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Title: The Yin and Yang of RNA surveillance in B lymphocytes and antibody-secreting plasma cells

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ABSTRACT

Random V(D)J junctions ensure that the diversity of the primary immunoglobulin (Ig) repertoire is adapted to the huge heterogeneity of antigens. In two thirds of cases, imprecise recombination between variable (V), diversity (D) and joining (J) segments induces a frameshift in the open reading frame that leads to the appearance of premature termination codons (PTCs). Thus, many B lineage cells harbor biallelic V(D)J-rearrangements of Ig heavy or light chain genes, with a productively-recombined allele encoding the functional Ig chain and a nonproductive allele potentially encoding truncated Ig polypeptides. Since the pattern of Ig gene expression is mostly biallelic, transcription initiated from nonproductive Ig alleles generates considerable amounts of primary transcripts with out-of-frame V(D)J junctions. How RNA surveillance pathways cooperate to control the noise from nonproductive Ig genes will be discussed in this review, with regard to the benefits of nonsense-mediated mRNA decay (NMD) activation during B-cell development and to the detrimental effects of nonsense-associated altered splicing (NAS) in terminally differentiated plasma cells.

INTRODUCTION

To ensure the fidelity of gene expression and prevent translation of truncated proteins, many cellular RNA surveillance pathways have been developed to distinguish between normal transcripts and those harboring premature termination codons (PTCs). RNA surveillance mechanisms have been extensively studied in lymphoid cells that naturally acquire PTCs at high frequencies during the error-prone V(D)J recombination process [1]. Through the analysis of nonproductive (PTC+) immunoglobulin (Ig) and T-cell receptor (TCR) transcripts, it has been demonstrated that transcriptional silencing, splicing inhibition or suppression (SOS), alternative splicing and mRNA degradation cooperate to limit the amount of potentially deleterious truncated proteins [2–12].

The nonsense-mediated mRNA decay (NMD) pathway is very active in lymphoid cells and ensures rapid degradation of PTC-containing mRNAs [1,9,13]. The nearly complete absence of PTC+ Ig or TCR mRNAs, due to their strong NMD degradation, leads to the assumption that transcription of PTC+ alleles is safe. Accordingly, nonproductive alleles are often considered as passengers, in comparison to the driving effects of productively-rearranged Ig or TCR alleles during lymphoid development. Whereas benefits of NMD activation are clearly established in lymphoid cells [14,15], less is known about the impact of nonsense-associated altered splicing (NAS) with regard to the production of truncated Ig or TCR polypeptides. In this review, we summarize our current knowledge on the opposite effects of NMD and NAS during B cell development. This paradox will be discussed in line with our recent findings showing that NAS events can lead to the production of truncated Ig κ polypeptides that blunt plasma cell (PC) differentiation [16]. Challenging the classical antigen-driven PC differentiation model, this new PC checkpoint suggests that the expression of PTC+ Ig genes can sometimes be disabling.

THE GENERATION OF ANTIBODY REPERTOIRE: A RISKY DIVERSITY**The error-prone V(D)J recombination process**

Ig genes are good candidates to study nonsense RNA surveillance because the generation of primary Ig repertoire in early B-cell development and the process of somatic hypermutations (SHM) in germinal center B cells frequently generate PTCs [1].

V(D)J recombination of Ig genes takes place in the bone marrow and assembles the variable region from germline variable (V), diversity (D), and joining (J) gene segments. Control of V(D)J recombination occurs at several levels including cell-type specificity, intra- and inter-locus sequential rearrangements, and allelic exclusion [17]. Although DNA rearrangements in the Ig heavy (IgH) and light (*i.e.* Igκ and Igλ) loci occur in a precise order, the selection of gene segments within each locus is random which allows for combinatorial diversity. The mechanism used by lymphoid cells to successfully rearrange their antigen (Ag) receptor genes requires the use of recombinase enzymes RAG1 & RAG2, which are only active in lymphocytes [18,19]. Recombinases act at early stages of lymphoid cell development in order to bring two segments into close proximity, forming a loop of intervening DNA which can then be excised. The ends of the segments are annealed to form a newly rearranged DNA sequence. To increase diversity, joining of V, D and J segments is imprecise and contains nucleotide deletions or insertions. Non-template (N) nucleotide additions are introduced by Terminal deoxynucleotidyl Transferase (TdT) and palindromic (P) insertions occur after asymmetric hairpin opening. Random N-additions cannot be attributed to any other genomic sequences and rarely exceed a dozen nucleotides [20,21]. They are polymerized by TdT which is, with RAG1 and RAG2, the third lymphoid-specific protein involved in V(D)J recombination [22–25]. P insertions rarely exceed two nucleotides and form a palindrome with respect to the sequence at the end of the coding strand [26–28]. Although nucleotide deletions and insertions greatly enlarge the diversity of the Ig repertoire, only one third of all

V(D)J junctions are in-frame, with the other two thirds being out-of-frame due to frameshift mutations that create PTCs.

Frequency of PTC-containing Ig genes in B-lineage cells

Clonal selection implies that each B cell clone expresses a unique receptor, and hence that one of the two inherited Ig alleles is functionally rearranged. This allelic exclusion associates asynchronous V(D)J recombination events at Ig loci, and receptor-mediated inhibitory feedback control [29]. At the pro-B cell stage, VDJ recombination is initiated by biallelic D to J rearrangements at IgH loci, followed by a monoallelic V to DJ recombination. A productive VDJ junction encodes the variable (V) region of the μ heavy chain that can associate with the surrogate light chain to form the pre-BCR (pre-B cell receptor). Regulatory mechanisms mediated by pre-BCR signaling prevents further V to DJ rearrangements on the second IgH allele and initiates VJ recombination at Ig light chain loci lacking D segments. By contrast, when the VDJ junction on the first IgH allele is nonproductive, the lack of the pre-BCR inhibitory signal allows V to DJ recombination on the second allele. If this second attempt is successful, a pre-BCR-mediated proliferation wave generates abundant B cell clones with biallelic VDJ rearrangements. Roughly half of mature B cells harbor biallelic VDJ-rearrangements with a nonproductively-recombined IgH allele [30–32]. If another nonproductive VDJ junction occurs on the second IgH allele, cells are eliminated through apoptosis. As mentioned above, pre-BCR signaling stimulates recombination of Ig light chain genes. The presence of two Ig κ and Ig λ light chain isotypes permits multiple VJ recombination events. Again, expression of a functional BCR precludes further recombination in immature B cells. In humans, ~50% of mature B cells express Ig λ whereas in mice, recombination takes places preferentially at the Ig κ locus and only 5% of B cells express Ig λ .

isotypes [32]. Hence, B cells harbor numerous nonproductive VJ-recombined Ig light chain alleles (Figure 1).

After their exit from bone marrow, alternative splicing of constant $C\mu$ and $C\delta$ exons ensures the co-expression of IgM and IgD at the surface of naïve B cells [33]. Upon antigen encounter, IgD expression is downregulated and activated B cells can be subjected to a second wave of Ig gene diversification by SHM in germinal centers (GCs). Frequent nonsense mutations can arise during this affinity maturation process that requires transcription of the target region and enzymatic activity of the B-cell-specific activation-induced deaminase AID [34]. This process leads to the introduction of multiple nucleotide changes in the V exon (*i.e.* VDJ or VJ) and few hundred base pairs in the downstream intron [35]. Nucleotide insertions and deletions (indels) have been also observed [36,37]. SHM leads to the expression of a secondary repertoire from which those B cells carrying a mutated BCR with improved Ag-binding affinity can be selected [38]. We previously observed that SHM occurs at similar levels on productive and nonproductive VDJ-rearranged IgH alleles [39]. If a nonsense codon appears on the productive allele, the lack of Ag-binding activity provokes a rapid elimination of the mutated B cell clones within GCs [40–42]. The occurrence of SHM on nonproductive Ig alleles can introduce additional nonsense codons, modifying the PTC position within the V exon. Class switch recombination (CSR) also occurs in germinal centers and this second round of IgH intragenic rearrangements replaces the $C\mu$ exons with a downstream constant gene [43]. GC B cells can differentiate into memory cells, or terminally differentiated plasma cells (PC) secreting substantial amounts of antibody [44]. The PC transcriptional program induces major changes including a transcriptional boost of Ig gene transcription and activation of the unfolded protein response (UPR) to ensure proper Ig folding [45]. In PCs, the use of secreted polyadenylation signal (PAS) instead of downstream membrane PAS allows

alternative IgH pre-mRNA processing in order to switch from membrane to secreted Ig isoforms [46].

Taken together, the vast majority of B-lineage cells harbor PTC⁺ Ig alleles in their genome with nonsense codons introduced in the V exon or in the adjacent constant exon, during the V(D)J recombination or SHM (Figure 1).

Transcriptional control of PTC-containing Ig genes

The high frequency of PTC⁺ V(D)J-rearranged Ig alleles in B-lineage cells needs additional mechanisms in order to downregulate these nonsense transcripts. A transcriptional silencing of PTC⁺ Ig genes has been proposed as a primary mechanism preventing their expression. This mechanism called “nonsense-mediated transcriptional gene silencing” (NMTGS) involves chromatin remodelling and “heterochromatinization” of the PTC⁺ DNA sequence. NMTGS can be inhibited by the overexpression of exonuclease but the involvement of siRNA like molecules has not yet been elucidated [4]. NMTGS is also impaired upon knock-down of the main NMD factor UPF1, suggesting a mechanistic link between NMD and NMTGS [47]. Although NMTGS has been demonstrated in Hela cells transfected with minigene constructs, the occurrence of such a quality control mechanism needs to be determined in B cells. Instead of active silencing, many studies including ours have shown a biallelic transcription pattern for productive and nonproductive Ig alleles in B cells [9,39,48–51]. To study the transcription and RNA surveillance of PTC⁺ IgH alleles during B cell development, we introduced a nonsense V exon in the IgH locus to specifically mark each allele in heterozygous mutants. Consistent with previous observations in a pro-B cell line [52], productive and nonproductive IgH alleles exhibit equivalent transcription rates with similar RNAPII loading in LPS-stimulated B cells [9,48]. This also confirms our earlier study in germinal center B cells

demonstrating that the frequency of transcription-dependent SHM is similar for productive and nonproductive VDJ-recombined IgH alleles (Delpy JI 2004).

RNA SURVEILLANCE IN B-LINEAGE CELLS

The machinery of RNA surveillance is now known to include various pathways controlling the quality of pre-mRNA and mRNA to limit the translation of truncated proteins. The molecular mechanisms involved in the activation of RNA surveillance pathways have been extensively reviewed in the literature [13,53,54].

Accumulation of PTC-containing Ig pre-mRNAs

Despite their active transcription, PTC⁺ Ig genes can be controlled at the pre-mRNA level. Previous studies performed in Milstein's lab have shown an accumulation of PTC⁺ Igκ unspliced or splicing intermediate RNA precursors, supporting the idea that nonsense codons can be recognized in the nucleus by a mechanism independent of protein synthesis [2,6]. In the simplified model of Igκ transcripts containing only three exons, splicing intermediates have eliminated the first intron (IVS1) but retained the intervening sequence between VJ and Cκ exons (IVS2). Upon transfection of B cell lines spanning the main developmental stages with PTC⁺ and PTC-free Igκ minigenes, we observed that the presence of PTC induced a ~2-fold increase for each unspliced and splicing intermediate RNA precursors [5]. Again, unspliced and partially spliced Igκ RNAs were mainly confined in the nucleus and the accumulation of PTC⁺ precursors was not affected by protein synthesis inhibitors [5]. In agreement with these data, RNA-FISH experiments performed in Sp6-derived hybridoma cells further demonstrated that PTC⁺ Ig-μ pre-mRNAs can accumulate near the site of transcription [55]. Recent findings obtained in *Drosophila* indicate that UPF1 can bind to nascent RNAs at RNAPII transcription sites, facilitating nuclear processes of gene expression

[56]. It has been demonstrated that UPF1 is needed for the release of poly(A) mRNAs from chromosomal transcription sites and for their export from the nucleus. It is tempting to speculate that these new nuclear scanning functions of UPF1 could be involved in the nuclear accumulation of nonproductive Ig RNA precursors or in NAS (see below). Interestingly, fluctuations in the steady state level of PTC⁺ IgH pre-mRNAs occurs during B cell development, with low accumulation in PCs compared to resting B cells [9]. These observations indicate that a high level of transcription precludes splicing inhibition and accumulation of PTC⁺ Ig pre-mRNAs. Thus, the efficient processing of PTC⁺ Ig transcripts in PCs needs additional RNA quality control to prevent the synthesis of truncated Ig polypeptides.

NMD of PTC-containing Ig mRNAs

NMD is tightly coupled to translation and the molecular mechanisms of the two prevailing exon-junction complex (EJC)-dependent and PTC-PABPC1 (Poly(A) Binding Protein Cytoplasmic 1) distance-dependent NMD activation models have been extensively characterized in the past decades [1,13,53,54,57,58].

According to the “50-55 nucleotide rule” for EJC-dependent NMD activation, the presence of a PTC more than 50-55 nucleotides upstream from the last exon-exon junction triggers efficient mRNA degradation by NMD. This mode of NMD activation is elicited to downregulate the level of nonproductively-rearranged IgH mRNA that contains PTCs in the V or in the first constant exon (CH1) and several constant exons downstream. Up to 100-fold accelerated degradation of these NMD candidates has been documented in B cell lines [59–61]. Consistent with a previous study using TCR β as gene models [62], we found that the extent of NMD was closely correlated to the splicing rate during B-cell development, with ~2 to 20 fold degradation of PTC⁺ IgH mRNAs in resting and LPS-stimulated B cells,

respectively [9]. Interestingly, the levels of nonproductive IgH mRNAs remained constantly low in those B cell populations, suggesting compensatory effects between nonsense-mediated splicing inhibition and NMD. Hence, qualitative changes in the RNA surveillance machinery occur during B-cell development and the cooperation between RNA surveillance mechanisms controls the amount of PTC+ IgH mRNAs.

For Igk alleles, the situation is completely different because a frameshift at the VJ junction leads to the appearance of a PTC within the last Ck exon, or less frequently at 3 nucleotides upstream from the last exon-exon junction [50]. Thus, the position of PTC on nonproductive Igk mRNAs does not meet the requirements for EJC-dependent NMD but rather belongs to the PTC-PABPC1 distance-dependent NMD activation model. The latter pathway occurs when the 3'UTR of an mRNA is abnormally long, as in the case of the presence of a PTC (Lejeune, 2017). NMD is activated when the 3'UTR structure precludes the normal interaction of PABPC1 with the release factors (eRF1 and eRF3) involved in translation termination. The spatial rearrangement of 3'UTR controlling the proximity of the termination codon and the poly(A) tail is a critical determinant for NMD [63]. Although UPF1 was previously assumed to be the essential player involved in the interaction with the release factors (eRFs), *in vitro* translation termination assays recently performed by Neu-Yilik et al. indicate that UPF3B (also named UPF3X) directly interacts with both RNA and eRF3 [64,65]. This NMD mechanism is most likely involved in the uptake of nonproductive Igk mRNAs, inducing a modest (~2-fold) NMD degradation in mouse B cells [5] (Figure 1).

NAS of PTC-containing Ig transcripts and production of deleterious truncated Ig polypeptides in plasma cells

Mutations within the sequence of exonic splicing enhancers (ESEs) and the presence of a PTC have been shown to elicit alternative splicing called class-I and class-II NAS, respectively

[8,11–13,66,67]. As an additional RNA surveillance pathway, class-II NAS (hereafter referred as NAS) can prevent the maturation of full length PTC-containing mRNAs by promoting alternative splicing to skip the offending PTCs. However, NAS can yield internally deleted mRNAs and proteins, and hence, exhibits opposite effects compared to NMD with regard to the production of truncated proteins. Although NMD and NAS use the common factor UPF1, these processes are mechanistically different and knockdown of several key NMD factors including UPF2, UPF3A, UPF3B and SMG1 showed no significant effects on NAS [13]. The intrinsic mechanisms responsible for the activation of NAS remain elusive and conflicting results have been obtained with regard to the sensitivity of NAS to translation inhibition or to frameshift mutations [5,13]. Future breakthroughs would indicate whether NAS, as well as nonsense-mediated SOS or splicing inhibition, are induced as a response to PTC recognition during translation, after nuclear degradation of spliced PTC-containing mRNAs, or by other nuclear-scanning mechanisms.

To study NAS of Igk transcripts, we transfected several B-cell lines with minigenes harboring frameshift mutations at the VJ junction that lead to the appearance of PTCs at the end of the V exon (V^{PTC}), or within the last C κ exon (C^{PTC}) [5,16]. Consistent with a reading frame-dependent NAS activation, the presence of V^{PTC} , but not of C^{PTC} , strongly induced skipping of the V exon. We also confirmed that V exon skipping occurs in mouse B cells, during splicing of both nonproductive Igk and IgH transcripts [5,16,48]. Interestingly, we found that skipping of the PTC-containing V exon was greatly increased in PCs compared to B cells, and this was correlated with a transcriptional boost of Ig genes during PC differentiation [48]. Thus, a fast RNAPII elongation rate enhances skipping of PTC-containing V exons in PCs, whereas a slow elongation rate authorizes their splicing as full-length mRNAs in B cells (or splicing inhibition as mentioned before). These observations are in agreement with the known

influence of RNAPII elongation rate on alternative splicing [68], suggesting that PTC-independent and PTC-dependent exon skipping events can be governed by some similar rules. Until recently, the consequences of NAS with regard to the production of truncated Ig chains have been overlooked. Interestingly, we observed that exon skipping of V^{PTC} Ig κ pre-mRNAs encodes V domain-less κ light chains (Δ V- κ LCs) that induce the death of PCs through endoplasmic reticulum (ER) stress-associated apoptosis [16]. Revealing a new PC checkpoint referred to as Truncated Immunoglobulin Exclusion (TIE)-checkpoint, the production of Δ V- κ LCs dampens PC differentiation by eliminating cells expressing nonproductively-rearranged V^{PTC} Ig κ alleles (Figure 2). In addition, the conditional expression of Δ V- κ LC mRNAs in ‘inducible-TIE’ (iTIE) knock-in mice reproduces the physiological TIE checkpoint and affects the survival of long-lived PCs and antibody production [16]. Thus, the transcription of nonproductive Ig alleles is not as safe as previously assumed and activation of NAS can drive the elimination of PC clones harbouring biallelic Ig κ rearrangements. Remarkably, the TIE-checkpoint reduces the magnitude of humoral responses and shapes the antibody repertoire independently of the classical constraints related to Ag specificity.

Closing remarks

Aberrantly rearranged Ig alleles are abundant in B-lineage cells but the cooperative action of nonsense-mediated splicing inhibition (or SOS) and NMD limits the amount of deleterious truncated Ig polypeptides. However, activation of the NAS pathway exerts opposite effects with exon skipping-mediated production of V domain-less Ig chains. In PCs with biallelic Ig κ rearrangements, the good news from the transcriptional boost of Ig genes is that it sustains massive Ig synthesis, but the bad news is that it also promotes NAS activation and exon skipping during splicing of nonproductive transcripts. Because the survival of normal and malignant PCs is tightly controlled by ER stress and proteasome activity [69], high amounts

of truncated Ig provoke PC death as a consequence of a deregulated proteostasis. According to an efficient TIE-checkpoint, alternatively spliced mRNAs encoding structurally abnormal Ig chains are hardly detectable in non-malignant PCs and can only be observed in some lymphoproliferative disorders, including Multiple Myeloma and Burkitt lymphoma [70–72]. Previous findings indicate that activation of the TIE checkpoint eliminates ~20–25% of PCs, *i.e* those expressing nonproductive V^{PTC} Igκ alleles [16]. If a similar disappearance of PC harboring nonproductive VDJ rearrangements is observed for the IgH repertoire, this would indicate a broad TIE-checkpoint blunting the terminal differentiation of many PCs with biallelic IgH and/or IgL rearrangements. Altogether this should have major consequences in our classical mindset usually considering nonproductively V(D)J-rearranged Ig alleles, as passengers rather than drivers.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1: Abundance of nonproductive V(D)J rearrangements in B-lineage cells

(A) Schematic representation of productive and nonproductive V(D)J rearrangements during the generation of primary antibody repertoire. V(D)J recombination is initiated by a monoallelic V to DJ recombination at the IgH locus (biallelic D-J rearrangements are not depicted). If successful, then V to J recombination occurs at Ig light chain (IgL) loci. Successive IgL rearrangements are possible due to the fact that there are two Igκ and two Igλ alleles (not depicted). Pre-B cell receptor (pre-BCR) or BCR-mediated feedback signalling upon in-frame rearrangement of one IgH or IgL allele (*i.e.* VDJ⁺ or VJ⁺) prevents V(D)J recombination on the second allele [32]. By contrast, a nonproductive V(D)J recombination on one Ig allele (*i.e.* VDJ⁻ or VJ⁻) induces rearrangement on the second allele. The imprecise nature of V(D)J junctions generates ~1/3 of productive and ~2/3 of nonproductive V(D)J-rearranged alleles. Hence, most B-lineage cells harbor nonproductively-recombined Ig alleles in their genome (red parts in pie charts). If, the two attempts on both Ig alleles are unsuccessful, the cell is programmed to die by apoptosis (dashed circles). (B) PTCs introduced during the error-prone V(D)J recombination process (red stars) or by somatic hypermutations (SHM; yellow stars) can activate different modes of NMD degradation. Frameshift V(D)J junctions can lead to the appearance of PTCs in the variable (V) exon or in the downstream adjacent constant exon. SHM can lead to the appearance of PTCs in the first leader exon (L₁: L-part1) or in the V exon, with a greater abundance in the complement-determining regions (CDRs). For IgH mRNAs, PTC introduced by SHM or during V(D)J recombination elicit exon junction complex (EJC)-dependent NMD. EJCs that remain bound to mRNAs after a pioneer round of translation are depicted (blue ovals). As good NMD candidates, PTC-containing IgH mRNAs are strongly degraded by NMD (up to 100-fold) [1,9,59]. However, it has been demonstrated that some nonsense codons in the 5' half of the VDJ exon did not elicit strong NMD degradation [73]. Similarly, PTC close to the initiation codon are NMD resistant in other models, likely due to a critical interaction between PABPC1

with the translation initiation complex [74,75]. For nonproductive Igk alleles, PTCs are located at the end of the V exon or within the last constant C κ exon. Hence, these PTC-containing IgL mRNAs does not elicit EJC-dependent NMD degradation but are likely targeted by a PTC-PABC1 distance-dependent mode of NMD, which induces a less efficient degradation (~2-fold) [5].

Figure 2: A Truncated Immunoglobulin Exclusion (TIE) checkpoint during terminal differentiation plasma cell differentiation

(Top) Schematic structure of the Igk locus showing the various classes of V-J rearrangements. In-frame and out-of-frame VJ junctions with the position of PTCs from the latter are depicted. Any VJ recombination involving mouse Jk1 segments leads to the appearance of PTCs in the last constant exon (C κ). For other Jk segments, the addition of 1 nucleotide (nt) (*i.e.* $3n + 1$ nt) at the VJ junction also leads to the appearance of a PTC in the C κ exon, whereas the addition of 2 nt (*i.e.* $3n + 2$ nt) creates a PTC at the end of the variable (V) exon, 3nt upstream the exon-exon junction [50]. (Middle) Whereas nonsense-associated altered splicing (NAS) is not activated by the presence of a PTC within the C κ exon (C^{PTC} class), PTCs in the V exon (V^{PTC} class) strongly promote exon skipping and translation of V domain-less κ light chains (Δ V- κ LCs) [5,16]. (Bottom) The production of Δ V- κ LCs is innocuous in B cells but provokes endoplasmic reticulum (ER) stress-associated apoptosis in plasma cells (PCs), precluding they differentiation as long-lived PCs. Overall, significant numbers of PCs harboring biallelic VJ recombination, in a V^{PTC}/VJ⁺ configuration, at the Igk locus are eliminated through activation of a Truncated Immunoglobulin Exclusion (TIE) checkpoint [16]. The TIE checkpoint exhibits dual effects on antibody responses: i) on one hand, its activation blunts the PC repertoire that can emerge from mature B cells, thus limiting the extent of antibody responses, and ii) on the other hand, the TIE-checkpoint favors the selection of long-lived PCs with

limited basal ER stress that support further elevation of Ig secretion. (GL: germline; VJ+: productively-recombined Ig κ allele; V^{PTC}: nonproductively-recombined Ig κ allele harboring a PTC within the V exon; C^{PTC}: nonproductively-recombined Ig κ allele harboring a PTC within the C κ exon)

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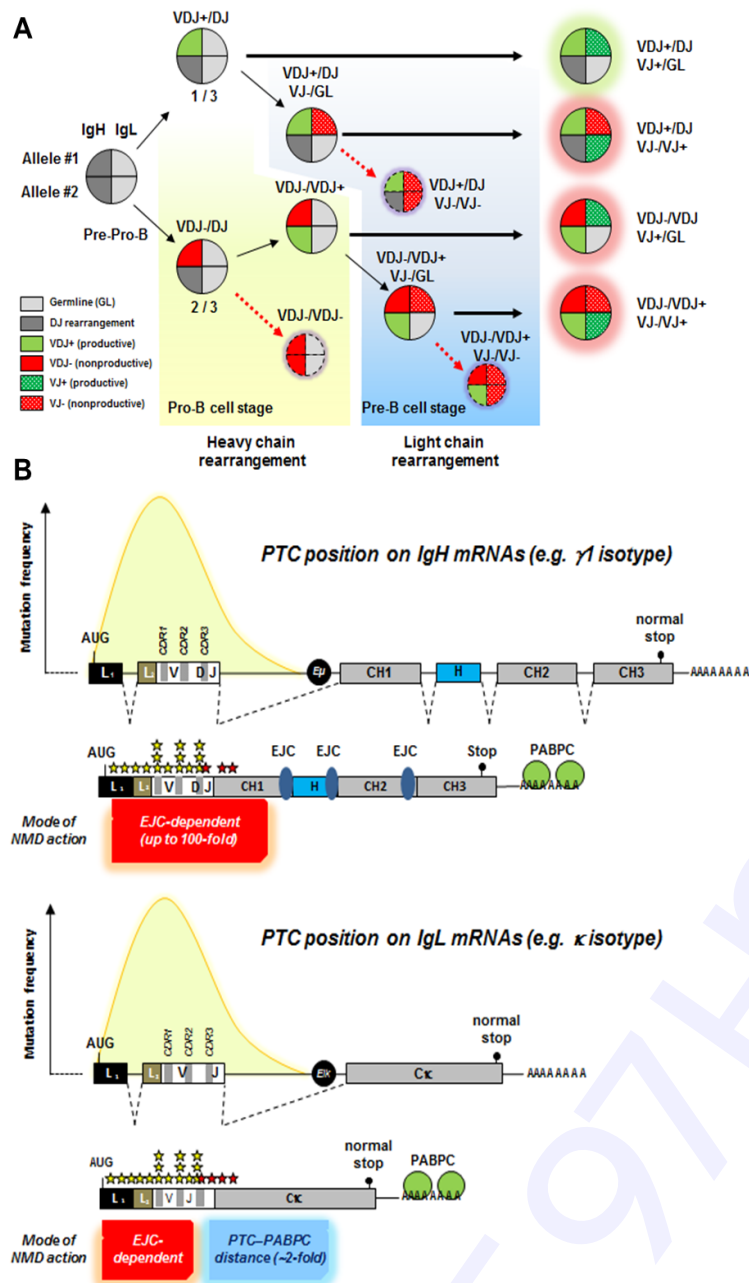


Fig. 1. Figure 1

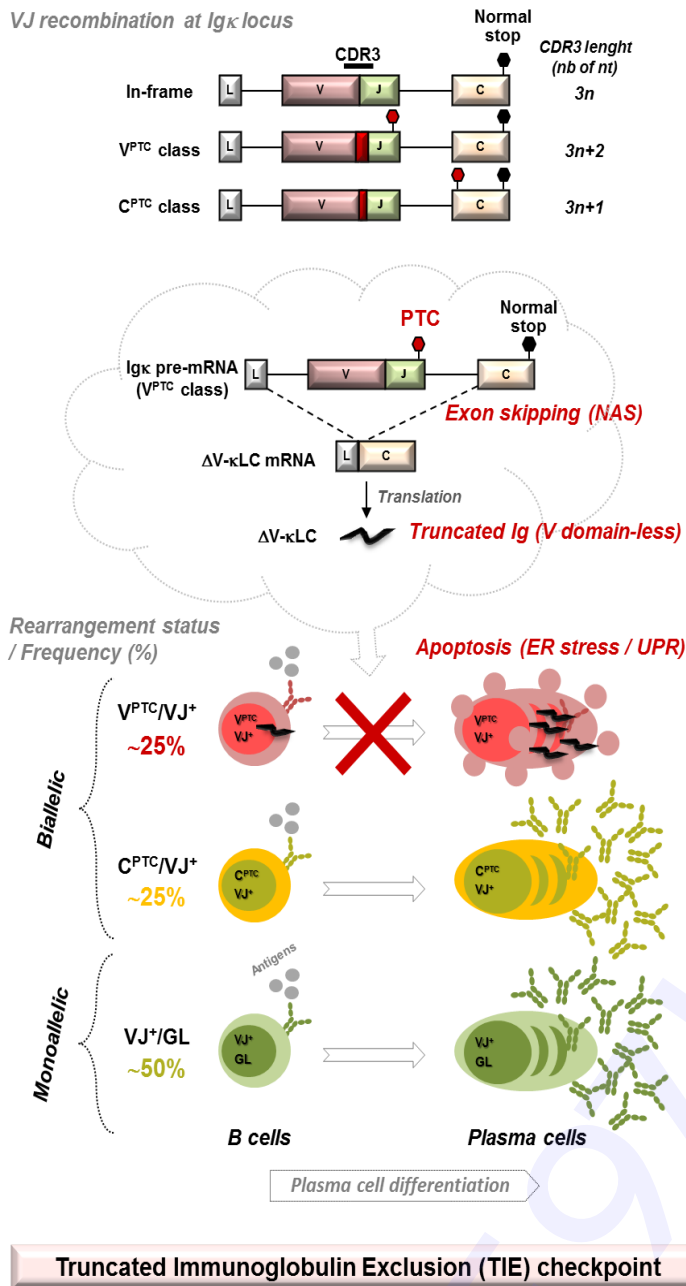
VJ recombination at Ig κ locus

Fig. 2. Figure 2