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Review

Selection events in directing B cell development

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Summary. Homeostasis in the B cell compartment (as well as in T cells) is controlled by tightly regulated selection events. Throughout their life span, B cells are subjected to selection signals determining not only developmental progression, but also maturation and survival. It is now clear that most of these signals require the expression of B cell antigen receptor (or preB receptor) with functional signaling capacity. The administration of numerous mutations into the mouse germline enabled us to identify several checkpoints along the B cell developmental pathway, and provided us with powerful experimental tools to probe for selection events regulating developmental progression. In here, we will discuss recent studies in this field.

Key words: B cell development, B cell receptor, Receptor editing, Negative selection, Positive selection, Secondary immunoglobulin rearrangement

Selection at early stages of B cell development

The development of B cells in the BM is guided by successive immunoglobulin (Ig) gene rearrangements and antigen receptor assembly (Hardy et al., 1991; Boekel et al., 1995; Grawunder et al., 1995). However, commitment to the B cell lineage occurs early in development, prior to the initiation of Ig gene rearrangements. This commitment depends on the expression of Pax5 transcription factor (Nutt et al., 2001), but E2A and early B cell factor (EBF) are also required for B lymphopoiesis (Bain et al., 1994; Lin and Grosschedl, 1995). Mice deficient of Pax5 have complete block in B cell development at the preB-I stage (Nutt et al., 2001). Pax5-deficient B cell progenitors can grow in vitro in the presence of IL-7 and stromal cells, and can give rise to cells of other hematopoietic lineages such as T lymphocytes, in vivo and in vitro (Rolink et al., 1999). Commitment to the B cell lineage is followed by initiation of Ig gene rearrangements at the H chain locus. The construction of the H chain variable domain requires DNA recombination and ligation of V_H, D_H and J_H elements to form a functional and productive VDJ (Fig. 1A). It is allowed and directed by asymmetric recombination signal sequences (RSS), which are linked to the recombining elements (Brodeur et al., 1988). The RSSs are specifically recognized by the recombinase machinery, which is an enzymatic complex composed from several enzymes including recombination activating gene 1 and 2 (RAG-1 RAG-2), Terminal deoxynucleotidyl transferase (TdT), DNA ligase IV, Ku proteins and XRCC4 (Bassing et al., 2002). Most of these proteins are essential to the recombination process, and mice deficient of these enzymes have complete block or severely impaired B cell development (Bassing et al., 2002).

Productively rearranged VDJ enables preB-I cells to synthesize μH protein that can pair with the surrogate light (SL) chain components λ5 and VpreB to form the preB cell receptor (preBCR) (Rajewsky, 1996; Rolink et al., 1999). Surface expression of the preBCR requires association with the signal-transducing molecules Igα and Igβ (Rajewsky, 1996). The assembly and expression of the preBCR with signaling capacity confer an important checkpoint in B cell development. A functional preBCR, composed from μH and surrogate light chain (SL), is required for positive selection of preB I cells, and to signal for proliferative expansion (Rajewsky, 1996; Rolink et al., 2001). It also signals for down regulation of the V(D)J recombinase activity and for the establishment of allelic exclusion at the heavy chain locus (Grawunder et al., 1995; Loffert et al., 1996; Melchers et al., 1999). It is not clear what triggers preBCR signaling. Several studies have used signaling mutated or deficient mice and suggested that some form of preBCR aggregation is required to achieve both developmental progression and allelic exclusion (Meffre et al., 2000). Other studies suggested a putative preBCR ligand that is expressed on stromal cells (Bradl and Jack, 2001). Hence, it appears that preB-I positive selection is

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regulated by the assembly and expression of the preBCR. This is supported by additional in vivo and in vitro data, showing that not all μH chains are able to pair with the SL chain, resulting in formation of incompetent preBCRs (ten Boekel et al., 1997; Minegishi and Conley, 2001). Such preB-I cells are developmentally arrested and undergo ongoing V(D)J recombination at the H chain locus, attempting to express a new μH chain that pairs with the SL chain, and is capable to promote positive selection (Kitamura and Rajewsky, 1992; Ehlich et al., 1993; Papavasiliou et al., 1995; Loffert et al., 1996). Evidence for such secondary recombination is provided by detection of many peripheral B cells, in normal and γ2b transgenic (Tg) mice, carrying two in-frame VDJs (Roth et al., 1993; ten Boekel et al., 1998). In addition, some VH genes, such as V81X, are counterselected in adult BM, probably as a result of TdT activity in the CDR3 region, which limits the interaction with the SL chain and preventing preBCR formation. In contrast, V81X is positively selected in fetal liver, where TdT activity is not detected (Meffre et al., 2000; Rolink et al., 2001). Hardy and colleagues suggested that fetal liver B cells, in contrast to BM B cells, counterselect VH genes that associate with the SL chain (Wasserman et al., 1998). Collectively, positive selection promotes preB-I cells utilizing μH chains capable to pair with SL chain, and there would be a selection against μH genes that pair poorly with SL chain. In contrast, preB-I cells that fail to express appropriate preBCR, do not suppress V(D)J recombination and attempt to revise their preBCR by further DNA recombination and expression of a new μH (Kitamura and Rajewsky, 1992; Loffert et al., 1996; Melchers et al., 1999).

Positively selected preB-I cells undergo several divisions and enter the small non-cycling preB-II stage (Rolink et al., 1999). These preB-II cells re-activate V(D)J recombinase machinery and direct its activity to the light chain loci, first to kappa and later to lambda (Boekel et al., 1995; Grawunder et al., 1995; Rajewsky, 1996; Constantinescu and Schlissel, 1997; Engel et al., 1999). DNA recombination at the light chain loci aims to ligate VL and JL elements, using their asymmetric RSSs, to form the light chain variable domain VJ (Fig. 1B). This can occur by deletion or inversion of intervening DNA, depending on the transcriptional orientation of the recombining VL (Fig. 1B). Productive light chain recombination leads to BCR assembly and replacement of surrogate light chain in the BCR by Igκ or Igλ, and progression into an immature stage (Boekel et al., 1995; Melchers et al., 1999; Rolink et al., 1999). However, because V(D)J recombination is error prone, most of VJ ligations are out of frame and only 1/3 encode a productive light chain protein (Coleclough et al., 1981). Failure to express complete BCR (sIgM) blocks

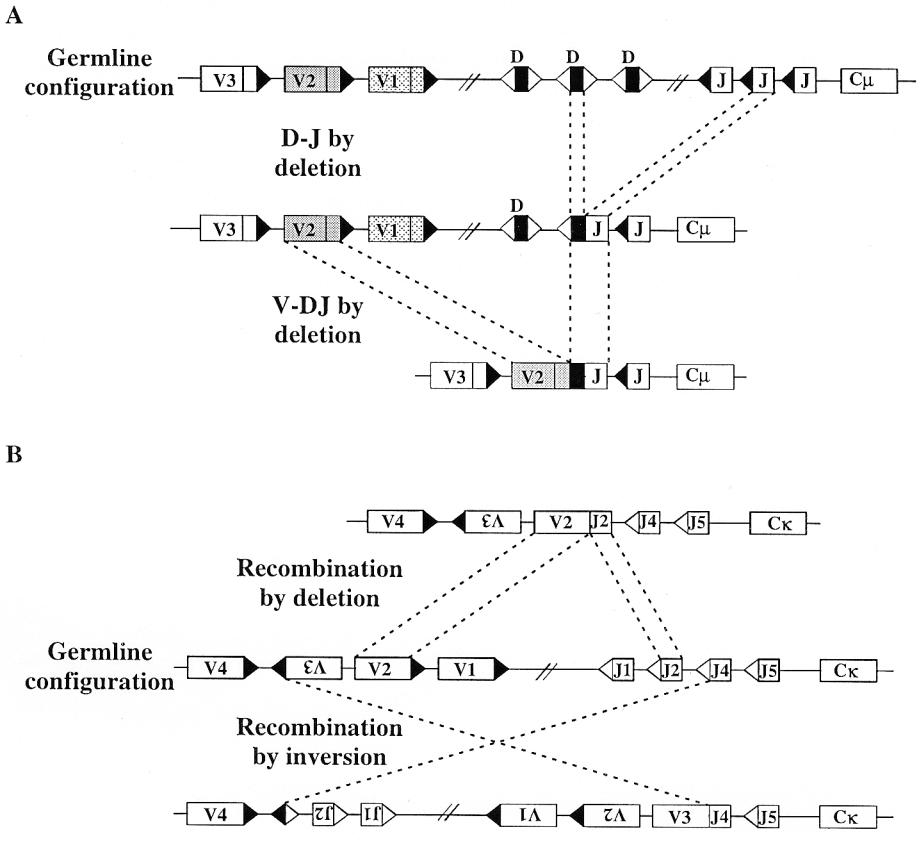

Fig. 1. Heavy and light chain DNA rearrangements. **A.** Germline configuration of heavy chain locus includes VH genes, DH genes and JH genes, all in same transcriptional orientation (top). Recombination is initiated at the proB stage by ligation of a DH and a JH elements to form a DJ, with deletion of the intervening DNA (middle). Subsequent recombination of a VH to DJ, to form a VDJ, occurs by deletion of the intervening DNA at the preB-I stage (bottom). **B.** Light chain recombination occurs at the small preB-II stage. Germline configuration of the kappa locus includes VL genes and JL genes (middle). Part of the VL genes are in the same transcriptional orientation as J such as V1 V2 and V4, and others are in different transcriptional orientation as J such as V3 (middle). VL and JL genes in same transcriptional orientation rearrange by deletion of the intervening DNA to form a VJ (top). VL and JL genes in different transcriptional orientation rearrange by inversion of the intervening DNA to form a VJ (bottom).

developmental progression into the immature stage (Meffre et al., 2000). Such developmental block may also be imposed as a consequence of miss pairing of particular heavy and light chain proteins, as 20% of preB-II cells express light chain protein in the cytoplasm but not on surface (Rolink et al., 2001). Cells that fail to express sIgM undergo secondary DNA recombination on the same or the other kappa allele, or at the lambda locus, in an attempt to productively rearrange and express light chain protein (Rolink et al., 1999). This is evident by the detection of 50% peripheral B cells carrying at least two rearranged kappa or lambda alleles. In most of these B cells, one allele has out of frame rearrangement or inactivated by recombining sequence (RS) recombination, whereas in 10% of them two productively rearranged alleles have been detected (Yamagami et al., 1999). Thus, because B cell development is programmed to fulfil preBCR and BCR assembly and expression, secondary DNA recombination at early stages of B cell development aims to facilitate the generation of competent receptor that can promote positive selection.

Selection in immature B cells

The expression of sIgM signals for termination of V(D)J recombination and establishment of allelic exclusion at the light chain loci. As this is the earliest point in B-cell development, at which complete surface IgM (sIgM) is expressed on the plasma membrane, immature B cells are now subjected to negative selection imposed by immune tolerance (Nossal, 1983; Monroe, 1996; Radic and Zouali, 1996). The process of negative selection occurring in bone marrow is very important in limiting autoantibody production. The clonal selection theory proposed by Burnet on 1959 predicted that such autoreactive cells will be deleted upon binding self-antigen. Early experiments with normal mice treated with anti IgM antibodies or with Ig transgenic (Ig Tg) mice supported this idea (Lawton et al., 1972; Nemazee and Burki, 1989; Hartley et al., 1991). It was later shown that negative selection and deletion are critically depending on avidity interactions and BCR signaling threshold (reviewd in Healy and Goodnow, 1998).

However, several studies with B cell lines and BM culture B cells showed that Ig gene recombination continues in high frequency despite the expression of BCR (Levy et al., 1989; Rolink et al., 1993). In addition, high frequency of DNA episomes, excised from the genome by V(D)J recombination as a result of nested κ rearrangements, contained VκJκ joints that were in frame (Harada and Yamagishi, 1991). These findings have raised the receptor editing hypothesis predicting that autoreactive immature B cells can undergo secondary Ig gene rearrangements to alter the BCR specificity. Receptor editing has been first studied in conventional immunoglobulin transgenic (Ig-Tg) mice expressing BCR specific to MHC or DNA (Gay et al., 1993; Radic et al., 1993; Tiegs et al., 1993). These studies showed that in the presence of self-antigen Tg B cells elevate recombinase genes and undergo secondary light chain recombination, effectively altering the B cell specificity (Gay et al., 1993; Radic et al., 1993; Tiegs et al., 1993; Hertz and Nemazee, 1997; Melamed and Nemazee, 1997). Lack of CD19 or CD45 did not have significant effect on receptor editing stimulated by multivalent membrane bound Ag (Buhl et al., 2000; Shivtiel et al., 2002). Subsequent studies have used targeted replacement mice (knock-in), in which Ig-Tgs were placed in their physiologic genomic context, to show that receptor editing is extremely efficient in altering autoreactive receptors (Chen et al., 1997; Pelanda et al., 1997; Pewzner-Jung et al., 1998). Secondary DNA rearrangements and receptor editing can occur at the heavy and the light chain loci, though through different mechanisms (reviewed in Meffre et al., 2000; Nemazee, 2000; Nemazee and Weigert, 2000). At the kappa locus, secondary recombination replaces the entire pre-existing VJκ by recombination of upstream Vκ with a downstream Jκ (Harada and Yamagishi, 1991; Yamagami et al., 1999), whereas cryptic recombination signal sequences, embedded in many heavy chain genes, allow upstream VH genes to recombine with pre-existing V(D)J to produce hybrid VH genes (Kleinfield et al., 1986; Reth et al., 1986) (Fig. 2). Another way that the organization of the kappa locus promotes receptor editing is by the existence of a conserved recombination sequences (RS), which upon recombination inactivates the active κ allele and directs ongoing gene rearrangements to the second κ or to the λ loci (Radic and Zouali, 1996; Hertz and Nemazee, 1998; Meffre et al., 2000). Studies both in normal and Ig targeted mice showed that RS recombination occurs frequently during B cell development (Pelanda et al., 1997; Retter and Nemazee, 1998), and 40-60% of sorted IgM+/λ+ B cells bear an in-frame Vκ-Jκ rearrangement inactivated by RS recombination (Retter and Nemazee, 1998). Thus, autoreactivity can be controlled by clonal selection, which determine death or survival of a clone, or by receptor selection, which regulates the process of V(D)J recombination and alter or fix receptor specificity.

Appreciation of receptor editing as a main mechanism in negative selection and in controlling of B cell autoimmunity has been established using in vitro BM culture systems. It has first been shown that autoreactive Ig-Tg immature B cells encountering self-antigen are developmentally arrested (Hartley et al., 1993; Melamed and Nemazee, 1997), and 50-66% of these cells undergo receptor editing (Melamed and Nemazee, 1997). Additional studies suggested that receptor editing and apoptosis are developmentally regulated in B cells. Using IL-7-driven BM cultures, prepared from anti MHC 3-83Tg mice, it was shown that immature Ig-Tg B cells progress form receptor editing competent, apoptosis resistant stage into receptor editing incompetent, apoptosis sensitive stage (Melamed et al., 1998). Other studies in non-Tg B cells suggested that negative selection, by receptor editing or apoptosis, is

determined based on site of antigen encounter and the presence of Thy 1^{dull} cells in the BM (Sandel and Monroe, 1999; Sandel et al., 2001). More recently, receptor editing was found to be the preferred mechanism of B cell tolerance in anti-DNA knock-in H+L transgenic mice, and that the efficiency of receptor editing was directly related to the number of available Jκ segments on the expressed Vκ allele (Yachimovich et al., 2002). Nussenzweig and colleagues proposed that receptor editing has also a major contribution to the antibody repertoire. Using mice carrying human and mouse Cκ genes they show that about 25% of the antibody molecules are produced by light chain gene replacement (Casellas et al., 2001). This is particularly important because accessibility to V(D)J recombination is affected by several factors such as differences within recombination signal sequences, promoter regions and other cis-acting regulating elements, resulting in overrepresentation of certain genes (Bassing et al., 2002). Thus, secondary recombination may promote higher degree of randomness in Ig gene usage (Casellas et al., 2001).

Selection in the periphery

B cell receptor expression and signaling is also critically involved in the process of maturation. Recent studies revealed significant stage-dependent differences in BCR signaling molecules (Benschop et al., 2001) and formation of lipid rafts (Sproul et al., 2000), between mature and immature B cells. Studies in BCR-signaling-deficient mice have shown that lack of Btk, SLP-65, Lyn, or Vav, and in the presence of non-functional Igß, B cells are developmentally arrested and/or have impaired maturation (Khan et al., 1995; Chan et al., 1997; Jumaa et al., 1999; Reichlin et al., 2001; Tedford et al., 2001). This implicates that only B cells with "appropriate" receptors can survive and be selected into the peripheral pool. In a normal mouse, about 90% of the produced B cells are lost at this stage of development (Lu and Osmond, 1997). This loss is attributed to both negative selection and/or lack of positive selection. Immature B cells encountering self-antigen in the periphery undergo apoptosis or become anergic (Goodnow et al., 1988; Russell et al., 1991). Those, however, can be rescued by appropriate T cell help (Fulcher et al., 1996), or by challenging with self-mimicking foreign antigens (Kouskoff et al., 2000). Also, mice deficient of syk or carrying Igα lacking the cytoplasmic tail have increased loss of B cells at this developmental check point (Torres et al., 1996; Turner et al., 1997), perhaps due to increased levels of tyrosine phosphorylation (Torres and Hafen, 1999). That B cell maturation is also regulated by positive selection is supported by the fact that VH gene repertoire in BM B cells is different than that found in peripheral B cells (Gu et al., 1991). It is now thought that proper assembly and expression of an oligomeric BCR generates some basal phosphorilation required for

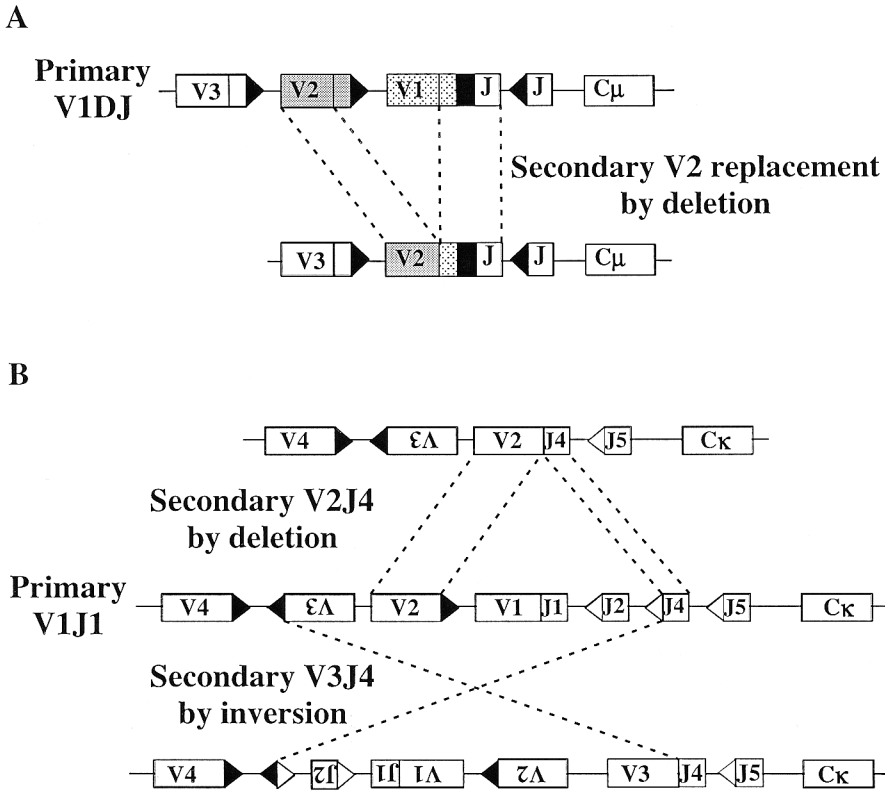

Fig. 2. Secondary recombination at the heavy and light chain loci. **A.** Secondary recombination at the heavy chain locus occurs by VH replacement, when upstream VH rearranges into embedded heptamers and replacing most of the VDJ. Primary V1DJ (top) is formed as described in figure 1A. Secondary recombination of V2 into the V1DJ replaces most of V1 and resulting in an altered VDJ (bottom). **B.** Secondary nested rearrangements at the kappa locus can occur by direct recombination of an upstream VL to downstream JL, extinguishing the primary V1J1 shown (middle). When VL genes are in the same transcriptional orientation as JL, recombination occurs by deletion of the intervening DNA fragment (top). If the VL has a different transcriptional orientation relative to JL, recombination occurs by inversion (bottom).

selection and splenic maturation (Loder et al., 1999; Meffre et al., 2000; Reth et al., 2000). In the absence of CD19, which is a positive regulator of BCR signaling, B cell maturation and selection into the marginal zone is impaired (Buhl et al., 2000; Martin and Kearney, 2000; Shivtiel et al., 2002). Using Ig-Tg mouse models, which limit the B cell repertoire, it was recently shown that B cells expressing signaling incompetent receptors, deficient of CD19 or Lyn, have increased number of edited cells in the periphery (Buhl et al., 2000; Meade et al., 2002; Shivtiel et al., 2002). In vivo and in vitro analysis of these mice revealed that positive selection of immature B cells is impaired due to incompetent BCR signaling (Shivtiel et al., 2002). Most interestingly, these B cells elevate V(D)J recombination and undergo antigen-independent secondary DNA rearrangements (Shivtiel et al., 2002). It appears therefore, that competent BCR signaling is critical for positive selection at this developmental checkpoint. B cells that fail to fulfil appropriate signaling requirements re-activate the V(D)J recombination machinery in an attempt to rearrange new receptor that is capable to promote positive selection. Hence, secondary Ig gene rearrangements may be attributed to both negative and positive selection of B cells.

Surprisingly, secondary V(D)J recombination was also detected in mature germinal center (GC) B cells following antigenic immunization or upon stimulation with a combination of IL-4 and LPS, or IL-4 and CD40 (Han et al., 1996, 1997; Hikida et al., 1996; Papavasiliou et al., 1997). These Ig gene rearrangement attempts were termed receptor revision (Nemazee and Weigert, 2000). The GC is an anatomical site where activated B cells proliferate and differentiate to antibody producing cells, undergo heavy chain isotype switch and form immunological memory. It is also the site where somatic mutations and affinity maturation take place (Rajewsky, 1996). It is thought that receptor revision is activated in GC B cells exposed to antigens to which their receptors bind at low affinity (Nussenzweig, 1998), and that receptor revision has an important contribution to the generation of high affinity antibodies during affinity maturation (George and Gray, 1999; Nemazee and Weigert, 2000; Magari et al., 2002). That receptor revision is firmly linked to the germinal center reaction is supported by recent findings showing that GC B cells underwent receptor revision after somatic mutation was initiated (Brard et al., 1999; de Wildt et al., 1999; Wilson et al., 2000; Bellan et al., 2002). Such secondary recombination can occur at the light chain (Casellas et al., 2001; Li et al., 2001; Magari et al., 2002) or at the heavy chain loci (Chen et al., 1995; Itoh et al., 2000; Wilson et al., 2000), as described in Fig. 2. However, while receptor revision can significantly expand the B cell repertoire, expression of a new heavy or light chain may also provide these B cells with self-reactivity, as it has been shown in Fas-deficient (lpr/lpr) mice (Brard et al., 1999), lupus (Dorner et al., 1998) and RA (Itoh et al., 2000; Meffre et al., 2000).

Analysis of GC B cells undergoing receptor revision revealed many similarities to BM B cells including the expression of GL-7, IL-7R, SL chain components, and TdT (Han et al., 1997; Hikida et al., 1998; Meffre et al., 1998). These similarities raised the possibility that secondary recombination is going on in immature B cells that have migrated to the periphery. Using RAG-indicator mice, in which the expression of RAG can be monitored by co-expression of the green fluorescent protein (GFP), three independent studies showed that RAG/GFP appears only in peripheral B cells with immature $IgM^{lo/neg}$ phenotype (Kuwata et al., 1999; Monroe et al., 1999; Yu et al., 1999). More recently these RAG-indicator mice were used to show that RAG expressing immature B cells are recruited into the germinal center after immunization, but this recruitment is antigen-independent (Gartner et al., 2000; Nagaoka et al., 2000). Thus, the conclusion made by these studies is that most of RAG expression and activity in GC arise from immature B cells migrating from the BM and recruited into the spleen and lymph nodes. To reconcile this conclusion with studies demonstrating receptor revision in cells undergoing hypermutation it is necessary to assume that immature B cells can participate in the germinal center reaction, or that receptor revision in GC B cells is a rare event. Several studies in Fas-deficient mice showed that sIg- B cells, generated by a stop mutation in its expressed Ig-k gene, were able to participate in the germinal center response (Brard et al., 1999), and that μ-deficient proB cells could undergo isotype switch and mature in μMT/lpr mice (Melamed et al., 2000). In addition, sequencing analysis revealed that light chain revision may occur at very low frequency (>3%), and that V_H gene replacement does not accompany somatic mutations or that such cells are counterselected (Goossens et al., 2001).

Alternatively, it is possible that V(D)J recombination in GC B cells reflects lack of positive selection. B cell receptor expression and signaling is crucial for survival of mature B cells, because ablation of the BCR results in rapid death (Lam et al., 1997). Thus, somatic hypermutation in CDR or FW, which may affect the interaction between heavy and light chain proteins, may result in improper assembly of the BCR and failure to maintain continues positive signaling required for survival. There are several evidence that changes applied to CDR3 region (Meffre et al., 2000; Melchers et al., 2000; Minegishi and Conley, 2001; Rolink et al., 2001), or mutation in the lambda 1 constant region (Sun et al., 2002), affect the heavy and light chain pairing, the process of B cell selection and the antibody repertoire. In developing B cells, these changes stimulate secondary Ig gene rearrangement. It is yet to be determined whether these changes can activate V(D)J recombination and receptor revision in GC B cells. If true, than receptor revision can be considered as another salvage mechanism for B-lymphocytes, in addition to its contribution to the antibody repertoire.

Conclusions

B cell receptor signaling governs multiple developmental checkpoints determining both negative and positive selection of B cells. These checkpoints regulate developmental progression, differentiation and maturation of B cells in the bone marrow and the periphery. While the contribution of secondary Ig gene rearrangement to negative selection is well described, its contribution to positive selection is yet to be determined.

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