Allelic exclusion of immunoglobulin genes: models and mechanisms

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Summary

The allelic exclusion of immunoglobulin (Ig) genes is one of the most evolutionarily conserved features of the adaptive immune system and underlies the monospecificity of B cells. While much has been learned about how Ig allelic exclusion is established during B-cell development, the relevance of monospecificity to B-cell function remains enigmatic. Here, we review the theoretical models that have been proposed to explain the establishment of Ig allelic exclusion and focus on the molecular mechanisms utilized by developing B cells to ensure the monoallelic expression of Igκ and Igλ light chain genes. We also discuss the physiological consequences of Ig allelic exclusion and speculate on the importance of monospecificity of B cells for immune recognition.

Keywords

Monospecificity of B lymphocytes and Ig allelic exclusion

Since Burnet's clonal selection theory of the adaptive immune system, the monospecificity of B lymphocytes has been a central paradigm in explaining the pathogen-specific production of antibodies (1). This paradigm, also known as the 'one B cell – one antibody' rule, is supported by a great body of experimental evidence (2-6). According to Burnet's theory, antibodies displayed as B-cell antigen receptors (BCRs) at the surface of a single B cell contain only one particular antigen-binding site, allowing for the clonal selection of antibody-producing cells by their respective pathogen-associated antigens. Ideally, the monospecific expression of BCRs by B cells and the highly specific BCR/antigen interaction result in an antibody response that targets the pathogen, while avoiding wasted resources and collateral damage.

The genetic basis of monospecificity of B cells is the allelic exclusion of immunoglobulin (Ig) heavy (H) and light (L) chain genes. Studies over the past decades demonstrated that allelic exclusion of Ig genes is established during the rearrangement of V, (D), and J gene segments that encode the variable region of the antibody molecule – a process termed V(D)J recombination. Due to the imprecise and random nature of V(D)J recombination, only a fraction of the resulting Ig genes is functional, i.e. they contain a productive V(D)J exon (in the correct reading frame) that encodes a pairing Ig chain that can be assembled into a surface-expressed BCR. While the somatic generation of functional Ig genes by V(D)J

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recombination is subject to allelic exclusion, the expression of Ig loci *per se* does not appear to be monoallelic. This is best illustrated by genetically modified mice that carry two different, fully recombined functional IgH alleles and give rise to allelically included B cells, thus demonstrating the principal ability of B cells to express Ig heavy chains (HCs) from both alleles (7).

Ig genes markedly differ from other monoallelically expressed genes, such as X-chromosomal genes, the odorant receptor genes (8,9), the interleukin-4 (IL-4) gene (10), the Ly49 natural killer (NK) cell receptor gene (11), the Toll-like receptor-4 (TLR4) gene (12), and the H19/insulin growth factor (Igf) 2 genes (13,14), all of which are regulated by monoallelic silencing mechanisms (Fig. 1). Monoallelic silencing leads to the exclusive expression of transcripts from only one of several alleles which is chosen either stochastically or through parental origin ('genetic imprinting'). The expression of the other allele(s) is suppressed by a variety of epigenetic mechanisms (reviewed in 15).

In contrast, Ig transcripts are expressed from both alleles; yet under normal circumstances, only one of the two Ig alleles is functional, as defined above. To facilitate allelic exclusion, the second allele is kept or rendered non-functional for any of the three following reasons (Fig. 1). (i) The non-functional allele is unrearranged and thus produces only sterile germline transcripts. (ii) The non-functional allele is incompletely rearranged ($D_H J_H$) or non-productively rearranged [out-of-frame V(D)J exon] and thus produces only transcripts encoding a truncated Ig chain. In addition, transcripts from non-productively rearranged Ig alleles usually contain a premature stop codon and thus are degraded by the nonsense codon-mediated mRNA decay (NMD) pathway. (iii) The non-functional allele is productively rearranged but encodes only a non-pairing (dysfunctional) Ig chain, i.e. one that cannot be assembled into a surface-expressed BCR or antibody molecule.

In summary, monospecificity of B cells is effected by limiting the number of functional Ig alleles to one per B cell. This unique characteristic separates Ig allelic exclusion from other modes of monoallelic gene expression. In this article, we review the models that have been proposed to explain the establishment of Ig allelic exclusion during B-cell development. We then discuss the mechanisms that regulate V(D)J recombination to bring about the allelic exclusion of Igκ and Igλ light chain genes. Finally, we speculate on the relevance of monospecificity to B-cell function within the adaptive immune system.

Ordered rearrangement of Ig genes during B-cell development: an overview

The variable portions of Ig genes are assembled through V(D)J recombination during early B-lymphocyte development in the bone marrow. The process of V(D)J recombination results in the random selection of single V, (D), and J segments from large pools of gene segments and additionally generates imprecise coding joints, thereby establishing diversity in the antibody repertoire. V(D)J recombination is mediated by the lymphocyte-restricted recombination-activating gene (RAG) 1 and 2 proteins, which cleave recombination signal sequences (RSSs) that flank the rearranging gene segments (reviewed in 16). RSSs consist of a conserved nonamer and heptamer sequence, separated by a spacer of either 12 or 23 nucleotides in length. Only gene segments with RSSs of dissimilar spacer length can be joined by RAG. This restriction is known as the 12/23 rule and instructs IgH rearrangement by preventing direct V_H to J_H joining, since these two gene segments are flanked by RSSs of similar spacer length. To join Ig gene segments, RAG proteins need to collaborate with additional enzymes, in particular with the DNA endonuclease artemis and the factors of the non-homologous end joining (NHEJ) DNA repair pathway (ligase IV, Ku70/80, and XRCC4).

The tightly restricted access of the RAG proteins to RSSs within chromatin structure is widely accepted to be responsible for the lineage- and developmental stage-specific regulation of V(D)J recombination [referred to as the accessibility hypothesis (17)]. Limited RSS accessibility explains why complete Ig gene rearrangements occur only in developing B cells that fully activate the Ig chromatin, even though RAG is expressed in both T- and B-lineage cells (18). The rearrangement of IgH, Igκ, and Igλ genes in B-lineage cells follows a relatively strict developmental order (19-23): Early in B-cell development, pro-B cells activate the IgH locus and first recombine D_H and J_H segments. This is followed by recombination of a V_H segment to generate a complete $V_H D_H J_H$ exon that encodes the variable region of a HC protein. While D_H-to-J_H recombination occurs on both IgH alleles, the subsequent V_H-to-$D_H J_H$ joining step occurs on only one allele, and thus is allelically excluded. Only in the event that the $V_H D_H J_H$ exon on the first allele is non-productive or encodes a non-pairing HC does the cell rearrange the second IgH allele.

Pro-B cells with a productive (in-frame) $V_H D_H J_H$ exon express a μHC that is tested for functionality by pairing with the surrogate light chain (SLC) composed of the invariant polypeptides VpreB and λ5 (6,24-27). The assembly of μHC, SLC, and Igα/Igβ forms the pre-B-cell receptor (pre-BCR) that orchestrates survival, proliferative expansion of early pre-B cells expressing a functional μHC, and the subsequent developmental transition to the late pre-B-cell stage where Igκ recombination is initiated (reviewed in 28). Once pre-B cells have successfully joined a $V_κ$ and a $J_κ$ gene segment to generate a productive $V_κ J_κ$ exon, they express a κLC that is tested for functionality by pairing with μHC, thus forming a BCR.

Pre-B cells expressing an κLC that is unable to pair with a μHC or that forms an autoreactive BCR undergo secondary rearrangements – a process termed receptor editing (29-33). Receptor editing can replace the $V_κ J_κ$ exon by joining upstream $V_κ$ segments with downstream $J_κ$ segments in an attempt to create a different, potentially non-autoreactive BCR specificity (34-37). Pre-B cells can also inactivate Igκ genes by deletion and switch to rearrangement of Igλ genes as a last attempt to generate a non-autoreactive functional BCR. Since BCR signals are essential to the survival of mature B cells, developing B cells that fail to rearrange and express both a functional IgH and a functional IgL gene undergo apoptosis (38).

Despite the precise orchestration of Ig gene rearrangements in developing human and murine B lymphocytes, ordered recombination of IgH and IgL gene segments *per se* is not required for Ig allelic exclusion. Studies in chicken demonstrated that Ig allelic exclusion remains intact, despite the fact that IgH and IgL genes are rearranged at the same developmental B-cell stage (39). Similarly, skates and sharks display IgH allelic exclusion, even though they carry multiple IgH loci in their genome that are activated for rearrangement at the same stage and can undergo V_H-D_H before D_H-J_H joining (40).

Genetic models for the allelic exclusion of Ig genes

The current genetic models to explain the establishment of Ig allelic exclusion fall into three categories: asynchronous recombination models, the stochastic model, and feedback inhibition models.

Asynchronous recombination models

Asynchronous recombination models explain how the recombination process prevents the simultaneous rearrangement of the two Ig alleles in one cell. These models rely on mechanisms that control the accessibility of Ig alleles within chromatin structure, thereby assuming that the tightly controlled and precisely timed access of the RAG recombinase to RSSs helps to preclude biallelic rearrangements.

In the probabilistic model, asynchronous rearrangement of the two Ig alleles results from the low efficiency of the recombination process due to limitations of chromatin accessibility (41-43). This is based on the notion that if the probability of a recombination event is sufficiently low for each allele (e.g. 0.05), the incidence of biallelic rearrangements, calculated as the square function of this probability (e.g. 0.0025), will be negligible. Thus, the slow, inefficient activation of Ig gene chromatin could limit the frequency of recombination events at any given time point to one per single cell.

The instructive model attributes the asynchronous rearrangement of the two Ig alleles to their asynchronous replication timing (44). Asynchronous replication of Ig alleles is established during early embryogenesis and maintained as an epigenetic mark in all cell types. As a result, the early replicating Ig allele would be the first allele available for recombination in B-lineage cells, and only in the case of an unsuccessful rearrangement would the second, late replicating allele undergo rearrangement after some time. In contrast to the probabilistic model, the instructive model assumes that once asynchronous allelic replication has been established in the early embryo, the two Ig alleles have different probabilities of being chosen for the first recombination attempt in pre-B cells. Since paternal and maternal Ig alleles have an equal chance of becoming the early replicating allele during embryogenesis, however, the instructive model predicts that 50% of the resulting B cells express either allele, a prediction also supported by the probabilistic model.

Similar to the probabilistic model but based on a different concept of probability, the stochastic model proposes that Ig rearrangement is maximally efficient, but recombination infrequently results in more than one functional Ig allele per cell (45,46). In this model, Ig allelic exclusion is a statistical consequence of the low probability of rearranging an allele in the correct reading frame that encodes a pairing Ig chain, given the high probability of generating a non-productive (out-of-frame) allele or an allele encoding a non-pairing Ig chain. Since no coordination is required between the two Ig alleles, asynchrony of allelic recombination or feedback inhibition are irrelevant to the stochastic model. Based on the assumptions of this model, one can deduce the theoretical upper limit for IgH allelic inclusion in newly developing B cells to be approximately 20% in the absence of any other regulation (47) (i.e. if both IgH alleles were rearranged simultaneously and there was no feedback inhibition) (Fig. 2). However, since the observed frequencies of IgH and IgL allelic inclusion among peripheral B cells are in the order of 1% or less (3,5), additional mechanisms must exist to prevent biallelic Ig rearrangements (or to eliminate Ig allelically included B cells) for the stochastic model to be correct.

Feedback inhibition models

Feedback inhibition models propose that the gene products or intermediates of Ig gene rearrangements inhibit the recombination process.

The classical feedback inhibition model poses that the cells can sense successful Ig gene rearrangements, because functional Ig gene products are assembled into either pre-BCRs or BCRs that initiate signals to suppress allelic recombination (19,48,49). An elegant aspect of this model is that if the first rearrangement is non-productive or gives rise to a non-pairing Ig chain, the lack of feedback inhibition signals will allow further recombination. This model explains the empirically observed ~60/40 ratio of peripheral B cells that have the IgH loci in either $V_H D_H J_H^+/D_H J_H$ or $V_H D_H J_H^-/V_H D_H J_H^+$ configuration (+, productive; –, unproductive). (19) (Fig. 2). Similarly, peripheral B cells show ~60/40 ratio of $V_\kappa J_\kappa+/\kappa^o$ to $V_\kappa J_\kappa^-/V_\kappa J_\kappa+$ configurations at the Igκ locus, suggesting that the feedback model also applies to Igκ rearrangements (46,50).

The more recently proposed allelic communication model is a feedback inhibition model based on the physical interaction and direct communication between the two Ig alleles (51). Allelic communication might act through signaling pathways triggered by RAG cleavage on one Ig allele, leading to the relocation of the second allele to a recombination-suppressive nuclear compartment. This may enforce or stabilize the asynchrony of recombination between the two Ig alleles and thus provide the cell with a greater window of time for monoallelic rearrangements, until classical feedback inhibition signals originating from pre-BCRs or BCRs eventually terminate recombination.

While asynchronous recombination models focus on the monoallelic onset of Ig gene rearrangements, feedback inhibition models focus on the mechanisms that stabilize the asynchrony between the two rearranging alleles or impose a final time limit on the ongoing recombination process. Thus, these two types of models account for complementary aspects relevant to the establishment of Ig allelic exclusion (Fig. 3).

Cellular selection model: historical debates and current repercussions

Apart from the genetic models above, the cellular selection model claims that cells with allelically included IgH genes are generated initially but subsequently counter-selected and thus purged from the B-cell repertoire (52,53). Historically, this model was based on the notion of 'heavy-chain toxicity', assuming that a diploid IgH gene dosage confers a growth disadvantage to B cells (54). The cellular selection model was refuted in terms of its general applicability, since B cells are capable of developing under the condition of IgH allelic inclusion and can express functional HCs from both alleles (7). It should be noted, however, that little is known about the relative fitness of IgH or IgL allelically included B cells under competitive conditions within a diverse B-cell repertoire.

The cellular selection model might explain how pre-B cells co-expressing one pairing and one non-pairing HC (the latter of which is unable to signal feedback inhibition of allelic recombination) are lost from the repertoire during their transition to mature B cells (6,25,55,56). One potential mechanism could be the induction of apoptosis in these cells through the unfolded protein response (UPR) initiated by an overload of the endoplasmic reticulum (ER) with non-pairing and thus incompletely folded HC proteins (57). Similarly, B cells co-expressing one high-affinity autoreactive and one innocuous HC might be deleted from the repertoire to establish self-tolerance (1). These examples illustrate that, apart from 'IgH gene dosage', there might be other selection mechanisms that could aid in maintaining Ig allelic exclusion, based on the impaired functionality of some, but perhaps not all, Ig allelically included B cells. This may result in the under-representation of Ig allelically included B cells within a diverse B-cell repertoire (discussed in the last section).

From a different perspective, the relevance of IgH gene dosage for allelic exclusion was recently revisited by studying mice that carry a functional IgH knockin gene but lack the Eμ enhancer (58). Interestingly, the lower expression of HCs from an allele lacking the Eμ enhancer leads to a sharp increase in the frequency of allelically included B cells. However, this is not solely due to impaired feedback inhibition signals, as one may expect from the lower IgH expression level but rather the outcome of positive selection favoring HC double producers at the transition from immature to mature B cells. These data demonstrate the power of selection in shaping the B-cell repertoire and suggest that a certain threshold of IgH gene expression is required for B-cell survival and maturation. Thus, a critical role of the Eμ enhancer is to ensure that sufficient amounts of HCs can be expressed from one functional IgH allele to sustain allelically excluded B cells during their development.

Mechanisms activating Igκ genes for recombination

Igκ genes are silent in non-B-lineage cells and become progressively activated for recombination in developing B cells (18). The controlled release of repression of Igκ chromatin became the cornerstone of asynchronous recombination models, since the efficiency, pace and timing of this process may limit the frequency of biallelic recombination events in a single pre-B cell.

Early Igκ activation events: central nuclear location and altered chromatin structure

The activation of Igκ genes for recombination starts with the re-localization of both alleles from the suppressive environment of the nuclear periphery to a more central, euchromatic region in pro-B cells (59). At this time, the two Igκ alleles already show asynchronous replication that is established during mid-gastrulation (44); however, this epigenetic mark is not immediately used to instruct the monoallelic activation of Igκ chromatin. Instead, following re-positioning to a central location, both Igκ alleles lose repressive histone marks, such as the methylation of histone H3 at lysine 9 ($H3K9^{me2}$, $H3K9^{me3}$) and lysine 27 ($H3K27^{me2}$), and acquire activating histone marks, such as the acetylation of histone H3 at lysine 9 ($H3K9^{Ac}$), the acetylation of histone H4 ($H4^{Ac}$), and the methylation of histone H3 at lysine 4 ($H3K4^{me2}$, $H3K4^{me3}$) (60-63). The diminished methylation of H3K9 prevents the interaction with the chromodomain of HP-1 (Swi6), a non-histone component of constitutive heterochromatin (64,65). In parallel, $H3K9^{Ac}$ and $H4^{Ac}$ recruit bromodomain-containing proteins, such as the ATP-dependent hSWI/SNF complex that actively remodels chromatin structure by altering the position of nucleosomes, generating nucleosomal DNA loops and exchanging histone dimers and octamers (66-72).

Altogether the epigenetic reorganization of Igκ genes results in an open chromatin state, as measured by greater sensitivity to cleavage by DNase I (61). The altered epigenetic structure of Igκ chromatin also has direct implications for its sensitivity to RAG cleavage, since RAG enzymes cannot cleave RSSs that are positioned over unmodified nucleosomes *in vitro* (73-75). However, hSWI/SNF-mediated chromatin remodeling renders nucleosomal DNA accessible to RAG, suggesting that changes in nucleosome structure or nucleosome position are required for efficient V(D)J recombination (76-78). This could be supported by an ISWI-containing chromatin-remodeling complex that directly associates through its plant homeodomain (PHD) finger with $H3K4^{me3}$ (78,79). Additionally, RAG-mediated cleavage of an RSS positioned over a nucleosome is stimulated by the non-histone chromatin protein HMG1 that bends DNA and unwinds chromatin loops (75).

Different histone modifications likely cooperate in increasing the chromatin accessibility to RAGs (a concept termed 'the histone code'), since histone acetylation alone is not sufficient to allow efficient recombination *in vivo* (80,81). In summary, the structural constraints on RAG cleavage of RSSs imposed by unmodified nucleosomes provide a critical layer of regulation that restricts the efficiency of Igκ recombination. According to the probabilistic model of allelic exclusion, this could limit the incidence of biallelic Igκ rearrangements in a single pre-B cell.

Germline transcription

The changes in Igκ chromatin structure are accompanied by the pre-BCR-mediated upregulation of transcription factors such as SpiB, IRF4, IRF8, and E2A, thereby enabling germline transcription of unrearranged V_κ and J_κ gene segments, which correlates with the activation of Igκ chromatin for recombination in pre-B cells (82-85). The correlation between germline transcription and recombination has fueled many studies trying to decipher how these two processes are connected: Are they simply consequences of a

common underlying cause, the open chromatin state? For example, the PHD finger of the basal transcription initiation factor TFIID directly binds to the H3K4^{me3} mark associated with open chromatin (86). Or, is active, ongoing germline transcription a pre-requisite for efficient recombination? Gene-targeting studies in mice demonstrated that the two transcriptional enhancers (Ei$_\kappa$, 3'E$_\kappa$) and the intergenic proximal and distal germline promoters (Fig. 4) are crucial for efficient Igκ transcription and recombination (87-89). Thus, the binding of transcription factors to *cis*-regulatory elements (promoters and enhancers) in the Igκ locus is key to recombination. However, since these factors may directly affect the chromatin state by recruiting histone-modifying enzymes (90), the impact of the transcriptional elongation process itself on recombination remained elusive for some time.

Preliminary evidence for a causal connection of recombination with transcription came from T cells where germline transcription through RSSs enhances TCRβ recombination, likely through the recruitment of histone modifiers (histone acetylases, H3K4 methyltransferase) by the Polymerase II elongation complex (91-94). Histone marks deposited during transcriptional elongation might enforce the association of hSWI/SNF and ISWI chromatin-remodeling complexes that alter nucleosome positions and generate DNA loops on the nucleosome surface, as described above. It is currently unknown whether other activities of the Polymerase II elongation complex, such as the transient disruption of nucleosome structure by eviction of individual histones or the complete removal of nucleosomes, play a direct role in activating recombination (95-97). It is also an open question whether the germline transcripts themselves are functionally relevant to Ig recombination or allelic exclusion.

Previous findings from our laboratory suggested that infrequent, monoallelic transcription from the Igκ proximal germline promoter (pGP), which is located immediately upstream of the J$_\kappa$ gene segments (Fig. 4), may contribute to the allelic exclusion of Igκ genes (41). Monoallelic transcription could be mediated through the limited availability of transcription factors that drive germline transcription and activate Igκ chromatin for recombination. However, other studies revealed that the Igκ distal germline promoter (dGP), located ~3.5 kb upstream of the pGP, is highly active on both Igκ alleles in virtually all pre-B cells, suggesting that germline transcription *per se* is neither limiting nor allelically excluded (98-100). Similarly, germline and antisense transcription of IgH loci are biallelic (101,102), underscoring that, in principle, IgH and IgL genes can be expressed from both alleles.

H3K4^{me3} and RAG recruitment

Recent publications reveal another interesting aspect of the link between germline transcription, chromatin structure, and V(D)J recombination by demonstrating that histone H3 modified by trimethylation at lysine 4 (H3K4^{me3}) can directly interact with the PHD finger in the RAG2 protein (103-105). H3K4^{me3} not only aids in recruiting the RAG complex to the RSS but also functions as an allosteric activator of enzymatic cleavage and hairpinning activities (106). Therefore, the introduction of H3K4^{me3} correlating with the activation of Igκ germline promoters may enhance chromatin accessibility, thereby recruiting RAGs and stimulating the cleavage of J$_\kappa$ RSSs. Accordingly, gene-targeting in mice demonstrated that the C-terminal region of RAG2 (containing the H3K4^{me3} binding PHD finger) is required for efficient rearrangements at some Ig loci *in vivo* (107,108).

H3K4^{me3} is considered to be a universal epigenetic mark associated with active promoters, but it is usually restricted to a ~1.5 kb region upstream and downstream of the transcription start site (109,110). Given the large distance between the transcription start site of the distal GP and the J$_\kappa$ segments (~3.5 kb) (Fig. 4), it is possible that the efficiency with which Igκ gene segments are activated for RAG cleavage is limited by a relatively low density of the

H3K4^{me3} mark at J$_\kappa$ RSSs. In support of this idea, promoter location, rather than promoter orientation or histone acetylation, is the primary determinant for recombination efficiency of stably integrated reporter constructs (81). Thus, inefficient RAG recruitment and cleavage at J$_\kappa$ RSSs could ensure a low frequency of Igκ rearrangements in pre-B cells, as postulated by the probabilistic model. It remains to be tested whether the precise distribution of H3K4^{me3} and/or additional histone modifications that directly support interaction with RAG proteins, such as the methylation of histone H3 at arginine 2 (H3R2^{me2}) (111), play a crucial role with respect to the asynchronous recombination and allelic exclusion of Igκ genes.

Locus contraction and subnuclear repositioning

Along with the changes in histone modifications, the Igκ locus undergoes a large-scale contraction on both alleles in pre-B cells, likely facilitating synapsis between rearranging V$_\kappa$ and J$_\kappa$ segments (112). The contracted state is maintained throughout the early immature B-cell stage, which could aid in the editing of autoreactive Igκ genes. While in a contracted state in pre-B cells, one of the two Igκ alleles is repositioned to the repressive environment of centromeric heterochromatin, where it is bound by HP1 and Ikaros, whereas the other allele remains located in a euchromatic region of the nucleus and is enriched in acetylated histone H3 (60,112). Thus, the differential subnuclear repositioning of Igκ alleles in pre-B cells is the first striking allele-specific event that makes the two Igκ alleles epigenetically distinguishable and could impose an allelic order of recombination.

The association of unrearranged Igκ alleles with centromeric heterochromatin is mediated through a silencer sequence in the Igκ locus (designated Sis) that binds to the transcriptional repressor Ikaros (113,114). It is possible that the location of Sis in the V$_\kappa$-J$_\kappa$ interval (~7 kb upstream of the J$_\kappa$ cluster) may be important for its ability to suppress V$_\kappa$-to-J$_\kappa$ joining in pre-B cells by interfering with DNA loop formation or recruiting of chromatin modifiers. Moreover, the recent identification of a CTCF binding site in close proximity to Sis suggests the existence of an insulator element in the V$_\kappa$-J$_\kappa$ interval that could limit the scope of chromatin activation originating from the two Igκ enhancers located downstream of the J$_\kappa$ cluster (115). Sis might also mediate repositioning of the unrearranged Igκ allele to heterochromatin in activated mature B cells (116), although in these cells, Sis is a degenerate genetic mark for unrearranged Igκ alleles, since Igκ rearrangements can occur by inversion, thereby retaining the V$_\kappa$-J$_\kappa$ interval.

It is not completely clear whether the repositioning of one Igκ allele to centromeric heterochromatin prior to recombination in pre-B cells is predetermined by the later replication of this allele, as predicted by the instructive model (60). Even though the heterochromatic Igκ allele appears to be the late replicating allele in pre-B cells, it is difficult to distinguish between cause and effect, since the association of genes with heterochromatin can lead to late replication and/or delayed segregation of sister chromatids in S-phase (117). Therefore, the abrupt transition from biallelic to monoallelic positioning of the Igκ locus may well be a 'stochastic choice' made in pre-B cells. One possibility to explain the switch to monoallelic activation events could be a scenario in which the two pre-activated Igκ alleles compete for limited space and resources in certain euchromatic nuclear locations with high recombinase activity, as it was postulated for 'recombination factories' (118). If these recombination factories functioned under a 'winner takes all' principle, then the limitation of RAG activity to certain 'hot spots' in the nucleus could explain why the initiation of Igκ recombination is monoallelic, while the other allele is repositioned to heterochromatin. In support of this notion, homologous pairing of Igκ alleles prior to recombination suggests a more intimate physical interaction of the two alleles (51), thus facilitating direct spatial competition for limited resources of RAG activity.

Another explanation for the switch from biallelic to monoallelic activation of Igκ chromatin arose from a recent study in T cells, which provided new evidence for the probabilistic model of allelic exclusion: a surprisingly high percentage of double-negative T cells (in total 95%) had one (35%) or both (60%) TCRβ alleles associated with either the nuclear lamina or centromeric heterochromatin (42). Apparently, TCRβ alleles can be distributed independently of each other and with high frequency to recombination-suppressive nuclear compartments, suggesting a stochastic rather than instructive mechanism for the non-equivalent subnuclear localization of the two alleles in developing T cells. As a result, only a very small fraction of double-negative T cells (5%) contains two 'free' TCRβ alleles available for RAG-mediated cleavage, thereby restricting biallelic TCRβ rearrangements and presumably supporting TCRβ allelic exclusion. It will be interesting to explore whether similar mechanisms govern the subnuclear repositioning and activation of Igκ alleles for recombination in pre-B cells.

While the Igκ alleles change their nuclear position and acquire or lose histone modifications, both of them remain fully DNA methylated at their CpG dinucleotides in pro-B and early pre-B cells (60,119). DNA methylation has a dual role in suppressing V(D)J recombination (119-121). First, DNA methylation within the RSS heptamer markedly reduces RAG cleavage without inhibiting RAG/DNA complex formation (122). Second, methylated DNA recruits methyl-CpG binding-domain (MBD) protein that directly inhibits the binding of RAGs to DNA and serves as a loading platform for histone deacetylases (HDACs), thereby restricting chromatin accessibility (122,123). Therefore, late in the ontogeny of pre-B cells, one of the two Igκ alleles must undergo DNA demethylation prior to recombination. This was thought to be mediated by the preferential binding of NFκB transcription factors to the Igκ allele with more permissive histone modifications (such as acetylated H3), that is located in an euchromatic region of the nucleus (60,124,125). Recent findings in gene-targeted mice, however, refuted the idea that NFκB activation is essential to Igκ recombination, since the genetic ablation of all three IκB kinases did not impair the development of B cells expressing κLC-containing BCRs (126). Additional transcription factors may therefore be involved in demethylating one Igκ allele prior to rearrangement.

The question remains whether monoallelic DNA demethylation and the sequential order of recombination of the two Igκ alleles is indeed pre-programmed by their asynchronous replication, as suggested by the instructive model (44). Both DNA methylation status and replication timing correlate with the V(D)J recombination status of Igκ alleles in mature B cells, but does this mean that early replication dictates the choice of the first Igκ allele to undergo recombination in pre-B cells? Most of the initial epigenetic and conformational changes that predispose Igκ genes for recombination occur on both alleles, demonstrating that the time lag resulting from late replication does not immediately translate into a delayed activation of the late replicating Igκ allele. Moreover, the instructive model estimates that the probabilities of undergoing DNA demethylation for the early and the late replicating Igκ allele are 0.9 and 0.3, respectively (119). Thus, up to 27% of pre-B cells might demethylate the DNA simultaneously on both Igκ alleles ($0.9 \times 0.3 = 0.27$), suggesting that additional mechanisms are required to guarantee asynchronous allelic recombination. One such mechanism could be limiting the efficiency of Igκ rearrangements, as postulated by the probabilistic model.

In comparing the probabilistic and instructive models, there is another scenario in which the instructive model falls short: how to prevent secondary recombination events targeting the same Ig allele? Once a productive V(D)J exon has been formed and the gene products are tested for functionality through the assembly of pre-BCRs or BCRs, the existing V(D)J exon must not be rapidly deleted by subsequent rearrangements of the same Ig allele. Otherwise the cells that receive pre-BCR or BCR signals would no longer sense their actual Ig gene

configuration. The obsolete sensing of functional Ig rearrangements would cause severe complications at the pre-BCR checkpoint that positively selects pre-B cells expressing a functional μHC and at the central tolerance checkpoint that positively selects immature B cells expressing non-autoreactive BCRs. However, allelic differences in replication timing, nuclear localization, and DNA demethylation cannot account for the exclusion of secondary rearrangements on the same Ig allele. Thus, during the time lag before classical feedback inhibition takes effect, a low efficiency of recombination, as suggested by the probabilistic model, may be required to prevent the rapid destruction of newly formed V(D)J exons. This holds true not only for Igκ genes, where upstream V_κ segments can recombine with J_κ segments downstream of the initial $V_\kappa J_\kappa$ exon, but also for IgH genes, where an initial $V_H D_H J_H$ exon can be modified through recombination with upstream V_H segments by using a cryptic RSS found in the 3' end of most V_H segments (V_H replacement) (127-130).

Mechanisms involved in feedback inhibition of Igκ recombination

Feedback inhibition models for allelic exclusion postulate that developing B cells sense the presence of a functional Ig allele and subsequently suppress recombination to prevent the rearrangement of a second functional Ig allele or the deletion of the initially generated V(D)J exon. In support of this idea, early studies using genetically modified mice demonstrated that the expression of V(D)J-recombined Ig transgenes prevents the rearrangement of endogenous Ig loci (131-135). Similarly, the insertion of a productively rearranged, functional V(D)J exon into an endogenous Ig allele precludes the rearrangement of the other allele (7,35,136). According to the classical feedback inhibition model, functional Ig gene products are assembled into surface-expressed pre-BCRs or BCRs that trigger signals to inhibit further recombination. The importance of pre-BCR signaling for the allelic exclusion of IgH genes was established by using mice with a targeted disruption of the Igμ transmembrane exons (55): These mice display IgH allelic inclusion, since secreted μHCs are not assembled into signaling-competent pre-BCRs (as the μHC transmembrane domain is required for the association with the signal transducers Igα/Igβ). The feedback signaling pathways that mediate Ig allelic exclusion remain poorly defined; yet the activation of the Syk family kinases Syk and/or ZAP70, both of which are partially redundant and bind to phosphorylated ITAMs in Igα/Igβ, is required to establish IgH and IgL allelic exclusion (137,138).

Downregulation of RAG expression by pre-BCR and BCR signals

One of the mechanisms thought to suppress V(D)J recombination after the first successful Ig rearrangement is the downregulation of the lymphocyte-specific components of the recombination machinery, RAG1 and RAG2, mediated by pre-BCR or BCR signals (139-141). The downregulation of RAG1 and RAG2 in early cycling pre-B cells upon expression of a functional μHC is accomplished through transcriptional repression, likely by modulating the activity of various lymphocyte-specific transcription factors such as Pax5, c-Myb, LEF1, and FoxO1 that bind to the RAG locus (142-146). In addition, RAG2 protein is actively degraded in cycling pre-B cells at the G1/S transition following phosphorylation of Thr490 by cyclinA/CDK2 and ubiquitinylation by Skp2-SCF ubiquitin ligase (147-149). Since the half-life of RAG1 is prolonged in the absence of RAG2 (150), this pathway probably leads to degradation of both proteins, thereby decreasing the overall abundance of the active RAG holoenzyme.

The expression of a stabilized RAG2 mutant in transgenic mice, however, demonstrated that the degradation of RAG2 in cycling cells is not essential to enforce allelic exclusion of TCRβ genes in T-lineage cells but rather serves to synchronize RAG cleavage with DNA repair mediated by NHEJ factors that are preferentially active in G0/1 phase (151). Accordingly, the constitutive expression of RAG1/2 transgenes driven by the lck promoter

in T cells does not abrogate TCRβ allelic exclusion (152). By analogy, this finding suggests that the rapid downregulation of the RAG proteins in cycling pre-B cells may be less critical than previously thought for establishing IgH allelic exclusion. Assuming that the pace of cell cycle-dependent RAG2 degradation determines the abundance of active recombinase, it would take proliferating pre-B cells about 30 min to eliminate 50% of their RAG activity after the successful completion of a functional rearrangement on one IgH allele: 5 minutes for the production, assembly and surface transport of a pre-BCR (discussed in 153), presumably 5 minutes for the transduction of feedback signals and shutting down of transcription of the RAG locus, and 10-20 minutes half-life of RAG2 in S-phase (149,151). In fact, the remaining amount of RAG2 is about 20% of the starting amount, even after 90 minutes of incubation with S-phase extract (149). Thus, the downregulation of RAG activity in pre-B cells might be too slow to effectively suppress the recombination of the second IgH allele.

Further, the RAGs are re-expressed in late, non-cycling pre-B cells to enable the recombination of IgL genes, suggesting that other mechanisms – apart from RAG downregulation – are required to maintain IgH allelic exclusion (139). Expression of the RAG recombinase is eventually terminated after a successful IgL rearrangement; yet, RAG transcripts and proteins are detectable in immature IgM^{low} B cells (139,154,155), which could be partly due to the fact that immature B cells do not enter the cell cycle upon BCR expression and may need to undergo receptor editing. This calls into question a major role for rapid RAG downregulation in establishing IgL allelic exclusion. The constitutive expression of RAG1 and a stabilized RAG2 mutant in transgenic mice by using a B-lineage-specific promoter might be helpful to determine whether the impaired downregulation of RAG activity leads to a violation of IgH or IgL allelic exclusion.

Regulating chromatin accessibility and locus contraction following pre-BCR and BCR signals

Within the classical feedback inhibition model, pre-BCR signals are thought to lead to recruitment of the non-functional IgH allele to centromeric heterochromatin, perhaps by attenuating IL7R-signaling (112,116). This could keep the non-functional IgH allele inaccessible to RAG proteins after the successful rearrangement of the other allele and thus enforce allelic exclusion. One candidate for inducing a closed chromatin state at the Ig loci is the histone methylase G9a that inhibits RAG cleavage by introducing H3K9^{me3}, thereby creating docking sites for the heterochromatin protein HP-1 (156). Somewhat puzzling, however, is the observation that both the functional and non-functional (germline or non-productive) IgH allele are accessible to Polymerase II and transcribed at similar rates in B-lineage cells (101). This finding suggests that the monoallelic recruitment to heterochromatin does not completely silence the recruited IgH allele.

In contrast to IgH alleles, one of the two Igκ alleles is already associated with centromeric heterochromatin prior to (or concomitantly with) the onset of recombination (60,62), making it unlikely that feedback signals originating from BCRs are essential for the centromeric recruitment of Igκ genes. In addition, recent experiments conducted in our laboratory by using a novel Igκ locus reporter mouse showed that about 90% of mature B cells in heterozygous C$_κ$-IRES-EYFP mice expressed EYFP (98). Since EYFP can be expressed from both functional and non-functional (germline or non-productive) Igκ alleles, these data demonstrate that the non-functional Igκ allele associated with heterochromatin in B cells is not permanently silenced (in case of random monoallelic silencing of the nonfunctional Igκ allele upon BCR expression, the percentage of EYFP-positive B cells should be ~50%). There is some evidence, however, that additional changes in chromatin accessibility might be involved in suppressing allelic Igκ recombination after the expression of a BCR, since V$_κ$

segments show a lower sensitivity to DNase I in mature B cells compared to that in pre-B cells (61).

Other data support the view that the decontraction of IgH loci in response to feedback signals triggered by surface-expressed pre-BCRs decreases the likelihood of allelic recombination after a successful rearrangement event (112). This decontraction separates distal V_H segments from $D_H J_H$ joints, thereby preventing further V_H-to-DJ_H rearrangements following the re-expression of RAGs in late pre-B cells. Interestingly, allelic inclusion of endogenous IgH loci is sometimes observed for proximal V_H segments in Igμ-transgenic mice, even though the endogenous IgH loci are in a decontracted state (112,157). Therefore, feedback inhibition by locus decontraction is a leaky mechanism, and the distance along the chromosome between rearranging gene segments may control the recombination efficiency. Accordingly, rare IgH allelically included B cells found in wildtype mice show a bias towards IgH rearrangements involving proximal V_HQ52 and V_H7183 segments (5).

Locus decontraction appears to be less critical for establishing Igκ allelic exclusion, since Igκ genes remain in a contracted state throughout the early immature (IgM^{low}) B-cell stage, presumably to enable the editing of autoreactive Igκ genes (112). Thus, additional mechanisms are required to ensure the generation of only one functional Igκ allele during receptor editing (discussed below). Decontraction of Igκ genes eventually occurs in late immature (IgM^{high}) B cells, suggesting a role for locus decontraction in maintaining Igκ allelic exclusion in cells that have passed this developmental stage.

Direct allelic communication through an ATM-mediated DNA damage response

A strikingly different model to explain Ig allelic exclusion (the allelic communication model) was proposed recently in a study revealing that RAG1 mediates the homologous pairing of Ig alleles prior to recombination (51). Following the generation of double-stranded DNA breaks by RAG proteins on one of the two Ig alleles, the other allele is repositioned to centromeric heterochromatin as part of an ATM-mediated DNA damage response, suggesting a direct way for B-lineage cells to sense recombination intermediates and thus ongoing Ig rearrangements. Since ATM-mediated sensing of DNA breaks can only stabilize or enforce the asynchrony between the two rearranging Ig alleles after the onset of recombination, the allelic communication model is formally a feedback model. Hence, it does not provide an explanation as to how the cells initially achieve monoallelic RAG cleavage, which could be controlled by either probabilistic or instructive mechanisms. Nonetheless, the frequency of biallelic RAG cleavage, measured as the frequency of colocalization of Ig alleles and γH2AX, a histone variant deposited at sites of double-stranded DNA breaks, is increased in ATM-deficient cells (1.1% of pro-B cells and 4.8% of pre-B cells) compared to that in wildtype cells (0.2% of pro-B cells and 0.6% in pre-B cells) (158). Feedback inhibitory signals triggered by ATM might restrict the number of RAG-mediated DNA breaks in a single cell at any given point in time, thereby limiting the frequency of biallelic Ig rearrangements and minimizing the risk of genome damage.

Testing theoretical models in genetically modified mice

As outlined in the previous section, there is ample genetic evidence supporting the classical feedback inhibition model for Ig allelic exclusion including numerous gain-of-function and loss-of-function studies in genetically modified mice, even though key mechanistic aspects remain to be elucidated. In contrast, critical loss-of-function studies addressing the *in vivo* relevance of asynchronous recombination models are largely absent. Moreover, the allelic communication model for Ig allelic exclusion appears to be somewhat inconsistent with data from genetically modified mice, since it would predict that mice lacking key factors of the

DNA damage response display Ig allelic inclusion at the level of BCR surface expression, which is not the case, at least with respect to ATM-deficient mice (51).

Most available experimental data supporting the probabilistic and instructive models for Ig allelic exclusion are correlative findings that link rearrangement status, chromatin state, nuclear localization, and replication timing to the choice of the first allele for recombination. It is an open question, however, as to whether the proposed mechanisms for the allelic asynchrony of Ig gene rearrangements are indeed essential to allelic exclusion. The prediction that overriding these mechanisms would lead to simultaneous biallelic Ig recombination and thus to a substantial violation of Ig allelic exclusion has yet to be tested in mice. For the probabilistic model, the future challenge will be to establish appropriate mouse models that display enhanced recombination efficiencies for Ig genes, for example by overexpressing transcriptional activators, tethering of chromatin modifiers to RSSs, or by modifying the Ig locus structure, and to demonstrate that higher recombination efficiencies lead to a violation of Ig allelic exclusion. For the instructive model, similar efforts are required to demonstrate that biallelic DNA demethylation or the lack of asynchronous replication timing of the two Ig alleles brings about a violation of Ig allelic exclusion.

With regard to the latter, there appears to be an interesting experimental possibility to distinguish between the instructive and the probabilistic models, since the probabilistic model does not rely on asynchronous replication timing of the two Ig alleles to establish allelic exclusion. Asynchronous replication requires that Ig alleles are located within separate units of replication, i.e. on homologous chromosomes. Therefore, repositioning of both Ig alleles in tandem on the same chromosome would be expected to synchronize their replication, erase differential DNA methylation marks, and thus create two epigenetically equivalent Ig loci. According to the instructive model, this should result in Ig allelic inclusion, whereas the probabilistic model would predict that Ig allelic exclusion remains intact by virtue of a low recombination efficiency. Preliminary results obtained by using multiple copies of rearranging Ig minilocus transgenes indicate that allelic exclusion might be violated for Ig genes located *in cis* (on the same chromosome), while remaining intact for Ig genes located *in trans* (on homologous chromosomes) (159). However, due to gross differences in size and structure of Ig minilocus transgenes, these results are difficult to extrapolate to full-size Ig loci. Thus, we will need to utilize new strategies in gene-targeting, genome engineering, and BAC technology to perform genetic loss-of-function experiments in mice in order to further test asynchronous recombination models in the future.

Receptor editing and Igκ allelic exclusion

Autoreactive BCRs are rapidly downmodulated from the cell surface after binding to self-antigens (160), thereby interrupting feedback signals in immature B cells, and thus prolonging RAG expression while maintaining a contracted state of the two Igκ alleles (112,161,162). This enables immature B cells to continue to rearrange Igκ genes with the potential outcome that the newly generated Igκ chain assembles with the available HC into a non-autoreactive BCR, a process called receptor editing. The discovery of receptor editing raised the question as to which mechanisms maintain Igκ allelic exclusion, or more specifically, whether and how secondary rearrangements are targeted to the previously recombined Igκ allele, a process eliminating $V_\kappa J_\kappa$ exons that give rise to autoreactive BCRs (34,35,163).

The instructive model posits that asynchronous replication timing governs the choice of which Igκ allele to edit, thereby ensuring the deletion of autoreactive $V_\kappa J_\kappa$ exons (164). This does not account, however, for the fact that about half of all B cells that produce a κLC have rearranged the second, late replicating Igκ allele, since the rearrangement of the first, early

replicating allele was non-productive, and thus the cells show a $V_\kappa J_\kappa^-/V_\kappa J_\kappa^+$ configuration (50).

Other studies suggest that the choice as to which Igκ allele undergoes rearrangement during receptor editing occurs stochastically (165). In pre-B cells from mice carrying one wildtype and one autoreactive knockin Igκ allele, both alleles have an equal chance of becoming rearranged. Although this does not rule out the instructive model, since the autoreactive knockin Igκ allele is the early replicating allele in only 50% of pre-B cells, it raises the possibility that receptor editing can either delete the autoreactive allele or lead to Igκ allelic inclusion. Indeed, allelically included mature B cells expressing one autoreactive and one innocuous Igκ chain were identified in mice (160,165,166). Under normal circumstances, however, most allelically included B cells show phenotypic Igκ allelic exclusion with regard to BCR surface expression and antibody secretion, because one of the two κLCs is expressed at much lower levels or pairs less efficiently with the available HC (3,165). Another mechanism to maintain phenotypic allelic exclusion could be that autoreactive BCRs are selectively downmodulated from the cell surface by receptor-mediated endocytosis upon chronic exposure to self-antigens (160). In accord with this idea, low-affinity autoreactive BCRs are frequently polyreactive and bind to a variety of self-antigens including the BCR itself, resulting in constitutive BCR self-oligomerization that likely supports the internalization of autoreactive receptors. Similar mechanisms (binding of self-antigens and receptor self-oligomerization) also result in the low-surface density of pre-BCRs (167-169), suggesting an evolutionary relationship between pre-BCRs and autoreactive BCRs (discussed in: Vettermann and Jäck, Trends in Immunology, 2010, in press).

Analogous to primary Igκ rearrangements, a low frequency of secondary Igκ rearrangements in pre-B cells that already express a functional Igκ allele could restrict the generation of allelically included B cells during receptor editing, as proposed by the probabilistic model. In theory, the number of J_κ segments determines an upper limit of canonical secondary recombination attempts on each Igκ allele. Interestingly, the number of J_κ segments in the Igκ locus (4 functional J_κ) is much smaller than the number of J_α segments in the structurally related TCRα locus (~40 functional J_α). Therefore, the theoretical upper limit of secondary recombination events for each TCRα allele is much higher than the theoretical upper limit of secondary recombination events for each Igκ allele during receptor editing. In developing T cells, secondary rearrangements at the TCRα locus help to efficiently replace $V_\alpha J_\alpha$ exons encoding TCRs that do not interact with self-MHC molecules and thus fail positive selection (170,171). This comparison gains some significance, since TCRα genes, in contrast to Igκ genes, are largely allelically included. Up to 30% of T cells carry two productively rearranged TCRα alleles (172) and about 10-20% of all T cells express two different TCRα chains at the cell surface (173-175). Thus, there is a positive correlation between the maximal number of canonical secondary recombination attempts and the frequency of allelic inclusion. The limited number of secondary rearrangements available for each Igκ allele might therefore control the overall frequency of recombination events that occur during the lifespan of a single pre-B cell. This could potentially limit the incidence of biallelic Igκ rearrangements during receptor editing.

Rearrangement and allelic exclusion of Igκ genes

If pre-B cells have exhausted all possibilities to generate a functional and non-autoreactive Igκ gene, they generally inactivate the Igκ locus by deletion before they switch to Igλ rearrangement (176-179). Inactivation of Igκ is accomplished by RAG-mediated joining of the non-coding recombining sequence (RS) located ~25 kilobases downstream of C_κ with either an upstream V_κ RSS or the intronic RS (IRS) located in the J_κ-C_κ interval (Fig. 4), thus deleting the C_κ exon and creating a permanently silenced Igκ allele (reviewed in 180).

Whether signals originating from autoreactive BCRs govern the switch from conventional V_κ-J_κ recombination to RS recombination is not clear. It is known, however, that RS recombination promotes the formation of Igλ-expressing B cells (181).

The activation of the Igλ locus appears to be independent of κLC-BCR signaling (178), supporting the view that Igλ rearrangements are controlled by a cell-autonomous timing mechanism. This appears to require the presence of the transcription factor NFκB that induces the anti-apoptotic factor Pim2, thus providing essential survival signals to aging pre-B cells or autoreactive immature B cells (126). This facilitates Igκ recombination by controlling the lifespan of those cells that fail to rearrange a functional, non-autoreactive Igκ gene, and thus do not receive survival signals through the κLC-BCR.

The fact that Igλ-expressing B cells often inactivated the Igκ locus by deletional RS recombination may contribute to isotype exclusion of Igλ and Igκ genes. Far less is known, however, about how allelic exclusion of Igλ genes is established. Igλ genes can give rise to four different λLC isoforms (λ1LC, λ2LC, λ3LC, and λxLC) that are encoded by two independently rearranging clusters of gene segments (182,183) (Fig. 4). Rearrangements across the two clusters are very infrequent (184). Based on the organization of the Igλ locus, it can be deduced that rearrangements leading to the production of λ2LC (V_λ2-J_λ2) and rearrangements leading to the production of λxLC (V_λx-J_λ2) are mutually exclusive. Similarly, rearrangements leading to the production of λ1LC chains (V_λ1-J_λ1) exclude rearrangements coding for λ3LC (V_λ1-J_λ3). Why is it that only one of the two clusters, either Igλ2/x or Igλ1/3, undergoes a functional rearrangement? Thus, any model for Igλ allelic exclusion needs to explain two different phenomena: (i) isoform exclusion of Igλ2/x and Igλ1/3 clusters located on the same chromosome (*in cis*), (ii) allelic exclusion of the two Igλ alleles located on homologous chromosomes (*in trans*).

The two Igλ alleles on homologous chromosomes show asynchronous replication, suggesting that this epigenetic mark could instruct their order of recombination (44). Differences in replication timing cannot be invoked, however, to explain isoform exclusion of Igλ2/x and Igλ3/1 clusters, since they are located in close proximity, and thus most likely within the same chromosomal replication domain. Intriguingly, 97% of B cells producing Igλ chains carry only one Igλ rearrangement, suggesting that allelic rearrangements occur very infrequently following a non-productive rearrangement attempt (177). According to the probabilistic model, this could be attributed to a low efficiency of activating the Igλ locus for recombination, thereby precluding the simultaneous rearrangement of Igλ2/x and Igλ1/3 clusters. This may be due to the lower affinity of RAG for Igλ RSSs compared to Igκ RSSs (185), resulting in a lower frequency of $V_\lambda J_\lambda$ joining events (186). In addition, since Igλ genes are activated relatively late during pre-B cell ontogeny, the remaining lifespan for these aged pre-B cells to produce a functional Igλ allele could be very short, restricting the total frequency of Igλ rearrangements to one per single pre-B cell (46). After the generation of a functional Igλ allele, feedback signals triggered by surface-expressed λLC-BCRs inhibit allelic recombination, as was demonstrated by studies using Igλ-expressing transgenic mice (187,188).

With regard to receptor editing of autoreactive λLC-BCRs, Igλ genes are neither modified by secondary $V_\lambda J_\lambda$ recombinations targeting the same Igλ cluster nor inactivated by deletion, as is the case at the Igλ locus (189). Therefore, B cells with autoreactive λLC-BCRs undergo apoptosis, become anergic, or find alternative ways of revising their BCR specificity. One interesting possibility for receptor editing utilized by autoreactive Igλ-expressing immature B cells is the generation of additional functional IgL alleles, leading to Igλ allelic inclusion or Igλ/Igκ isotype inclusion (189-191). For example, transgenic mice expressing the autoreactive 3H9/56R HC that binds to DNA have a large population of B cells that express

two different LCs (190). One LC (usually the λ1LC) permits DNA binding, if paired with the 3H9/56R HC, while the other LC (usually a κLC) serves as an 'editor' and confers an innocuous specificity. Similarly, some B cells edit autoreactive λLC-BCRs by isoform inclusion of Igλ1 and Igλx (189,192). LC double-expressing B cells might be refractory to activation, because the expression of the innocuous BCR may dilute the surface density of the anti-DNA BCR to the point that binding of self-antigen is no longer efficient. This may be influenced by a greater ability of the innocuous LC to pair with HC, leading to selectively reduced surface presentation of the autoreactive BCR (29). In addition, the remaining low self-reactivity of Igλ/Igκ isotypically included or Igλ allelically included B cells may support the homing of these cells to the marginal zone (191). The restriction of these cells to this location may help to prevent them from undergoing affinity maturation and developing into autoreactive plasma cells and memory B cells.

Monospecificity of B cells and immune recognition

While much has been learned about the mechanisms establishing Ig allelic exclusion, the importance of monoallelic Ig expression to B-cell function remains enigmatic. In principle, Ig allelic exclusion controls the effective gene dosage and thus gene expression levels. Even though this appears to be critical for other genes, such as those on the X chromosome, which are expressed in a monoallelic fashion in females, it is not obvious why a twofold higher amount of functional Ig chains would be detrimental to B-cell function. Accordingly, B cells expressing functional HCs and LCs from both alleles undergo normal development and are capable of participating in a humoral immune response, suggesting that B cells can tolerate different Ig expression levels (7,193).

As mentioned previously, Ig allelic exclusion serves as the genetic basis of monospecificity of B cells, a phenomenon comparable to the monospecificity of olfactory sensory neurons. Hundreds of odorant receptor genes are found scattered throughout the genome, each encoding an odorant receptor that binds to a different odorant molecule (reviewed in 194). Single neurons, however, express only one type of odorant receptor, allowing them to sense exposure to one particular odorant molecule. Monospecificity of olfactory sensory neurons may be critical to receive and process information about odors, especially with regard to the ability to distinguish between different types of odors and to recognize changes within the complex composition of scents. The sensing of differences between environmental stimuli could be the common requirement that governs the monospecific expression of cell surface-expressed recognition receptors on olfactory sensory neurons and B cells: the monospecificity of B cells and thus Ig allelic exclusion could be critical for immune recognition, i.e. the ability of the adaptive immune system to sense and discriminate different antigens. This might aid in choosing an appropriate response (tolerance/anergy or immune activation) and in efficiently defending against pathogens, as outlined in the following paragraphs.

One crucial aspect of immune recognition is self/non-self discrimination. In the adaptive immune system, self/non-self discrimination is established by various selection mechanisms operating during lymphocyte development to guarantee self-tolerance, among them receptor editing. As described previously, violations of IgL allelic exclusion during receptor editing allow for the escape of B cells co-expressing IgL alleles that give rise to one autoreactive and one innocuous BCR specificity. It is currently unclear whether incomplete receptor editing resulting in IgL allelic inclusion is indeed an efficient way to functionally inactivate the autoreactive BCR or whether it poses an autoimmune hazard.

In support of the first possibility, the innocuous LC chain is thought to compete with the autoreactive LC for binding of free HCs and thus suppress the assembly and surface

transport of the autoreactive BCR, thereby diminishing the recognition of self-antigens (29,195). In support of the latter, however, IgL allelic inclusion clearly increases the risk that B cells will secrete autoreactive antibodies, even if the cells are activated by the binding of antigens to the non-autoreactive BCR specificity. Therefore, IgL allelic exclusion could be critical for maintaining self-tolerance of the B-cell system. Accordingly, T cells frequently express allelically included TCRα chains, which may contribute to autoimmunity based on the dual specificity of their surface-bound TCRs (196).

In the complete absence of Ig allelic exclusion, each B cell would be capable of producing up to 12 different BCR specificities (polyspecificity), given that there are 2 IgH alleles whose products can be combined with 2 Igκ and 4 Igλ gene products (Fig. 5). Even though this would theoretically increase the representation of BCR diversity within the B-cell repertoire, once a polyspecific B cell is activated, the information regarding which BCR specificity is engaged by cognate antigen cannot be stored by the immune system, since all BCRs on the surface of one B cell use the same intracellular signaling pathways. Consequently, the activation of each polyspecific B cell would result in the upregulation and secretion of one antigen-targeted antibody and potentially up to 11 'passenger antibodies'. Some of these passenger antibodies may, by chance, be reactive against the same antigen (in particular if they share the same HC or LC with the primarily engaged antibody). More importantly, however, passenger antibodies may encode useless specificities, thus wasting production capacities and diluting the effective dose of the antigen-targeted antibody. In the worst case, passenger antibodies may be harmful, if they contain specificities against neutral antigens and self-antigens, which may cause allergies and autoimmunity, respectively.

Notwithstanding the above arguments, polyspecific B cells may be somewhat refractory to antigen-mediated activation of BCR signaling when compared to monospecific, Ig allelically excluded B cells (193). One reason for this could be that the effective density of each BCR specificity on the cell surface of a polyspecific B cell is diluted by the presence of the other specificities (Fig. 5). The strength of BCR signals determines not only the developmental fate of peripheral B cells (197,198) but also their relative fitness with regard to the competition for survival niches in germinal centers where hypermutated B cells are positively selected according to their affinities for antigens presented by follicular dendritic cells. Thus, polyspecific, Ig allelically included B cells may compete less efficiently and become under-represented within the peripheral B-cell repertoire.

In addition, given the higher number of expressed Ig alleles, polyspecific B cells would have a higher risk of generating at least one autoreactive BCR specificity during somatic hypermutation, which may lead to counter-selection in germinal centers. Indeed, multiple Igκ transgene copies are 'inactivated' during affinity maturation of B cells, suggesting that Igκ allelic exclusion is maintained through peripheral failsafe mechanisms involving the selection against allelically included B cells in favor of monoallelic Igκ expression (199). All of these selection mechanisms might prevent the accumulation of Ig allelically included B cells that may occasionally arise during the putative re-expression of RAG in peripheral B cells (200-203).

The monospecificity of B cells guarantees the monospecificity of secreted antibodies. In their monomeric form, antibodies contain two identical antigen-binding sites (bivalent), but higher order structures, such as IgM pentamers, occur frequently and contain up to 10 identical antigen-binding sites (multivalent). The bivalent to multivalent structure of antibodies serves to increase the avidity for their cognate antigens, which is especially crucial for antigen binding by primary IgM antibodies that have not yet been subjected to affinity maturation. In addition, multivalency enables antibodies to crosslink antigens, supporting the formation of immune complex aggregates. This enhances the activation of the

complement system and Fc-binding receptors and thus aids in initiating an effective immune response. In the absence of Ig allelic exclusion, B cells would be unlikely to efficiently produce multivalent antibodies, since the probability of incorporating the same antigen-binding site (i.e. HC/LC combination) more than once in one antibody molecule is much lower for polyspecific B cells than for monospecific B cells. For example, given that there can be up to 12 different HC/LC combinations in polyspecific B cells, the probability of containing the same antigen-binding site on both F(ab) arms of one antibody monomer would be only $12/12^2 = 0.083$. The probability of containing the same antigen binding site on each of ten F(ab) arms of one antibody pentamer would be only $12/12^{10} = 1.9 \times 10^{-10}$. Therefore, the majority of antibody monomers secreted by Ig allelically included B cells would be bispecific and thus monovalent, and most higher order antibody structures would have a lower degree of multivalency with regard to each of their multiple antigen specificities (Fig. 5). We think that the loss of multivalency could impair the efficiency with which antibodies bind, opsonize, neutralize, and eliminate invading pathogens. Interestingly, this consideration is irrelevant to TCRs that have a monovalent structure and are not secreted, perhaps providing an explanation as to why TCRα allelic exclusion in T cells is less stringently regulated than Ig allelic exclusion in B cells. Therefore, Ig allelic exclusion may have evolved to enhance the ability of immune recognition in B cells and to enforce the effectiveness of antibody-mediated immune responses.

Acknowledgments

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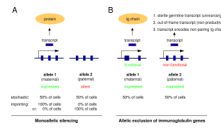
Fig. 1. Modes of monoallelic gene expression
(A) Monoallelic silencing can govern monoallelic gene expression. The choice as to which allele is silenced or expressed can be stochastic, resulting in 50% of cells expressing the paternal allele and 50% of cells expressing the maternal allele (e.g. IL4, Ly49 NK cell receptor, and TLR4 genes). Alternatively, the choice as to which allele is silenced or expressed can be 'imprinted' by the parental origins of the two alleles, resulting in 100% of the cells expressing either the maternal or the paternal allele (e.g. H19/Igf2 gene). (B) Allelic exclusion of Ig genes is not regulated by monoallelic silencing. On the contrary, Ig transcripts are expressed from both alleles; however under normal circumstances, only one transcript encodes a functional Ig chain. Functionality is defined by the ability of an Ig chain to become assembled into a surface-expressed BCR or pre-BCR. Non-functional Ig alleles are either unrearranged (encoding only sterile germline transcripts), non-productively rearranged (encoding out-of-frame transcripts), or productively rearranged but encoding a non-pairing Ig chain that is not assembled into a BCR or pre-BCR.
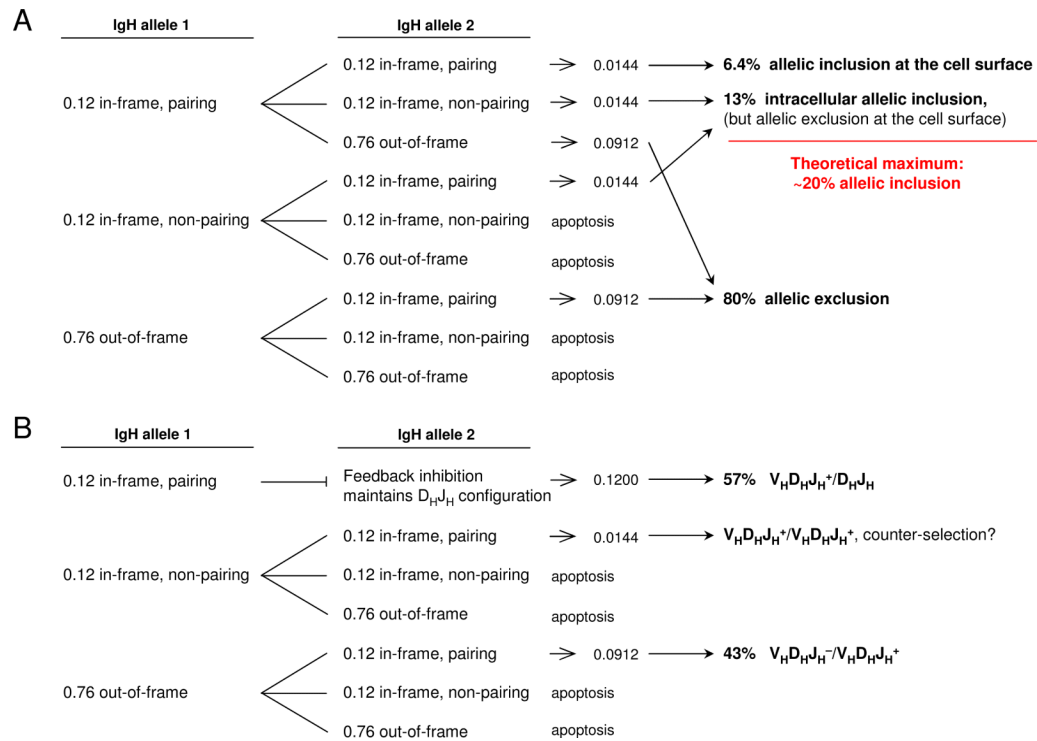
Fig. 2. Theoretical maximum of IgH allelic inclusion and the configuration of IgH loci in B cells
(A) The theoretical maximum of IgH allelic inclusion in B cells as predicted by the stochastic model (in the absence of any other regulation): Assuming that the probability of joining V_H-to-D_HJ_H in the correct reading frame is 0.33 (one out of three possible reading frames) and that all D_H segments can be used in reading frames one or two, but 80% of D_H segments carry stop codons in reading frame three (204,205), the probability of rearranging an in-frame $V_HD_HJ_H$ exon is $0.33 - (0.8 \times 0.11) = 0.24$. However, only half of all in-frame $V_HD_HJ_H$ exons encode a HC that is capable of pairing with SLC or LC (206). Therefore, the probability of generating a functional $V_HD_HJ_H$ exon (in-frame and pairing) for each IgH allele is 0.12. Consequently, the maximal frequency of IgH allelically included B cells is about 20%, since cells that do not rearrange at least one functional IgH allele die by apoptosis. Thirteen percent of B cells would produce two HCs, only one of which is capable of pairing with LC, thus showing intracellular IgH allelic inclusion but maintaining allelic exclusion at the level of BCR surface expression. (B) The configuration of IgH loci in B cells as predicted by the feedback inhibition model. Assuming the same probability of rearranging a functional $V_HD_HJ_H$ exon (in-frame and pairing) for each IgH allele as in (A), this model attributes the presence of B cells carrying the $V_HD_HJ_H^+$/D_HJ_H configuration to feedback inhibition signals triggered after the successful rearrangement of the first allele, thereby preventing V_H-to-D_HJ_H joining on the second allele. This results in a ~60/40 ratio of B cells with $V_HD_HJ_H^+$/D_HJ_H versus $V_HD_HJ_H^-$/$V_HD_HJ_H^+$ configuration. Notably, this model predicts that some cells (~6%) with intracellular IgH allelic inclusion ($V_HD_HJ_H^+$/$V_HD_HJ_H^+$) expressing one non-pairing and one pairing HC are generated initially. Indeed, these HC double-producers are detected among bone marrow pre-B cells but are under-represented among mature B cells ($< 1\%$), suggesting that they are counter-selected.
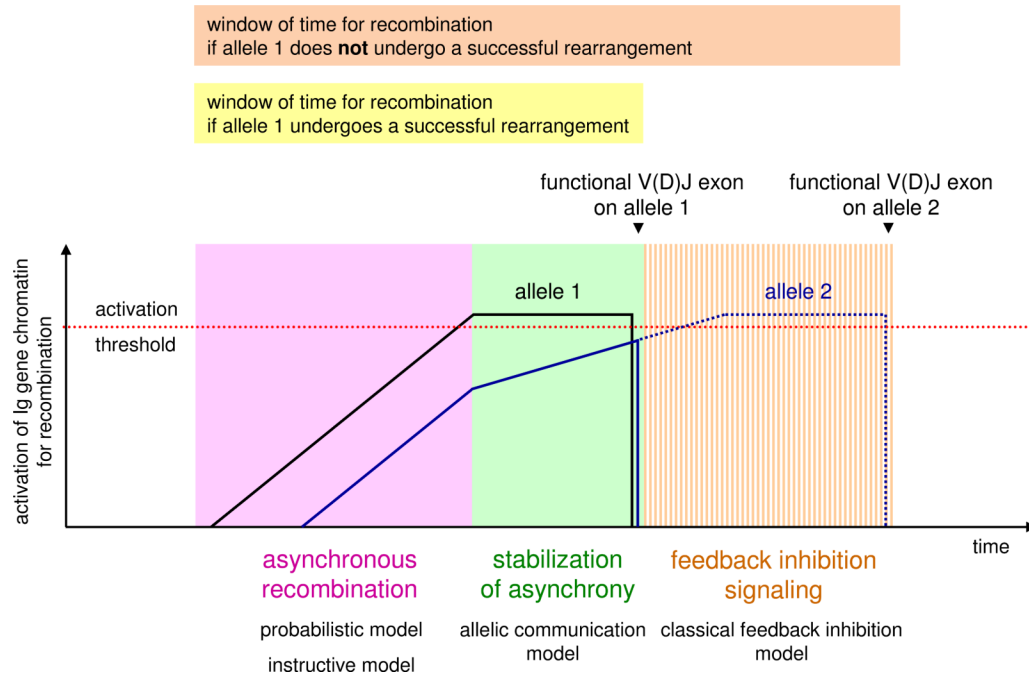
Fig. 3. Models to explain the establishment of Ig allelic exclusion

Ig allelic exclusion is established through asynchronous allelic recombination and feedback inhibition. Asynchronous allelic recombination can be achieved through slow, inefficient activation of Ig gene chromatin (probabilistic model, represented by a high activation threshold (red dotted line) and a low slope of the activation graphs for allele 1 and 2 (black and blue line, respectively). Alternatively, asynchronous allelic recombination can be achieved through ordered allelic recombination instructed by asynchronous replication timing of the two alleles (instructive model, represented by the offset between the activation graphs for allele 1 and 2). During recombination of allele 1, additional mechanisms may stabilize or enforce the asynchrony of allelic recombination (allelic communication model, represented by a diminished slope of the activation graph for allele 2). Following the successful rearrangement of allele 1 (i.e. upon generation of a functional V(D)J exon), feedback inhibition signals originating from surface-expressed BCRs or pre-BCRs suppress further recombination (represented by the drop in the activation graphs for allele 1 and 2). In the event that a non-functional V(D)J exon (out-of-frame or encoding a non-pairing Ig chain) is generated on allele 1, allele 2 will continue to undergo recombination.
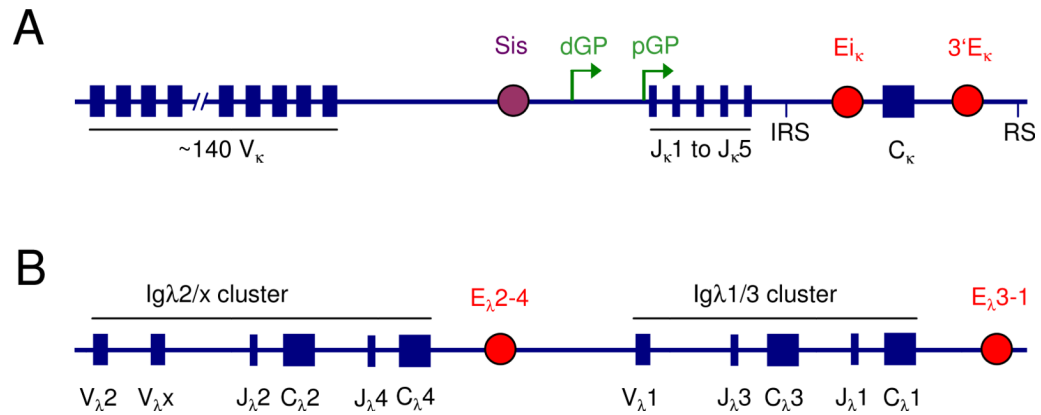
Fig. 4. Structure of Igκ and Igλ loci

(A) The Igκ locus contains 140 V_κ segments (~95 of which are functional), five J_κ segments (four of which are functional, since the $J_\kappa 3$ RSS is defective) and one C_κ exon. The recombination silencer sequence (Sis), the distal germline promoter (dGP), and the proximal germline promoter (pGP) are located within the V_κ-J_κ interval. There are two enhancers in the Igκ locus, one in the intron between the J_κ segments and the C_κ exon (intronic enhancer, Ei_κ) and the other downstream of the C_κ exon ($3'E_\kappa$). The intronic recombining sequence (IRS) and the recombining sequence (RS) ~25 kb downstream of C_κ can be utilized to inactivate the Igκ locus by deletional RS recombination (drawing not to scale). (B) The Igλ locus is comprised of two independently rearranging clusters of gene segments, the Igλ2/x cluster and the Igλ1/3 cluster. In contrast to the Igκ locus, the Igλ locus contains only three V_λ segments, each of which can be joined to only one or two J_λ segments, giving rise to a very limited λLC repertoire. Both the $V_\lambda 2$ and the (infrequently used) $V_\lambda x$ segments are rearranged to the $J_\lambda 2$ segment and utilize the $C_\lambda 2$ exon, giving rise to the λ2LC and λxLC isoforms ($J_\lambda 4$ lacks a consensus RSS, precluding the usage of the $C_\lambda 4$ exon). The $V_\lambda 1$ segment can be joined either to the $J_\lambda 3$ segment and utilize the $C_\lambda 3$ exon or to the $J_\lambda 1$ segment and utilize the $C_\lambda 1$ exon, giving rise to λ3LC and λ1LC isoforms, respectively. Each Igλ cluster is flanked by one downstream enhancer ($E_\lambda 2$-4 or $E_\lambda 3$-1) (drawing not to scale).
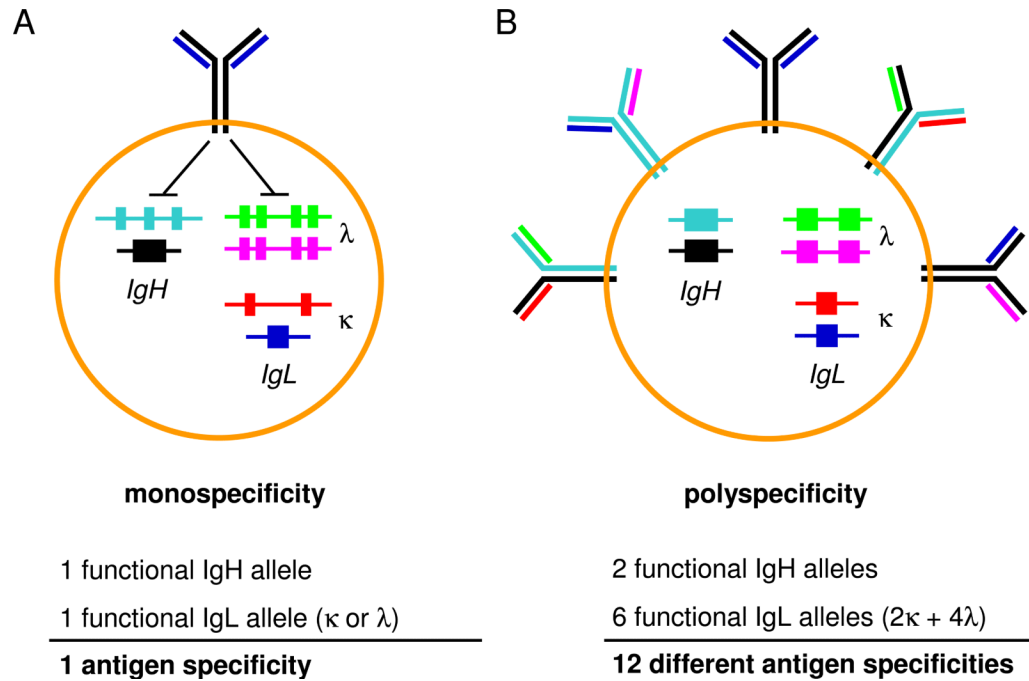
Fig. 5. Ig allelic exclusion and the monospecificity of B cells

(A) Ig allelic exclusion guarantees the monospecificity of B cells. Ig allelically excluded B cells produce only one functional HC and one functional LC, giving rise to BCRs or antibodies with only one particular antigen specificity (as there is only one possible HC/LC combination). (B) In the absence of Ig allelic exclusion, most B cells would be polyspecific, based on the capacity of Ig alleles in the murine genome to encode 2 different HCs and 6 different LCs, resulting in BCRs or antibodies with up to 12 different antigen specificities (as there would be $2 \times 6 = 12$ possible HC/LC combinations). Moreover, most BCRs and antibodies would be bi-specific, i.e. they would carry a different antigen binding site on each F(ab) arm.