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Immunogenetic Aspect of B-Cell Antigen Receptor Diversity Generation

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Abstract

The generation of antigen receptor diversity, and thus of the B-cell repertoire, is the result of very complex immunogenetic mechanisms. So, this chapter gives a brief overview on immunoglobulin (IG) gene organization and gene rearrangements, as well as the main mechanisms of immunoglobulin repertoire development.

Keywords: B-cell antigen receptor diversity, immunoglobulin gene organization, immunogenetic mechanisms

1. Introduction

The recognition specificity of different non-self-antigens or defective self-antigens (tumors) by a well-defined B-cell clone does not result from the presence of an extensive number of receptor genes, but rather from immunogenetic mechanisms affecting a limited number of IG genes, including mechanisms of genetic recombination, mutations, deletions or insertions, and gene repair, through very complex regulatory mechanisms that are responsible for a large B-cell repertoire.

This chapter focuses on the molecular description of the immunogenetic mechanisms responsible for the generation of B-cell antigen receptor diversity.

2. IG gene organization and gene rearrangements

2.1 Gene segments and generation of functional IG genes

In humans, the immunoglobulin heavy (IGH) locus is present at chromosome 14q32.33, and the IG light lambda (IGL) and kappa (IGK) loci are located at 22q11.2 and 2p11.2, respectively [1]. The immunoglobulin heavy chain variable region (IGH V) gene encodes VH framework regions 1-3 (FR1-3), VH complementarity determining region 1 (CDR1), and VH CDR2, while IGH joining (IGH J) gene encodes VH FR4. VH CDR3 is positioned at the IGH V-IGH D-IGH J junction [2]. The constant sequences of heavy (H) chain are arranged in the following order: $\mu \rightarrow \delta \rightarrow \gamma 3 \rightarrow \gamma 1 \rightarrow \alpha 1 \rightarrow \gamma 2 \rightarrow \gamma 4 \rightarrow \epsilon \rightarrow \alpha 2$ in humans (**Figure 1**), or $\mu \rightarrow \delta \rightarrow \gamma 3 \rightarrow \gamma 1 \rightarrow \gamma 2b \rightarrow \gamma 2a \rightarrow \epsilon \rightarrow \alpha$ in mice.

Similar to T-cell receptors (TCRs), functional genes of immunoglobulins (Igs) are the result of somatic recombination of DNA containing the relatively limited germinal genetic information, using the so-called V(D)J recombination process that occurs between individual genes (also referred to as gene segments) of the variable domains of the H and L chains (or α , β , γ , and δ chains of TCRs). Each of these genes is present in multiple copies in most antigen receptor loci. The locus of IGH genes (like TCR β and TCR δ loci) contains variable (V), diversity (D), and joining (J) genes, whereas the IGL and IGK loci (like TCR α and TCR γ loci) contain only V and J genes [3]. So, individual V, D, and J genes at the IGH locus, and V and J genes at either the IGL or IGK loci rearrange somatically at the DNA level to generate V-D-J and V-J regions that, after transcription and translation, encode the variable domains of the antibody [4] (for review see [1]).

Key Point 1|Germline.

- Germline DNA is a constitutional DNA because it is related to the DNA of tissues derived from reproductive cells, i.e., egg or sperm that is derived from stem cells, and incorporated into the DNA of each cell of the body of the offspring. Therefore, the mutated parental germline DNA can be passed to the offspring.
- Germline DNA can be extracted from bone marrow or peripheral blood nuclear cells.

Key Point 2|Somatic DNA.

Somatic DNA is found in all cells of the body (tissues, skin, organs, and blood), except germ cells and embryonic cells, which are the source of gametes. Therefore, a mutation in the somatic DNA is not transmitted to the offspring, but it can lead to the genesis of diseases, especially tumors. So, somatic DNA can be extracted from both tumor (or matched normal) tissue and plasma.

2.2 Diversity of B-cell receptors: primary/preimmune repertoire of Igs: V(D)J/ somatic recombination

2.2.1 Ordered rearrangement model

The ordered model was described by Alt and Baltimore in 1984 [5]. It states that during the V(D)J recombination, rearrangements at the H chain locus occur before those of the L chains.

2.2.2 Sequential recombination

The sequential recombination refers to the order of the stages of rearrangements from DH to JH occurring before rearrangements from VH to DJH [5]. In contrast, the genes of the TCR δ locus, in the precursors of T-cells, can recombine in any order. In the case of the IGH locus in B-cell precursors (and TCR β locus in T-cell precursors), one of the multiple DH segments (genes) recombines first with one of

Figure 1.
The organization of the IGH chain C-region genes in humans. E μ : intronic enhancer, S: switch region, IGH: immunoglobulin heavy chain.

Figure 2.
 Ordered rearrangement of gene segments (adapted from [3]). In the ordered rearrangement model, rearrangements of gene segments at the H chain locus precede those at the L chain locus. Sequential rearrangements lead first to the recombination of one of the DH segments with one of the JH segments, and then a VH segment is recombined with the DJH recombination. Although domain D is absent, the principle of IG light chain loci recombination is similar. Ig H chain: immunoglobulin heavy chain, VH: heavy chain variable genes, DH: heavy chain diversity genes, JH: heavy chain joining genes, CH: heavy chain constant genes

the multiple JH segments, by deletion of the DNA separating them. Then, one of the VH genes is recombined/juxtaposed with the rearranged DJ site, again by deletion of the intermediate DNA (**Figure 2**). After recombination, V, D, and J genes form a single exon [3].

2.2.3 Combinatorial diversity, “P” junctional variability, “N” diversity, and diversity by genetic substitution

Like the TCR, B-cell receptor (BCR) diversity results from (i) the choice of segments to recombine, (ii) the “P” junctional variability of the nucleotides at the V-D-J or V-J junction sites between gene segments during rearrangements, (iii) the “N” diversity by insertion or deletion of a nucleotide during recombination under the action of terminal deoxynucleotidyl transferase (TdT), but also from (iv) the recombination between VH genes by substitution of a or part of a second VH gene to an already recombined VH-D-JH segment. The diversity of the B-cell repertoire is also increased by the process of somatic hypermutation (SHM) of IG variable genes. The V(D)J recombination process generates a much greater diversity at the level of the H chain loci compared to those of the L chains, where simply a V region is joined to a J region. Thus, if the human IGH locus contained only about 40 functional VH segments, 27 DH segments, and 6 JH segments, V(D)J recombination would generate about 6480 ($40 \times 27 \times 6$) H chains, whereas, human IGK, which contains about 40 Vκ genes and 5 Jκ genes, would give rise to approximately 200 κ chains, following 200 (40×5) different combinations. On the other hand, the variability of nucleotides/point mutations can occur at the junction sites, and bases can be lost (by deletion) or added (by insertion), giving additional diversity of the hypervariable CDR3 region of the H and L chains, which is coded by an additional sequence of DNA created by the junction of V, D, and J segments, for the H chain, and V and J segments, for the L chain; such a phenomenon is responsible for the junctional

diversity. In total, molecular mechanisms of genetic recombination could result in a potential repertoire of at least 10^7 antigen-specific recognition sites/receptors. Each clone of such a cell repertoire contains only a few cells that are capable of recognizing only one antigen; exceptionally, a T-cell clone can express two different receptors and can therefore recognize up to two antigens.

2.2.4 Rearrangements to a nonfunctional allele

Two-thirds of rearrangements produce a nonfunctional allele for at least three main reasons: (i) the reading frame of V and C regions is correctly aligned in only one-third of the cases, (ii) the codons contain three-nucleotide, and (iii) the number of nucleotides inserted or eliminated in the junctions is essentially random.

2.2.5 Diversity of receptors in the case of loci with a single V, D, or J segment

Some loci include only one V, D, or J gene segment. In these cases, all diversity is derived from junctional diversity or subsequent mechanisms of diversity, such as somatic mutation in IG loci, or from gene conversion in IG loci of some species.

2.2.6 D and J segments-CDR3 loop/IgH chain

D and J segments/genes encode amino acid sequences of the third loop of the immunoglobulin domain, which corresponds to the CDR3 region. If they had the same reading frame, recombination can give rise to an IgH chain.

2.2.7 Transcription and generation of functional messenger RNA

The transcription of the recombined IG gene gives rise to a functional messenger RNA, after elimination of introns, including any J segment/gene located between that which is joined to D and C segments. A similar process takes place in L chain loci.

2.2.8 Molecular mechanisms of rearrangements: DNA double strand-breaks and repair

2.2.8.1 Enzymatic mechanisms of recombination: RAG and RSS

Rearrangements require two major steps: double strand-breaks (DSB) and repair of these breaks.

Figure 3. Cleavage site of RAG proteins at the V, D, and J gene segments of the IGH locus: RSS positions. RAG: recombination-activating gene, RSS: recombination sequence signal, IGH: immunoglobulin heavy chain locus.

Figure 4.
RSS motifs. There are two types of RSS, one includes a 12-nucleotide spacer (12-RSS), and the other includes a 23-nucleotide spacer (23-RSS). Both 12-RSS and 23-RSS include a highly conserved palindromic heptamer and nonamer sequences. Bp: base pairs, RSS: recombination sequence signal.

Both recombination-activating gene 1 (RAG-1) and RAG-2 recombinase enzymes, expressed exclusively in developing lymphocytes, are required to generate DSBs [6] at the level of recombinant signal sequences (consensus RSS, recombination sequence signal), flanking all functional V, D, and J gene segments, on the side that will be joined, *i.e.*, on the 3' side of V segments, on both sides of D segments (3', 5'), and on the 5' side of J segments [7] (**Figure 3**).

2.2.8.2 Architecture of RAG-induced DSBs: the 12/23 rule

The RSS motifs are composed of a conserved palindromic heptamer and conserved nonamer motifs, separated by an intervening variable-sequence spacer of fixed length corresponding to 12 or 23 nucleotides (the resulting signals are referred to as 12-RSS or 23-RSS, respectively). From the architectural point of view of the H chains, all the V segments are tracked by a 23-RSS (on the 3' side), the D segments are “framed” (on the 5' and 3' sides) by 12-RSS, and the J segments are preceded by 23-RSS (on the 5' side). Regarding the L chain genes, the V segments are tracked by 23-RSS (on the 3' side), and the J segments are preceded by 12-RSS (on the 5' side) [8]. Only dissimilar RSS associations are efficiently recombined. Thus, each recombination that joins two gene segments occurs between 12-RSS and 23-RSS: this is known as the 12/23 rule. In the H chain recombination, the fact that the V and J segments are naturally both flanked by 23 nucleotide spacers (23-RSS), a connection between these two segments is not possible directly, but is done indirectly if they recombine with D elements, which are flanked on both sides by 12-RSS. After 12-RSS recombination with 23-RSS, the intermediate DNA will either be deleted or inverted depending on the orientation of the two signals (**Figure 4**). RAG-induced DSBs are then resolved by nonhomologous end joining (NHEJ) pathway.

2.2.8.3 RAG action and NHEJ repair

The assembly between the V, D, and J segments is done according to the sequential model as reported above. The V(D)J recombination mechanisms can be

generated experimentally *in vitro* by mixing DNA (as an enzyme substrate) with the endonuclease RAG-1 and RAG-2 proteins.

The kinetics of main rearrangement events are described according to the following steps (**Figure 5**):

- a. *Formation of a synapse*. RAG forms synaptic complexes, only with one 12-RSS and one 23-RSS, according to a 12/23 dogma that governs the recombination fidelity, and then the rearrangements are started [9].
- b. *RAG and RSS binding*. A key role is attributed to RAG-1 in DNA binding and catalysis, as well as in interactions with RAG-2, high-mobility group box 1 and 2 (HMG1/2), and itself, given its structure that contains a nonamer binding domain (NBD) required for stable recruitment of RAG proteins into RSS [10]. So, RAG-1 recruit RAG-2 after binding of an RSS and then RAG-2 maintains the RAG-1-RSS complex and binds to a second RSS.
- c. *DSB generation*. The two RAG-RSS complex will generate DSB at the level of the two RSS (between a gene segment and an RSS), by first a DNA strand cleavage, between the 5' end of the RSS heptamer and the region encoding the antigen receptor and then the other DNA strand, after reaction of the free 3'-hydroxyl (3'-OH) group with a phosphodiester bond of this strand.
- d. *Formation of hairpin structures*. Nucleotides of the cleaved-end coding sequences are covalently bound, thereby forming hairpin structures, and will be maintained within synaptic complexes.
- e. *Hairpin opening-DNA synthesis and planning of extremities*. The hairpin ends are opened and rendered straight by DNA synthesis or nucleolytic cleavage planning using a nuclease, which would appear to be "Artemis." The hairpin cleavage reaction can be done in two points: it can either be cleaved in the middle or a few nucleotides on one side of the center, producing offset cuts.
- f. *Formation of N regions*. During IGH V-IGH D and IGH D-IGH J joining [2], TdT, an important enzyme required for the junctional diversity generation, can intervene, before the juxtaposition of the two coding regions, to generate a higher level of diversity (see above), by catalyzing the elimination of nucleotides and/or the addition of nontemplated nucleotides to the 3' ends, which will produce the so-called N regions (nongermine/nontemplated nucleotide) [11]. So N1 and N2 regions are created between the V and D genes and between the D and JH genes, respectively.
- g. *Deletion/insertion of P nucleotides*. Deletions or insertions of short complementary palindromic sequences, so-called P nucleotides, are generated through endonuclease activity and repair around the asymmetric opening of hairpin loops that form at the ends of the gene segments to be joined as part of the rearrangement process and produce short, self-complementary single stranded extensions that can be incorporated into junctions, or may alternatively be removed *via* exonuclease activity [12] (for review, see [13]).
- h. *NHEJ ligation*. The nonhomologous DNA ends of the two coding regions are repaired/ligated using an NHEJ repair system, generating, on the one hand, coding joints, in which the gene segments are joined [14, 15], and, on the other hand, RSS-containing signal joints, resulting from the direct joining of the DSB

Figure 5. Molecular mechanisms of V(D)J recombination and junctional diversity generation (adapted from [3]). (A) Main steps of V(D)J recombination. (B) Edge modification of coding regions and junctional diversity. DNA-PKcs: DNA-dependent serine/threonine protein kinase complex, DSB: double strand-breaks, N: nongermine/nontemplated nucleotide, P: short complementary palindromic sequences, XRCC4: X-ray cross-complementing gene 4.

bordering the RSS heptamer. When the two gene segments that are joined have the same orientation, which is observed in most cases, the signal joint is excised on a circular DNA segment and generates a circular episome (an extrachromosomal circular DNA) located between the two coding regions, which later disappears from the cell [16–18].

The junction system comprises a number of ubiquitous repair proteins (present in both B-cells and T-cells), which would allow rearrangement of IG genes in B-cell precursors, but rarely in T-cell precursors, and TCR gene rearrangements in T-cell precursors, but rarely in B-cell precursors. It includes in particular the catalytic subunit of a nuclear DNA-dependent serine/threonine protein kinase complex (DNA-PKcs), a member of the phosphatidylinositol 3-kinase-related (PIKK) family of protein kinases, composed of a heterodimer of Ku proteins that bind free DNA ends given their strong affinity (Ku70/Ku80 [encoded, respectively, by X-ray cross-complementing gene 5 (XRCC5) and XRCC6 genes in humans, and also called Ku86]) [19], XRCC4, DNA ligase IV and Artemis [3]. TdT is also recruited into the junction system and is involved in the formation of the coding joints, alongside Artemis and DNA-PKcs. Nevertheless, TdT is only rarely recruited into rearrangements that occur during fetal life, so that junctional diversity is limited.

2.2.9 Molecular mechanisms of regulation of V(D)J rearrangement

2.2.9.1 Transcription-sequential/ordered model-of germinal loci and accessibility by RAG-1 and RAG-2 proteins

It should be clearly noted that the VH segments are not silent before the V-DJH recombination steps or before their physical juxtaposition with the E μ enhancer. It has therefore been shown that they undergo active noncoding germline transcription in B-cell precursors. In addition, many other noncoding transcripts appear as rearrangements occur, in order to allow the opening of chromatin, and thus, the targeting and accessibility by RAG-1/RAG-2 complex, as well as the establishment of the three-dimensional structure of the locus considered. From a kinetic point of view, the first noncoding DH transcripts, also referred to as sterile transcripts (to differentiate them from the coding transcripts, which are initiated at the rearranged VDJ segments), are detected before the D-JH rearrangements and are initiated at JH-proximal DH gene (DQ52), which has both promoter and enhancer activities preferentially active in B-cell precursors [20], generating $\mu 0$ transcripts, and at downstream of intronic IGH enhancer E μ , generating I μ transcripts. Both $\mu 0$ and transcripts I μ are getting spliced and polyadenylated [21]. Once the DJH rearrangement is carried out, new noncoding germinal transcripts appear in VH regions (for review, see [21]).

2.2.9.2 Regulation of transcription and epigenetic modifications

Epigenetic modifications are necessary for the positive or negative regulation of the activities of different loci. Indeed, besides the presence of sites sensitive to DNase activity, other conditions, controlled by the activating elements and the promoters of the loci concerned, are necessary to initiate gene rearrangements and are correlated with the opening of chromatin to transcription, such as histone acetylation, DNA demethylation, and transcription itself.

- *Acetylation of histones.* It occurs prior to V(D)J recombination. It is associated with the VH region after IL-7 stimulation and DJ rearrangement before VH appendage to DJH and is accompanied by increased nuclease sensitivity and reorganization of nucleosome structure [22] (for review, see [23]).
- *DNA methylation/demethylation near RSS.* DNA methylation/demethylation is involved in regulating V(D)J rearrangement. DNA methylation around the RSS inhibits V(D)J cleavage activity of the RAG-1/RAG-2 complex (depending

on the position of the mCpG around the RSS) and induces an inaccessible chromatin configuration. Conversely, CpG demethylation in the heptamer of broken signal ends derived from the 3' D β 1 RSS has been shown to allow V(D)J cleavage in mouse [24].

2.2.9.3 Establishment of loops-Rosette locus-and contacting segments to recombine

The establishment of loops is necessary to bring into contact the different segments to recombine giving rise to an image of the so-called rosette locus, which represents one of the prerequisites for V(D)J rearrangements [25]. These loops take place through a number of factors, including transcriptional repressor CCCTC-binding factor (CTCF, also known as 11-zinc finger protein), Yin Yang 1 (YY1), a ubiquitously distributed transcriptional repressor/activator factor of a number of promoters, belonging to the GLI-Kruppel class of zinc finger proteins, and paired box 5 (Pax5), which is important regulators in early development, but not late stages of B-cell differentiation. Such different factors and particularly CTCF, by binding to cohesins, regulate the IG loci reorganization and contraction. This contraction/reorganization allows the juxtaposition of different gene segments (in particular the distal V genes) and thus facilitates rearrangements.

2.2.9.4 Regulation of loci position at the nucleus

The locus nuclear positioning is decisive for the rearrangement. Hence, the IGH locus, anchored *via* the distal VH genes at the nuclear periphery, migrates, in its extended chromatin state, to the center of the nucleus, which facilitates access of the V(D)J recombinase to proximal IGH domain and thus VH-DJH rearrangements.

3. Allelic exclusion

The allelic exclusion of IG of H and L chain genes allows the production of antibodies from a single chromosome located on 14q32.3 for the H chain [26], and one of the two chromosomes located on 2p12 or 22q11.2 for the L chain [27, 28] (respectively, the L κ and L λ chains). This phenomenon constitutes genetic basis of monospecificity of B-cells-a central paradigm in explaining the pathogen-specific production of antibodies (Burnet's clonal selection theory of the adaptive immune system), *i.e.*, each clone of B-cells generates a unique specificity for the appropriate antigen, which is established during the rearrangement of V(D)J gene segments of the variable regions.

During the differentiation of the B-cell, only one fraction of the IG genes, resulting from a first somatic V(D)J recombination on one of the two random chromosomes 14, is functional, *i.e.*, it contains a productive exon V(D)J. By cons, if the rearrangement is abortive (nonproductive), a new recombination is attempted on the other chromosome. The success toward a productive rearrangement of the H chain leads to a temptation to rearrange with the chromosomes encoding the L chains. The absence of rearrangements leads to the sterility of B-cell.

The mechanism of allelic exclusion uses pre-BCR-mediated signals. The pre-BCR consists of the H chain resulting from the productive rearrangement of an allele encoding the μ H chain associated with a pseudo-L chain (surrogate L chain). This chain comprises a V polypeptide (called V pre-B) and a type C polypeptide (called λ 5 in mice and λ -like in humans) that associate noncovalently. The signals

mediated by this pre-BCR block the accessibility of the RAG recombinases on the second allele of the nonrecombinant μ H chain and redirect them toward the $L\kappa$ chain locus to initiate the first recombinations. The formation of a complete BCR combining H and L chain blocks recombinations on other L chain alleles.

Importantly, it has been shown, using genetically engineered mice that carry two functional IGH alleles that are completely recombined and different, that the expression of IG loci does not appear to be monoallelic and that B-cells could have the ability to express H chains by both alleles [29] (for review, see [30]).

4. Isotypic exclusion

A single B-cell never expresses both a κ string and a λ string. The first recombination attempt for the L chains takes place at one of the two κ genes. In case of failure, the κ gene of the other chromosome 2 or the λ genes is used.

5. Mature B-cell activation: immunoglobulin class/isotype switching and somatic hypermutation

5.1 Isotype switching: a process affecting the constant region of the immunoglobulin H chain

The B-cell that initially produced the IgM isotype will subsequently produce other immunoglobulin isotypes (IgG, IgE, and IgA), thanks to a process termed Ig H chain class switching, isotype switching, or class switch recombination (CSR).

Figure 6. CSR process. Here is an example of switching to the IgG $_1$ isotype. First, AID induces DSB formation after deamination of S μ and S γ_1 regions. Subsequently, these two regions recombine by an intrachromosomal deletional recombination, and the expressed VDJ segment associates with the C γ_1 gene. AID: activation-induced cytidine deaminase, CSR: class switch recombination (also called immunoglobulin heavy chain class switching/isotype switching), DSB: double strand-breaks, E μ : intronic enhancer, S: switch region.

Such a phenomenon occurs after activation of a mature B-cell by an appropriate antigen, thereby generating different antibody isotypes that have the same variable domains as the original antibody generated in the immature B-cell during V(D)J rearrangement, but having distinct constant domains in their H chains.

CSR is instigated following conversion of deoxycytidines in S regions—a G rich with high density of WGCW (A/T-G-C-A/T) motifs—to deoxyuracil by activation-induced cytidine deaminase (AID) in the IG loci, which is also required for SHM. The presence of deoxyuracil promotes DNA mutagenesis through a subset of DNA repair proteins. Deoxyuracil residues are subsequently removed from DNA by enzymes of the base excision repair (BER) and mismatch repair (MMR) complex MSH2/6 pathways, leading to mutations, single-strand DNA breaks (SSBs), and the DSBs required for CSR. Recall that, in humans, nine functional CH genes are located downstream of the V, D, and J gene segments of antigen receptor loci. The V(D)J segment, initially rearranged in the bone marrow, can be juxtaposed, during B-cell activation, to one of these functional genes coding for another constant domain, depending on antigen and the cytokine milieu, and occurs between DSBs introduced into the donor μ S (S_{μ}) region and a downstream/acceptor S region located from ~65 to 160 kb downstream, which can subsequently recombine with an S region farther downstream (for review, see [31–33]). Finally, Igs resulting from the CSR process have the same specificity for the antigen responsible for the B-cell activation. They have also the same L chains as well as the same variable fragments of the H chains (**Figure 6**).

5.2 Somatic hypermutation: mutations take place on the variable segments of L and H chains

5.2.1 Brief anatomo-histological overview of B-cells

5.2.1.1 Primary follicles and interplay between B-cells and FDCs

Many resting B-cells, agglutinated around follicular dendritic cells (FDCs), harbor primary follicles. Thus, in the adult spleen, about 80% of B-cells are follicular B-cells.

A major role is attributed to FDCs, as prominent stromal cell constituents of B-cell follicles. These cells do not express major histocompatibility complex class II (MHC II) molecules nor do they have the capacity to phagocytose and process exogenous antigens for MHC I-restricted presentation [34]. Experiments using cryoimmunogold electron microscopy have demonstrated that the presence of MHC II molecules on their surface is passive and originate from microvesicles/exosomes they are attached to [35]. Additionally, their ontogeny remains controversial. They are not derived from the bone marrow hematopoietic stem cell, but they could originate from local mesenchymal precursors in lymphoid organs [36]. Moreover, FDCs promote the survival and continuous recirculation of naive B-cells and allow for the attraction of activated B-cells, as well as the selective process for affinity maturation within the GC of lymphoid follicles during humoral adaptive/antibody-mediated immune response, allowing activated B-cells to significantly improve the affinity of their BCR. Thus, they have the ability to retain on their Fc receptor (FcR) antigens in native form combined with antibodies (immune complexes [ICs]), for long periods of time, ranging from months to years, thus making them accessible to the centrocytes (CC) that enter light zone (LZ) and result from the proliferation of blasts in the dark zone (DZ; this zone has been designed because it contains a high cell density) (**Figure 7**).

Figure 7.

Schematic representation of the organization of the germinal center. The dark zone is occupied mainly by proliferating centroblasts. These are cells in which SHM would take place. After proliferation, B-cells, now called centrocytes, are in the adjacent LZ, which also contains Tfh cells and FDCs. Centrocytes that poorly link the antigen will die by apoptosis. Those that can bind the antigen and receive survival signals from the BCR and Tfh cells can either return to the dark zone for another cycle of proliferation, mutation, and selection or become memory B-cells or plasma cells that migrate to bone marrow to ensure prolonged antibody secretion [3]. BCR: B-cell antigen receptor complex, DZ: dark zone, FDC: follicular dendritic cell, LZ: light zone, M: mantle zone, SHM: somatic hypermutation, TBM: tingible body macrophages, Tfh: CD4⁺ T follicular helper cell.

5.2.1.2 Immune response, B-cell fates, and GC formation

Most protein antigens induce T-dependent (TD) antigen humoral immune responses (responses to T-independent [TI] antigen are not evoked in this chapter). Such responses require cooperation between the antigen-specific B-cell and the T-cell carrying a specific TCR of the same antigen. Nevertheless, the two cell types have different localizations within ganglion, and the probability for a B-cell to meet a T-cell with the same antigen specificity is extremely low and is about $1/10^8$ to $1/10^{12}$, knowing that the proportion of naive B-cell and T-cell that are specific for a given antigen is about $1/10^4$ to $1/10^6$ [37].

The search for the antigen by the appropriate B-cells is done through an immutable path. The blood enables the naive B-cells, *i.e.*, the mature peripheral B-cells (known as follicular B-cells), to be transported to the lymph nodes where they enter through the high endothelial venules (HEV), migrate across the T-cell area, and spend about 24 hours in the follicles before exiting through the efferent lymph and returning to the circulation [38] (for review, see [39]).

When B-cells expressing antigen-specific BCR encounter the appropriate antigen (acquired from FDCs) in secondary lymphoid organs (SLOs), within lymph nodes for the antigen that is carried into them from the tissues, or within spleen for the antigen that reach it from the bloodstream, they increase the expression of the C-C chemokine receptor type 7 (CCR7) on their surface and migrate to the T/B border [38] (comment in [40]) in the spleen and in the interfollicular region in lymph nodes, after both T-cells and B-cells have been primed with antigen. The activated B-cells follow one of the following two fates to trigger a TD humoral immune response to protein antigen (for review, see [41–44]):

- a. either they migrate to **extrafollicular foci (EF)** of SLOs, in the medullary cords of lymph nodes or in foci in the red pulp of the spleen [45], and

differentiate rapidly, as plasmablasts, in **short-lived antibody-secreting plasma cells (CLPCs)** [41], synthesizing IgM and IgG, which makes it possible to have rapidly circulating antigen-specific antibodies. Of note, the development of EFs in TD immune responses requires the help of CD4⁺ T-cells that share characteristics of CD4⁺ T follicular helper (Tfh) cells;

b. or they migrate to a primary **lymphoid follicle** (B-cell follicle) of the lymph nodes or spleen, where they undergo clonal expansion—a strong oligoclonal proliferation—to form, after a few days, the **GCs, then undergo somatic mutation to generate high-affinity memory B-cells and long-lived plasma cells (LLPCs)**. GC formation requires about 7 days during a primary immune response and about 36 h during a secondary response, which occurs following immunization and activation of memory B-cell with the same TD antigen. The activated B-cells receive signals from the CD4⁺ Tfh cells, proliferate, and undergo CSR. GCs persist until a few weeks. Of note, similar to naïve B-cells, memory B-cells can also be recruited into EF and give rise to immune responses that are associated with CSR but, at the most, only low-level SHM [45].

5.2.1.3 Immune responses to protein antigens and B-cell cooperation with CD4⁺ Tfh cells

Only CCs that express a high affinity receptor for epitopes of the antigen presented in its native form by the FDCs and that can capture it are selected efficiently. These selected CCs process the antigen and present antigen-derived peptides bound to MHC II molecules to antigen-specific CD4⁺ Tfh cells, which have been shown to develop immediately from naïve CD4⁺ T-cells during antigen priming by dendritic cells in T-cell zones [46] (comment in [47]). The Tfh cells then give survival and differentiation signals to B-cells, which can then undergo CSR and mature either in LLPCs or in memory B-cells.

5.2.1.4 Blast cell differentiation

- **GCs.** The GCs consist essentially of blast cells that divide every 6 h, reaching a number of 60,000 blasts after about 60 h, thus considerably increasing the number of antigen-specific B-cells. They are polarized into two cellular areas:
  - i. *Centroblasts (DZ)*, which no longer produce surface Igs because their genes undergo SHM.
  - ii. *CC (LZ)*, which correspond to smaller B-cells, expressing their new surface Ig, no longer proliferating, and are entangled in a large network of FDCs.
- **CCs.** The few CCs selected after contact with the antigen retained by the FDCs, allowing their affinity maturation, migrate to the apical part of the LZ, then undergo the CSR, and differentiate, as mentioned above, into memory B-cells or in LLPCs, through their cooperation with Tfh cells, which are present specifically within the LZ. DCs can also be selected for apoptosis elimination, if centroblast stage mutations that occur during the SHM process do not modify or even decrease the affinity of their BCR (see below Mechanisms of SHM process and Impacts of mutations on affinity of BCR and secreted Ig).

- *Lymphoid follicles fate.* The persistence of lymphoid follicles depends on antigenic stimulation. So they begin to reduce their size from the beginning of the 2nd week and disappear completely at the 3rd week in the absence of any further stimulation.
- *Nonspecific B-cells.* Small B cells that are not specific for the antigen are repelled from the primary follicles, as the blast cells divide, and thus form the perifollicular corona.
- *Memory B-cells.* Memory B-cells have the ability to respond rapidly to antigens that give rise to a primary response and to present it quickly and efficiently to T-cells during a secondary response, and also differentiate into plasma cells. Although they are a minority group of long-lived cells, they are able to persist in the quiescent state from several months to several decades in humans. They are usually IgD(–) and may have preferential localizations such as mucous membranes for cells that have switched to produce IgA.

5.2.2 SHM: features and molecular mechanisms

Following antigenic stimulation, B-cell IG genes undergo SHM and CSR within GCs.

5.2.2.1 Genes concerned by SHM

The process of somatic hypermutation-a process targeting the V genes of the H and L chains-is the basis for the antibody affinity maturation. It is induced during humoral responses of conventional B-follicular cells in response to TD antigen. In contrast to the somatic-V(D)J-recombination that takes place in the bone marrow, the SHM process takes place in the SLOs, in the DZ of the GCs, at a stage where the B-cell is called centroblast.

5.2.2.2 Roles and objectives of SHM

Since the specificity and affinity of the BCR/mIg of the B-cell that left the bone marrow, and subsequently the circulating antibody produced, are determined before the encounter with the antigen (antigenic epitope), the phenomenon of SHM occurs in the activated B-cell clone through mutations in the sequence of genes derived from V(D)J somatic recombination, in order to adjust the hypervariable regions to the epitope and thus to modulate/increase the antibody affinity and the effective and adapted recognition of the antigen triggering the humoral immune response. As mentioned above, such a phenomenon participates in the generation of Igs diversity.

5.2.2.3 Mechanisms of SHM process

5.2.2.3.1 SHM features

SHM introduces mutations that replace one or more amino acids in the Ig, producing closely related B-cell clones that differ subtly in terms of antigenic specificity and affinity [48]. Despite recent advances, the molecular mechanisms responsible for them remain little known. Nevertheless, the results of *in vitro* or *in*

vivo studies on mouse models expressing an IG transgene have made it possible to define certain characteristics of this process:

- a. It corresponds to an adaptive mutagenesis initiated by the action of the enzyme AID, which is expressed solely by the B-cells of the CG [48].
- b. It occurs during an extremely brief stage of differentiation of B-cells at the GCs.
- c. It occurs in the peripheral lymphoid organs in the DZ GC B-cells.
- d. Numerous point mutations are introduced in the hypervariable regions of the BCR or Ig H and L chain V gene following the activation of antigen-specific B-cell clones.
- e. The mutations extend over about 1 kb.
- f. Purine-purine or pyrimidine-pyrimidine mutations are mainly observed; A and G are more often mutated than T and C.
- g. Random mutations can enhance the affinity of the antibodies.
- h. It relates to TD antigen immune responses.
- i. The mutations essentially concern the variable gene and the adjacent 3' region of the rearranged V(D)J segment.
- j. The mutation domain extends in 3' from the promoter to the intronic J-C region.
- k. It also affects the DNA flanking the rearranged V gene, but does not generally extend to C region exons.
- l. Mutations are mostly nucleotide substitutions, but insertions and deletions are possible.
- m. SHMs only occur during the secondary response, but not during the primary responses.
- n. The cis sequences of IG locus are indispensable for triggering or regulating the hypermutation process.
- o. There is an important involvement of the IG promoter and enhancer sequences of the IG locus, suggesting a link between transcription and SHM [49].

5.2.2.3.2 AID, adaptive mutagenesis, and erroneous repair of uracil bases

- *Cytosine deamination.* AID initiates the SHM process within target sequences by cytosine deamination in the rearranged variable regions and focuses on 3–4 bp SHM motifs that are greatly enriched in S regions and in portions of variable region exons that encode antigen-binding sites (CDR region). SHM of V(D)J exons in GC B-cells require transcription for AID targeting, as during CSR, and begins ~150 bp downstream of the transcription start site (TSS) and extend

~1–2 kb further downstream [50] (for review, see [51–54]). Hypermutable DNA sequences are found in the regions of IG genes, preferentially in sequences containing mutational “hotspots” corresponding predominantly to RGYW/WRCY motifs (G: C is a mutable position, **R** = purine bases [A/G], **G** = guanine, **Y** = pyrimidine bases [C/T], **W** = A/T, **C** = cytosine) [55].

- *Mutagenic repair of U:G mismatches.* Uracil (U) generated from cytosine deamination creates U:G lesions/mismatches that will be repaired erroneously by introducing point mutations or, more rarely, insertions and deletions in V regions at one of 1000 bp per generation. Two possibilities of repair are possible:

a. If the U is targeted by the enzyme uracil-DNA N-glycosylase (UNG), which is usually followed by components of the base-excision repair (BER) pathway (an essential DNA repair pathway), it will be excised, and an abasic site appears in DNA following the action of apurinic/apyrimidinic endonuclease (APE), an enzyme that identifies damaged apurinic/apyrimidinic sites in DNA, cuts the phosphodiester bond in the backbone of the sites, and has critical roles in the base excision pathway. This site will, in turn, be mutagenically replicated by translesion DNA synthesis (TLS) polymerases, leading to a very high error rates.

Figure 8.

DNA deamination model of IG gene diversification by AID during SHM (adapted from [51]). AID creates mutations in DNA by deamination of cytosine base, which turns it into uracil, which is then targeted by BER or MMR mechanisms or simply replicated producing mutations. AID: activation-induced deaminase, APE: apyrimidinic/apurinic endonuclease, BER: base excision repair, bp: base pairs, CSR: class switch recombination, ExoI: mismatch repair exonuclease I, IG: immunoglobulin gene, IgV: immunoglobulin variable region, MMR: DNA mismatch repair, MSH2/6: mismatch recognition proteins, NHEJ: nonhomologous end joining, pol: polymerase, pol η: DNA polymerase eta, Rev1: Y-family DNA polymerase involved in DNA damage tolerance, SHM: somatic hypermutation, TLS: translesion DNA synthesis, UNG: uracil-DNA glycosylase, XRCC2/XRCC3: X-ray cross-complementing gene 2/3.

- b. If the U:G mismatch is recognized by the mismatch repair (MMR) protein pathway, the U:G lesion will be excised by Exonuclease I, resulting in degradation of the DNA strand surrounding the U, then the MSH2/MSH6 heterodimer induces the formation of a gap in the DNA. Unfaithful replication of a DNA strand will take place at the level of the gap by the TLS DNA polymerase η (pol η), a “wrong” TLS repair polymerase [56] (**Figure 8**).

5.2.2.3.3 Frequencies of mutations

The frequency of IG region V gene mutations corresponds to approximately one bp change per 1000 bp per gene and per cell division/generation, while that affecting the rest of the cell DNA is much lower, and corresponds to about one bp change per 10^{10} bp per cell and per division. Of note, there is approximately a 50% chance during each division of B-cells that a mutation leads to a change in BCR, since it is known that each V region is encoded by approximately 360 bp and that approximately $\frac{3}{4}$ basic changes modify the encoded amino acid [48].

5.2.2.3.4 Impacts of mutations on affinity of BCR and secreted Ig

The low affinity of antibodies produced during the primary immune response tends to increase as the immune response progresses, thanks to the numerous point mutations in hypervariable regions of the IG V gene of the antigen-specific B-cell clones.

Most mutations have no positive effect on the affinity of BCR or Ig produced, and frequently negatively affect their ability to bind antigen inducing B-cell activation.

Of the four types of possible mutations-silent, neutral, deleterious, and positive-only deleterious mutations and positive mutations have an effect on the affinity of the antigen for its appropriate BCR:

- i. The deleterious mutations are responsible for a decrease in the affinity of the antigen for its BCR, but also for the rapid division of B-cells (the expansion would overwhelm the lymphoid tissues), since they can induce modifications of the framework regions and thus disrupt the basic structure of Ig. The many B-cells that carry such mutations will be a target of apoptotic death by a negative selection process, either because they can no longer produce a functional BCR, or because they cannot efficiently internalize antigens through clonally distributed membrane BCRs. These apoptotic cells will invade GC and then be rapidly ingested by resident macrophages, giving rise to tingible body macrophages (TBM), containing dark nuclear debris in their cytoplasm [48].
- ii. Positive mutations are less common than deleterious mutations and are responsible for increasing antigen affinity for its BCR and improving its binding. The small portion of daughter cells with many nucleotide substitutions in the Ig V region encoding gene that are derived from B-cell clones undergoing such mutations will be positively selected and will therefore have an increased survival rate relative to the cells expressing a low BCR affinity. Positive selection would be a consequence of the accumulation and concentration of many amino acid substitutions in CDRs of the Ig V region, as a result of nucleotide changes that alter amino acid sequences and so the protein structure.

- iii. Silent or neutral mutations have no noticeable effect on antigen affinity. They preserve the amino acid sequence and do not alter the structure of proteins and are dispersed throughout the V region [48].

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