Louis*

Michael A. Mahowald  
*Washington University School of Medicine in St. Louis*

Clara Moon  
*Washington University School of Medicine in St. Louis*

Bernard Khor  
*Washington University School of Medicine in St. Louis*

Barry P. Sleckman  
*Washington University School of Medicine in St. Louis*

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs

Part of the [Medicine and Health Sciences Commons](https://digitalcommons.wustl.edu/open_access_pubs)

**Recommended Citation**

Mahowald, Grace K.; Mahowald, Michael A.; Moon, Clara; Khor, Bernard; and Sleckman, Barry P., "Out-of-frame T cell receptor beta transcripts are eliminated by multiple pathways In Vivo." PLoS One. 6,7. e21627. (2011).  
https://digitalcommons.wustl.edu/open_access_pubs/502

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.
Abstract

Non-productive antigen receptor genes with frame shifts generated during the assembly of these genes are found in many mature lymphocytes. Transcripts from these genes have premature termination codons (PTCs) and could encode truncated proteins if they are not either inactivated or destroyed by nonsense-mediated decay (NMD). In mammalian cells, NMD can be activated by pathways that rely on the presence of an intron downstream of the PTC; however, NMD can also be activated by pathways that do not rely on these downstream introns, and pathways independent of NMD can inactivate PTC-containing transcripts. Here, through the generation and analysis of mice with gene-targeted modifications of the endogenous T cell receptor beta (Tcrb) locus, we demonstrate that in T cells in vivo, optimal clearance of PTC-containing Tcrb transcripts depends on the presence of an intron downstream of the PTC.

Introduction

Lymphocyte antigen receptor chains are composed of N-terminal variable regions encoded by the first two exons of the antigen receptor gene and C-terminal constant regions encoded by the remaining exons [1]. The second exon of all lymphocyte antigen receptor genes is assembled by the V(D)J recombination reaction, which is initiated when the RAG endonuclease introduces DNA double strand breaks at the border of recombining variable (V), diversity (D) and joining (J) gene segments and their flanking RAG recognition sequences (recombination signals, RSs) [2]. These DNA double strand breaks are repaired by the non-homologous end-joining (NHEJ) pathway, joining the V, D and J gene segments [1]. NHEJ is imprecise and the random gain and loss of nucleotides that accompanies this joining process is essential for antigen receptor gene diversification and adaptive immunity. However, this diversification also leads to reading frame shifts and premature termination codons (PTGs) in two out of the three reading frames. These out-of-frame antigen receptor alleles are actively transcribed in lymphocytes, providing a rich source of PTC-containing transcripts that if not inactivated or destroyed by nonsense-mediated decay (NMD) could lead to the production of truncated antigen receptor peptides that could be deleterious to developing lymphocytes. Consistent with this notion the selective ablation of Upf2, a central mediator of NMD, in thymocytes leads to defects in T cell development, which could be due, in part, to the accumulation of T cell receptor gene transcripts with premature termination codons [3].

In lower eukaryotes, NMD is activated when PTCs are sensed in incompletely spliced, in the nucleus. Moreover, PTC-containing transcripts can be alternatively spliced removing the exon with the PTC, a process termed nonsense-associated altered splicing. How PTCs are sensed in these incompletely processed transcripts is not completely understood. Together, these mechanisms prevent transcripts with PTCs from encoding truncated proteins that could have detrimental effects.

T cell receptor beta (Tcrb) chain locus transcripts containing PTCs are readily destroyed by NMD in vivo [27]. Here, we develop an approach to directly determine the requirement for an intron downstream of the PTC in efficiently clearing transcripts.
Elimination of Out-of-Frame TCRβ Transcripts

Results and Discussion

Generation of the TcrbA and TcrbF alleles

The mouse Tcrb locus spans 0.7 Mb, with 34 Vb gene segments lying in a 0.4 Mb region upstream of two Db-Jb clusters, each with a single Db and 6 or 7 Jb gene segments (Fig. 1a) [28]. The second exon is completed when a Vb gene segment rearranges to a DJb rearrangement at either of the Db-Jb clusters. Four constant exons lie downstream of each Db-Jb cluster (Cb1 and Cb2) (Fig. 1a). VDJb rearrangements to DJb1 are transcribed with the four Cb1 exons; likewise, VDJb rearrangements to DJb2 are transcribed with the four Cb2 exons.

Multi-step gene targeting was used to generate mice with two modified endogenous Tcrb loci (TcrbA and TcrbF, Fig. 1b). Initially, the Db1 gene segment was deleted, limiting rearrangement of Vb gene segments to the Db2-Jb2 gene segment cluster (Step A, Fig. 1a, see Jb1M3 allele in ref. [29]). The Db2, Jb2.1, Jb2.2 and Jb2.3 gene segments were then replaced with a DJb2.3 rearrangement in its native configuration to generate the TcrbA allele (Step B, Figs. 1a and S1). The TcrbA allele is identical to the TcrbF allele except that the four Cb2 exons have been replaced with a DNA fragment containing a fusion of these exons without any introns (Step C, Figs. 1a and S2). Importantly, as the TcrbA and TcrbF alleles were generated through modest gene targeted modifications of the wild type endogenous Tcrb locus, the TcrbA and TcrbF alleles remain under the same cis-acting regulation (endogenous Tcrb promoters and enhancers) as the wild type Tcrb locus.

T cell development in TcrbAA and TcrbFF mice

Efficient assembly and expression of a productive Tcrb chain gene is required for the normal development of T cells. Several lines of evidence demonstrate that rearrangement of both the TcrbA and TcrbF alleles occurs with near normal efficiency. First, in lymph node TcrbAA and TcrbFF T cell hybridomas, complete rearrangements occurred at a high frequency on both the TcrbA and TcrbF alleles (Fig. 2a and Table S1). Moreover, analysis of TcrbAA and TcrbFF T cell hybridomas revealed that 32% and 33%, respectively, have complete VDJb rearrangements on both alleles (Fig. 2b and Table S2). This is close to the maximum 40% expected due to allelic exclusion [29,30]. Finally, analysis of TcrbAF T cell hybridomas with single VDJb rearrangements revealed approximately equal frequencies of VDJb rearrangements on the TcrbA and TcrbF alleles (Fig. 2c and Table S3).

Thymocyte development in TcrbAA and TcrbFF mice was indistinguishable from wild type mice (Fig. 2d and e). In this regard, wild type (TcrbAA), TcrbAA and TcrbFF mice had similar numbers of CD4+CD8– (double negative, DN), CD4–CD8+ (double positive, DP) and CD4+CD8+ or CD4+CD8+ (single positive, SP) thymocytes (Fig. 2d and e). Flow cytometric analysis of Tcrb chain expression revealed no significant differences between TcrbAA, TcrbFF and TcrbAF thymocytes (data not shown). Finally, TcrbAA, TcrbFF and TcrbAF mice have similar numbers of mature CD4+ and CD8+ splenic T cells (Fig. 2f). Taken together these data demonstrate that the TcrbA and TcrbF alleles are efficiently rearranged and expressed and can support normal T cell development.

Differential stability of PTC-containing TcrbA and TcrbF transcripts

Like the wild type Tcrb locus, TcrbA PTCs lie in the third of six exons while TcrbF PTCs lie in the third and final exon; thus, TcrbA PTCs have downstream introns whereas TcrbF PTCs do not

![Diagram of mouse Tcrb locus](image-url)
(Fig. 3a). Notably, TcrbA and TcrbF transcript PTCs lie at the same distance (0.7 kbp) from the poly(A) tract and have two upstream introns in the same locations (Fig. 3a). Thus, comparing the stability of PTC-containing TcrbA and TcrbF transcripts in TcrbA/A and TcrbF/F thymocytes allows us to determine the relative contribution of mechanisms that rely on introns downstream of the PTC in mediating degradation of PTC-containing Tcrb transcripts in vivo.

To this end, TcrbA/A and TcrbF/F CD25+ DN thymocytes were purified by flow cytometric cell sorting and VDJb rearrangements utilizing five different Vb gene segments were amplified and sequenced from genomic DNA and cDNAs generated from both Tcrb pre-mRNAs and mature transcripts. A total of 1592 sequences were analyzed to identify those that are in-frame (no PTC, PTC\(^{-}\)) and those that are out-of-frame (PTC-containing, PTC\(^{+}\)) (Table S4). As compared to genomic DNA, the fraction of PTC\(^{-}\) Tcrb pre-mRNAs was similar to the fraction of PTC\(^{-}\) Tcrb alleles in both TcrbA/A and TcrbF/F DN thymocytes (Fig. 3b, open bars and Table S4). As compared to genomic DNA, there is a twelve-fold reduction in the abundance of PTC\(^{-}\) mature TcrbA mRNAs (Fig. 3b, red bar and Table S4). In striking contrast, the abundance of PTC\(^{+}\) TcrbF mRNAs is reduced by only three-fold (Fig. 3b, red bar and Table S4). Thus, PTC-containing Tcrb\(^{+}\) mRNAs are eliminated more efficiently than PTC-containing Tcrb\(^{+}\) mRNAs.

**Concluding Remarks**

Here, we show that PTC-containing Tcrb\(^{+}\) and Tcrb\(^{+}\) transcripts having differing abilities to be eliminated in mammalian thymocytes in vivo. The only difference between the Tcrb\(^{+}\) and Tcrb\(^{+}\) alleles is the presence of introns downstream of the PTC. Thus, these findings demonstrate that these downstream introns are mechanistically important components in the efficient elimination of PTC-containing Tcrb transcripts in vivo, consistent with the notion that they are required to activate EJC-dependent NMD. Nevertheless, PTC-containing transcripts templated by the Tcrb\(^{+}\) allele are reduced three-fold in their abundance. Thus, mechanisms that do not rely on downstream introns are also capable of eliminating PTC-containing transcripts, although not to levels achieved when downstream introns are present. As transcripts templated by the Tcrb\(^{+}\) allele will have two introns it is conceivable PTC-containing Tcrb\(^{+}\) transcripts can also be inactivated by nonsense-associated altered splicing or other mechanisms that alter the splicing of PCT-containing transcripts. Notably, PTCs in
the endogenous immunoglobulin light chain kappa gene frequently reside in the last exon, and like Tcrbα transcripts in thymocytes, the abundance of PTC-containing immunoglobulin light chain kappa transcripts is also reduced by about three-fold in developing lymphocytes [33]. Embryonic stem cells (ES) were transfected, selected using a binomial test for pre-mRNA versus mRNA, and by Monte Carlo simulation for pre-mRNA and Tcrbα/F (script available on request).

Hybridomas

Hybridomas were generated and Tcrb gene rearrangements analyzed as previously described [23].

Flow cytometric analyses and cell purification

Flow cytometric analyses were performed on a FACSCalibur (BD Biosciences) using FITC-conjugated anti-CD25, PE-Cy5-conjugated anti-CD4 and FITC-conjugated anti-CD8. CD25+ DN thymocytes were purified from 4–5 mice for each genotype by flow cytometric cell sorting (FACSVantage BD Biosciences).

Sequence analyses

Genomic DNA and RNA were isolated as previously described from CD25+ DN thymocytes purified by flow cytometric cell sorting [33,34]. The SuperScriptII Reverse Transcriptase kit (Invitrogen) was used to synthesize cDNA using oligo-dT or the primers, the positions of the two potential PTCs (red octagons) and the normal terminal codon (TC, open octagon) are shown. Pre-mRNAs with exons (green bars) and introns (connecting black lines) are shown, as are completely processed PTC-containing Tcrbα and Tcrbβ transcripts (green bars) with exon junctions (EJ, purple dots). (b) Fold reduction (relative to PTC-containing alleles) in PTC-containing Tcrb pre-mRNA (open bars) and completely processed mRNA (red bars) in Tcrbα/F and Tcrbβ/F DN thymocytes. P-values were calculated by a binomial test for pre-mRNA versus mRNA, and by Monte Carlo simulation for Tcrbα/F versus Tcrbβ/F.

doi:10.1371/journal.pone.0021627.g003

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Washington University Animal Studies Committee (#20070109).

Generation of Tcrbα/F and Tcrbβ/F mice

The 5’ homology arm of pLNTK-DJ (Fig. S1) contains a Db2/Jb2.3 rearrangement amplified with 5’HDJ and 3’Jb2-3 and the Jb2.4-Jb2.7 gene segments amplified with 5’Jb2.4-US and 3’Jb2.7DS (oligonucleotide sequences are listed in Table S3). The Jb2.3 gene segment has a single G to C change to eliminate a PTC generated using the 5’Jb2.3, Jb2.3M1, Jb2.3M2 and 3’Jb2.3X oligonucleotides. The 3’ homology arm is a 3.2 kbp Clal/SpeI fragment downstream of Jb2.7. The 5’ homology arm of pLNTK-Ch2F (Fig. S2) is a 2.0 kbp Clal/PstI Tcrb fragment. The 3’ homology arm was generated by amplifying the constant region of a Tcrb cDNA with oligonucleotides A through F, 5’Ch2-3’H and 3’Ch2-3’H as shown in figure S2. Deletion of the Db1 gene segment is described elsewhere [32]. Embryonic stem cells (ES) were transfected, selected and injected into C57BL/6 blastocysts as previously described [32]. Intercrossing of Tcrbα/F and Tcrbβ/F mice led to the expected Mendelian ratios of Tcrbα/F and Tcrbβ/F mice, respectively.

Southern blotting

Southern blot analysis of ES cells targeted with pLNTK-DJ was performed on SacI-digested genomic DNA with probe A as previously described [33]. For ES cells targeted with pLNTK-Ch2F, PstI-digested genomic DNA was probed with a 400 bp HindIII/BamHI genomic fragment 3’ of the 3’ homology arm, and SacI-digested genomic DNA probed with probe A.

Supporting Information

Figure S1 Targeting strategy for generating the Tcrbα/F allele. Generation of the Tcrbβ/F allele. Shown is a schematic of
part of the Tcrb allele in which the Db1 gene segment has been deleted (top, Jb1\(^{+/-}\) in ref. [20]). The Db and Jb gene segments are shown as open rectangles (except for Jb2.3, shown as a shaded rectangle) and the RSs as open triangles. The Cb1 and Cb2 exons (blue rectangles) are also shown, as is the pLNTK-DJ targeting vector used to generate the Tcrb\(^{4+/-}\) allele, which has a targeted replacement of the Db2, Jb2.1, Jb2.2 and Jb2.3 gene segments with a Db2/Jb2.3 rearrangement and the loxP-flanked neomycin resistance gene (Neo\(^{K}\)). The Tcrb\(^{4}\) allele generated after Cre-mediated deletion of the neomycin resistance gene, leaving a single loxP site (filled oval), is shown. The relative positions of the different restriction sites are shown, as is probe A, which was used for Southern blot analysis of the different targeted alleles. Also shown is a Southern blot of genomic DNA from targeted ES cell lines digested with SaeI and hybridized to probe A. The molecular weight markers and relative positions of the bands generated by the different Tcrb alleles are indicated.

**Figure S2 Targeting strategy for generating the Tcrb\(^{F}\) allele.** a) Generation of the Cb2 fusion. Shown are schematics of the four Cb2 exons (labeled 1 through 4) in genomic DNA and in cDNA. b) Generation of the Tcrb\(^{F}\) allele. Shown is a schematic of the Cb2 region of the Tcrb\(^{4}\) allele (top) and the pLNTK-Cb2F targeting vector used to generate the Tcrb\(^{4+/-}\) allele, which has a targeted replacement of the four Cb2 exons with a DNA fragment containing a fusion of these exons and the loxP-flanked neomycin resistance gene. Also shown is the Tcrb\(^{F}\) allele generated after Cre-mediated deletion of the neomycin resistance gene, leaving a single loxP site. The relative positions of the different restriction sites are shown, as are probes A and B, which were used for Southern blot analyses. Southern blots of genomic DNA from targeted ES cell lines that were digested with PstI and hybridized to probe B, or digested with SalI and hybridized to probe A are shown. The molecular weight markers and relative position of the bands generated by the different Tcrb alleles are indicated.

**Table S1** Number of Tcrb\(^{4}\), Tcrb\(^{F}\) and Tcrb\(^{F}\) alleles in the VDJb configuration in the Tcrb\(^{A+/-}\) and Tcrb\(^{F+/-}\) T cell hybridomas analyzed. The total number (n) of hybridomas analyzed is indicated.

**Table S2** Number of Tcrb\(^{A+/-}\) and Tcrb\(^{F+/-}\) T cell hybridomas with Tcrb alleles in the VDJb/DJb and VDJb/VDJb configuration.

**Table S3** Number of Tcrb\(^{A+/-}\) T cell hybridomas with Tcrb alleles in the VDJb\(^{A+/-}\)DJb\(^{A+/-}\) and DJb\(^{A+/-}\)/VDJb\(^{A+/-}\) configuration.

**Table S4** Total number of sequences (n) and the number with (PTC\(^{+}\)) or without (PTC\(^{-}\)) PTCs from genomic DNA, pre-mRNA and mRNA from Tcrb\(^{A+/-}\) and Tcrb\(^{F+/-}\) DN thyromyces.

**Table S5** Oligonucleotide sequences.

**Acknowledgments**

We thank Dr. Eugene Oltz for critical review of the manuscript.

**Author Contributions**

Conceived and designed the experiments: BPS GKM BK. Performed the experiments: GKM MAM CM. Analyzed the data: GKM MAM BPS. Wrote the paper: BPS GKM.

---

**References**


