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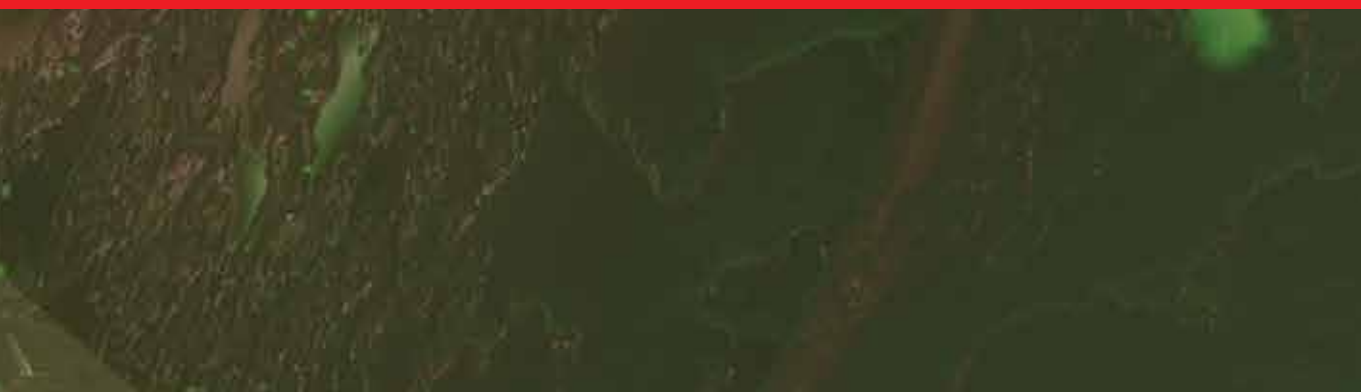
Normal and Malignant B-Cell

Edited by Mourad Aribi



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Meet the editor

Dr. Mourad Aribi, PhD, Dr. Hab., HDR, is a professor of immunology at the University of Tlemcen (Algeria), and the founder and director of the Laboratory of Applied Molecular Biology and Immunology (W0414100). His creativity allowed him to construct for the first time Master's and PhD training programs in immunology at his university, welcoming students and young researchers from different regions and countries. His current research focuses on the modulation of cell-mediated and inflammatory immune responses in autoimmune diseases, cancer diseases, and infectious diseases. Thanks to his interdisciplinary skills, he was able to develop numerous high-level bilateral and multilateral collaboration projects with large teams, in particular with partners from CNRS and INSERM (Montpellier, Marseille, Burgundy-Franche-Comté, France), Institute for Immunodeficiency (Freibourg, Germany), and, more recently, with partners from Harvard Medical School (Boston, USA). He is a regular reviewer for many international scientific journals and is also a member of the editorial board of Frontiers in Immunology (the official journal of the International Union of Immunological Societies).

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Preface

This book is a collection of harmonious chapters contributed by different authors. It sets out to describe the B-cell during different stages of ontogeny and the molecular mechanisms of its antigen receptor diversity. It also discusses the main clinical and etiopathogenic aspects when it is transformed into a malignant cell.

The current book is structured into seven chapters:

The Introductory chapter describes succinctly the main features of immunoglobulins, phases of B-cell ontogeny, and B-lymphoid lineage markers, as well as diseases related to B-cell abnormalities.

Chapter 2 focuses on the molecular description of the immunogenetic mechanisms responsible for the generation of B-cell antigen receptor diversity by giving a brief overview of immunoglobulin gene organization and gene rearrangements.

Chapter 3 focuses on clinical and etiopathogenic features, prognosis, treatment, and WHO classification of B-cell lymphomas.

Chapter 4 provides a general description of B-cell lymphomagenesis.

Chapter 5 reviews the morphological, clinical, and etiopathogenic aspects, as well as the therapeutic features, of diffuse large B-cell lymphoma.

Chapter 6 describes the importance of immunophenotypic characterization of B-cell precursors using flow cytometry in the detection of minimal residual disease in acute leukemia.

Chapter 7 specifically investigates the influence of splenectomy on the composition of B-cell subpopulations.

I really hope that this book will be interesting and useful for clinicians, biologists, researchers, teachers, and graduate students of both doctoral and master's degrees in the field of immunology.

I would like to thank all the collaborators who participated in the conception of this book. I would also like to express my warmest thanks to Ms. Sandra Maljavac, Author Service Manager, for her great patience in developing this work and the coordination with the authors. I would like to thank her and the team of IntechOpen for their trust and help in making this book.

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Section 1

B-Cells

Chapter 1

Introductory Chapter: B-Cells

Mourad Aribi

1. Introduction

Etymologically, the "B" from B-cells, also referred to as B lymphocytes, stands from the name of bursa of Fabricius, a lymphoid organ found only in birds, as reported, in 1956, by Bruce Glick and Timothy Chang [1, 2], but not from the bone marrow as it has been believed.

B-cells represent about 5–15% of circulating blood lymphocytes and are responsible for the humoral immune response, as a critical component of adaptive immune system. Their roles are not limited only to the production of antigen-specific antibodies after antigen binding with high affinity *via* their membrane Ig but also to antigen presentation. This allows them to interact with cells involved in cell-mediated immunity and to produce cytokines [3] within immunological synapses (IS) [4, 5] that they create with both $CD4^+$ T-cells [6] and $CD8^+$ T-cells [7] (for review, see [8–10]).

The current chapter presents a brief overview on Igs and phases of B-cell ontogeny and B-lymphoid lineage markers. The end of the chapter summarizes the main types of diseases related to B-cell abnormalities.

2. Immunoglobulins: transmembrane and secreted B-cell receptors

2.1 Structure of Igs

2.1.1 Ig chains

The most common form of Igs in the blood has a heterodimeric structure, about approximately 150 kDa [11], with two antibody sites—paratopes—that bind to the epitope of a specific antigen, located in the fragment antigen-binding [F(ab)]. This structure is composed of two identical heavy (H) and two identical light (L) chains, these being either kappa (Lκ) or lambda (Lλ). The H and L chains are associated with each other by disulfide bridges (**Figure 1**).

2.1.2 Ig domains

Each chain of Igs is composed functionally of constant (H; C_H, L; C_L) and variable (H; V_H, L; V_L) domains. The constant region of H chain is composed of three (for IgG, IgA, IgD) or four (for IgM and IgE) constant domains, designated, respectively, C_H1, C_H2, C_H3, and C_H4. Except for IgM and IgE, the region between C_H1 and C_H2 domains is called the hinge "H" region, permitting flexibility in the chain [12], which is longer and more flexible in IgG3 than the other IgG subclasses [13]. Ig L chains are composed of two separate domains, each having an approximate molecular weight of 12 kDa [14]. The association of the variable domains of the H and L chains defines the site of attachment to the antigen (**Figure 1**).

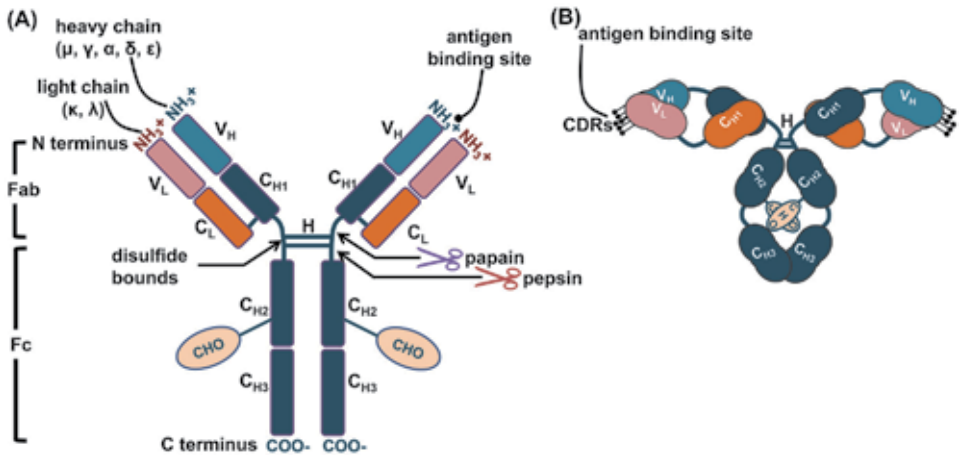


Figure 1.
Molecular structure of a typical Ig molecule. Ig molecules have a symmetric structure that is stabilized by interchain disulfide bonds. The heavy chain determines the isotypes, i.e., the classes (IgM, IgG, IgA, IgD, IgE) and subclasses (IgG1, IgG2, IgG3, IgG4, IgA1, IgA2) of Igs. Panel (A) shows a simplified schematic representation of an antibody molecule. Panel (B) illustrates a schematic representation of the four-chain composition and the separate domains comprising each chain. This representation is based on the X-ray crystallography of an IgG antibody. Three globular regions form a Y. The two antigen-binding sites are at the tip of the arms, which are attached to the trunk of the Y by a flexible hinge region [16]. Incubation of Igs with papain (papaya proteinase I), in the presence of a reducing agent, results in the production of two monovalent fab fragments (50 kDa each) and one intact fc fragment (about 50–70 kDa). Igs digestion by pepsin leads to the production of one F(ab')2 fragment and numerous smaller peptide fragments of the fc portion. CDRs, complementarity-determining regions; C_H, heavy chain constant domain; CHO, carbohydrate; C_L, light chain constant domain; fab, fragment antigen binding; fc, fragment crystallizable; H, hinge region; Ig, immunoglobulin; V_H, heavy chain variable domain; V_L, light chain variable domain.

Constant domains have specify effector functions such as activation of complement or binding to FcRs [15].

Each Ig domain contains roughly 100–110 amino acids long [14, 17] and consists of a two-layer sandwich of seven to nine antiparallel beta-strands arranged in two beta-sheets/-barrels with a Greek topology [18].

Ig domains play well-defined roles, which depend on the location of each one. So the C_H1 domain, located within the F(ab) region, interacts with the constant domain of L chains. The remaining C_H domains (C_H2-C_H3 or C_H2-C_H4) comprise the Fc region, which defines the isotype, classes, and subclasses of the Ig. The C_H2 (C_H3 for IgM and IgE) domain allows an important role in mediating the effector functions, including interaction with FcRs and antibody stability thanks to the presence of N-linked glycan, which is conserved in mammalian IgGs at Asn297 as well as in homologous regions of other antibody isotypes [19]. The importance of N-glycosylation is well-known for IgGs, but little is known for other isotypes [20]. The C_H3 domain allows dimerization and participates in the stabilization of the binding of the heavy chains to one another through interactions between the C_H3 domains. For both IgM and IgA, the C_H3 domains have short tailpieces to which the J-chain binds *via* disulfide bonds, whereas the secretory component is disulfide bonded to one of the C_H2 domains of the dimer. C_H3 domain of IgG binds to FcγR and of IgE to FcεRI and CD23 [15].

2.1.3 Framework and complementarity determining regions

Each Ig V domain contains three hypervariable regions, corresponding to the site of recognition of the antigen, thus forming the paratope (complementarity determining regions, CDR1, CDR2, and CDR3). CDRs separate four highly conserved segments with less variability, termed the framework regions and designated FR1, FR2, FR3, and FR4 [16] (**Figure 2**). As for the T-cell antigen receptor (TCR), the

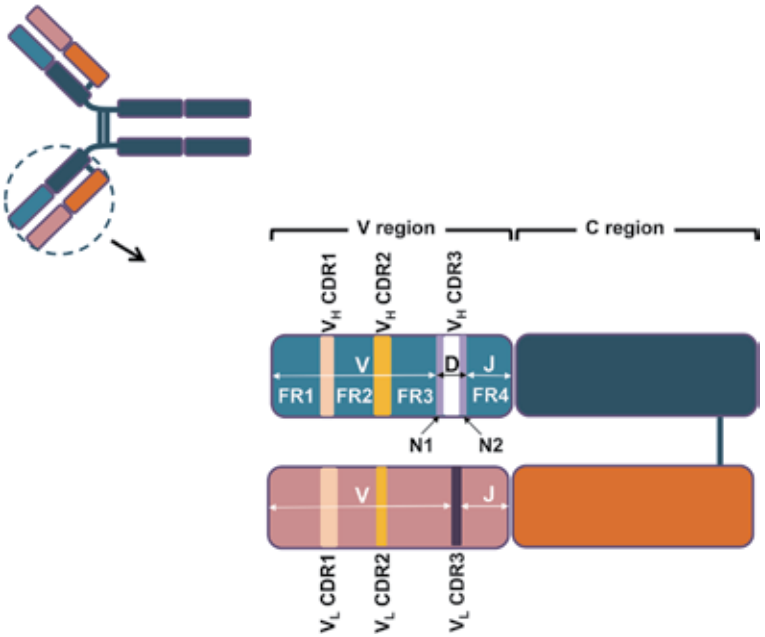
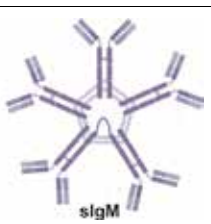


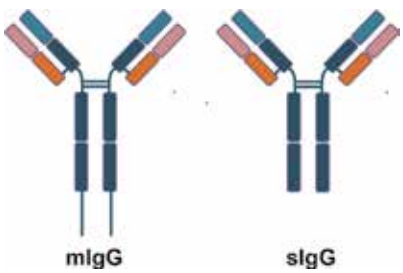
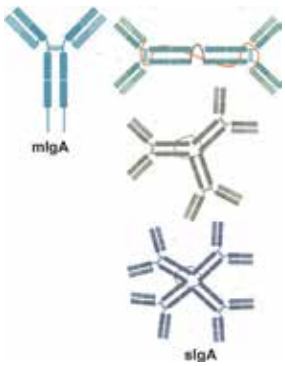
Figure 2.
Subdivision of the variable region of the Ig molecule. The Ig V region contains seven amino acid regions, four of which are FRs and three of which are CDRs. The FRs are located on the tips of the Y-shaped molecule and act as a scaffold for the CDRs. Of note, this representation shows the physical location of the V_H region and the V_L region. N region between the V and D regions is called N1, and that between the D and heavy J regions is called N2. CDR, complementarity determining region; FR, framework region; C, invariant constant domain; V, variable domain; D, diversity domain; J, joining domain.

binding specificity of the antigen is therefore determined by the loops present at one end of V_L and V_H domains of Ig chains; the difference in specificity between antibodies is therefore related to these loops [21].

2.1.4 Isotypes and subisotypes of Igs

There are five isotypes (classes) of Igs that are structurally and functionally distinct, IgM, IgD, IgG, IgA, and IgE. The difference between the classes of Igs lies in the constant portion of the H chains: mu (μ) determines the IgM class, gamma (γ) determines the IgG class, alpha (α) determines the IgA class, delta (δ) determines the IgD class, and epsilon (ε) determines the IgE class. So the DNA encoding the constant part of an H chain contains several constant sequences. Thus, there are four subclasses of IgG in both humans (IgG1, IgG2, IgG3, IgG4) and mice (IgG1, IgG2a, IgG2b, and IgG3) and two subclasses of IgA (IgA1 and IgA2) in humans, which are unequally distributed in the body fluids [22].

Key Point 1|Main features of human Ig classes and subclasses			
mIg	**sIg**	**Properties**	
		Molecular formula	$(\mu_2\kappa_2)_5$ or $(\mu_2\lambda_2)_5$
		Subclasses	None
		MW	900–970 kDa
		Valency	10
		Structure	mIgM; monomer, sIgM: pentamer
		H chain type|MW	μ|65–70 kDa

Key Point 1|Main features of human Ig classes and subclasses			
mIg	**sIg**	**Properties**	
		Serum concentration	0.5–2 mg/mL
		In vivo serum half life	5 days
		Relative serum abundance to total immunoglobulin	6–10%
		Carbohydrate content by weight (glycosylation)	12%
		Distribution	Mainly intravascular
		Crosses placenta	None
		Function	Primary antibody response
		Classical complement pathway activation	+++
		Molecular formula	$\gamma_2\kappa_2$ or $\gamma_2\lambda_2$
		Subclasses	Yes (IgG1, IgG2, IgG3, IgG4)
		MW	IgG1; 150 kDa, IgG2; 150 kDa, IgG3; 170 kDa, IgG4; 150 kDa
		Valency	2
		Structure	Monomer
		H chain type|MW	γ1|50 kDa, γ2|50 kDa, γ3|60 kDa, γ4|50 kDa
		Serum concentration	Total IgG: 10–16 mg/mL (IgG1: 9 mg/mL; IgG2: 3 mg/mL; IgG3: 1 mg/mL; IgG4: 0.5 mg/mL)
		In vivo serum half life	IgG1: 23 days; IgG2: 23 days; IgG3: 8 days; IgG4: 23 days
		Relative serum abundance to total immunoglobulin	75–80%
		Carbohydrate content by weight (glycosylation)	3%
		Distribution	Intra- and extravascular
		Crosses placenta	IgG1: +; IgG2: +/−; IgG3: +; IgG4: +
		Function	Secondary antibody response
		Classical complement pathway activation	IgG1: +; IgG2: +/−; IgG3: ++; IgG4: none
		Molecular formula	$(\alpha_2\kappa_2)_n$ or $(\alpha_2\lambda_2)_n$
		Subclasses	Yes (IgA1, IgA2) [in humans, serum IgA are mostly monomers, but a small amount of them is found in polymeric forms (pIgA), usually dimers, linked by disulfide bonds and by an additional, cysteine-rich polypeptide termed J chain (16 kDa). Serum monomeric IgA can also be found in most secretions. Mucosal secretory IgAs are mostly seen as a dimer with a secretory piece.]

Key Point 1|Main features of human Ig classes and subclasses			
mIg	**sIg**	**Properties**	
		MW	Secretory tetramer IgA1 or IgA2: 600 kDa; serum monomer IgA1 or IgA2: 160 kDa
		Valency	2, 4, 6 or 8
		Structure	Monomers, dimers (more common), and trimers and tetramers (both are rare)
		H chain type|MW	α1|55 kDa, α2|55 kDa
		Serum concentration	Total IgA : 1–4 mg/mL (IgA1: 3 mg/mL; IgA2 : 0.5 mg/mL)
		In vivo serum half life	IgA1: 6 days; IgA2: 6 days
		Secretion	Milk and secretions (tears, saliva, mucous, sweat, colostrum, and secretions from the genitourinary tract, gastrointestinal tract, prostate, and respiratory mucosa)
		Relative serum abundance to total immunoglobulin	13–15%
		Relative serum abundance to total IgA	IgA1; 85%, IgA2: 15% (higher in secretions)
		Carbohydrate content by weight (glycosylation)	10%
		Distribution	Intravascular and secretions
		Crosses placenta	None
		Function	Protects mucous membranes
		Classical complement pathway activation	None
		Molecular formula	$\delta_2\kappa_2$ or $\delta_2\lambda_2$
		Subclasses	None
		MW	180 kDa
		Valency	2
		Structure	Monomers
		H chain type|MW	δ|62–70 kDa
		Serum concentration	0–0.4 mg/mL
		In vivo serum half life	3 days
		Relative serum abundance to total immunoglobulin	0.2%
		Carbohydrate content by weight (glycosylation)	13%
		Distribution	Lymphocyte surface
		Crosses placenta	None
		Function	Somewhat unknown; while research have demonstrated its role in promoting immune defense related to inflammation and tissue damage by

Key Point 1|Main features of human Ig classes and subclasses			
mIg	**sIg**	**Properties**	
			inducing the activation and infiltration of immune cells
		Classical complement pathway activation	None
		Molecular formula	$\varepsilon_2\kappa_2$ or $\varepsilon_2\lambda_2$
		Subclasses	None
		MW	190–200 kDa
		Valency	2
		Structure	Monomers
		H chain type|MW	ε|70–73 kDa
		Serum concentration:	10–400 ng/mL
		In vivo serum half life	2.5 days
		Relative serum abundance to total immunoglobulin	0.002%
		Carbohydrate content by weight (glycosylation)	12%
		Distribution	Basophils and mast cells in saliva and nasal secretions
		Crosses placenta	None
		Function	Protects against parasites
		Classical complement pathway activation	None
mIg: membrane-bound immunoglobulin, sIg: secreted immunoglobulin (serum or secretory Ig).			

2.2 Transmembrane B-cell receptor

B-cells are defined by the presence of membrane-bound Igs (mIg) that act as specific receptors for the appropriate antigen in mature B-cell but, also as an excellent marker of the B-cell line. The mIg constitutes, with other glycoprotein chains, the B-cell antigen receptor complex (BCR). These correspond to non-covalently associated transmembrane disulfide-linked heterodimer phosphoprotein Igα/Igβ (CD79a and CD79b), which are encoded by *mb-1* and *B29* genes, and are structurally similar to CD3γ, δ, and ε chains on T-cells and therefore involved in signal transduction of B-cell [23] (**Figure 3**). The intracytosolic portion size of the heterodimer allows it to initiate signal transduction, following the binding of the specific antigen to the mIg.

2.3 Mobility of mIgs, capping, and antigenic modulation

Igs are very mobile on the surface of B-cells. In addition, various specific ligands cause what is called capping, *i.e.*, a rapid redistribution of complexes on the surface of the cell, followed by internalization of the complexes or their release into the surrounding environment. This transient disappearance of the mIg receptor *via* its binding to specific ligands, like anti-immunoglobulin antibodies or antigens, is called antigenic modulation, which constitutes the initial signal for B-cell activation. The modulated membrane receptor may also be synthesized by the cell and returned to the surface when the B-cell is metabolically active [24, 25].

2.4 Transmembrane *vs.* secreted B-cell receptor

The only structural difference between transmembrane and secreted B-cell receptors (soluble immunoglobulins, sIgs) is that the C-terminal region of the heavy

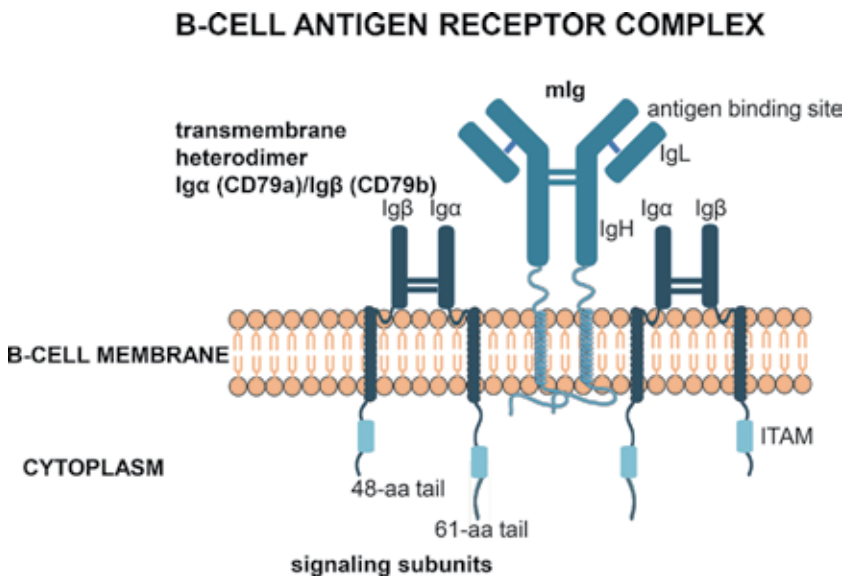


Figure 3.
BCR complex structure. The BCR complex is composed of a mIg non-covalently bonded to a transmembrane disulfide-linked heterodimer phosphoprotein composed of CD79a (Igα)/CD79b (Igβ). CD, cluster of differentiation; mIg, membrane-bound immunoglobulin.

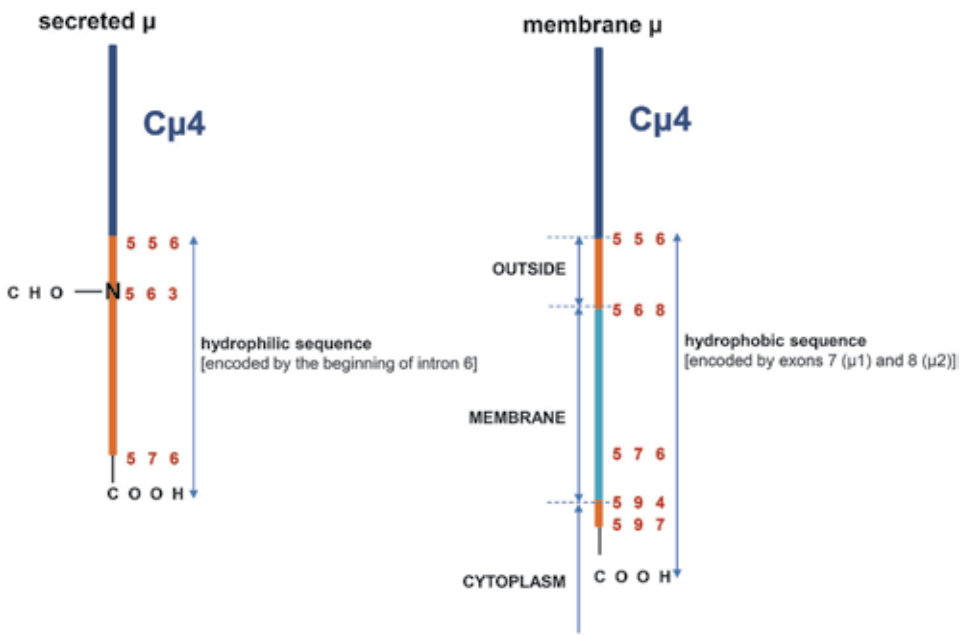


Figure 4.
Structural difference between the C-terminus of the H chain constant region of mIgM and sIgM. mIgM and sIgM differ simply by the COOH ends of the μ chain and therefore by mRNAs that differ at their 3′ ends. Membrane-bound IgM (membrane μ) is slightly larger than that of secreted IgM (secreted μ) [27]. The numbers indicate the position of amino acids. N-CHO represents the position of glycosylation of asparagine (N, Asn) (N-glycosylation site). mIgM, membrane immunoglobulin M; sIgM, secreted immunoglobulin M.

chains contains a short hydrophobic stretch which spans the lipid bilayer of the membrane [26] (**Figure 4**).

sIgs play a complementary role to that of T-cells. It needs to use other mechanisms for antigen removal. This implies that they bind other molecules, like complement molecules, or specialized receptors, called Fc receptors (FcRs, receptors of fragment crystallizable region), on the surface of effector cells that they activate, including phagocyte cells (**Figure 5**).

Antibodies allow B-cells to provide systemic protection of the host and immune surveillance through pathogen recognition and organization of immune reactions. Their expression varies according to the state of differentiation of B-cells. After activation, B-cells transform into plasma cells that secrete antibodies of the same specificity as their membrane BCR. Secreted antibodies are transported rapidly throughout the body by blood or lymph or secreted through the epithelia to protect the interface between the body and its environment. IgG antibodies also provide a mechanism by which acquired immunity can be transmitted from the mother to the fetus or infant, thus providing acquired immune protection during the critical period of early life. Nevertheless, some antibodies can bind to self-structures, referred to as autoreactive antibodies, and induce, under certain conditions, aberrant immune responses and tissue damage [28].

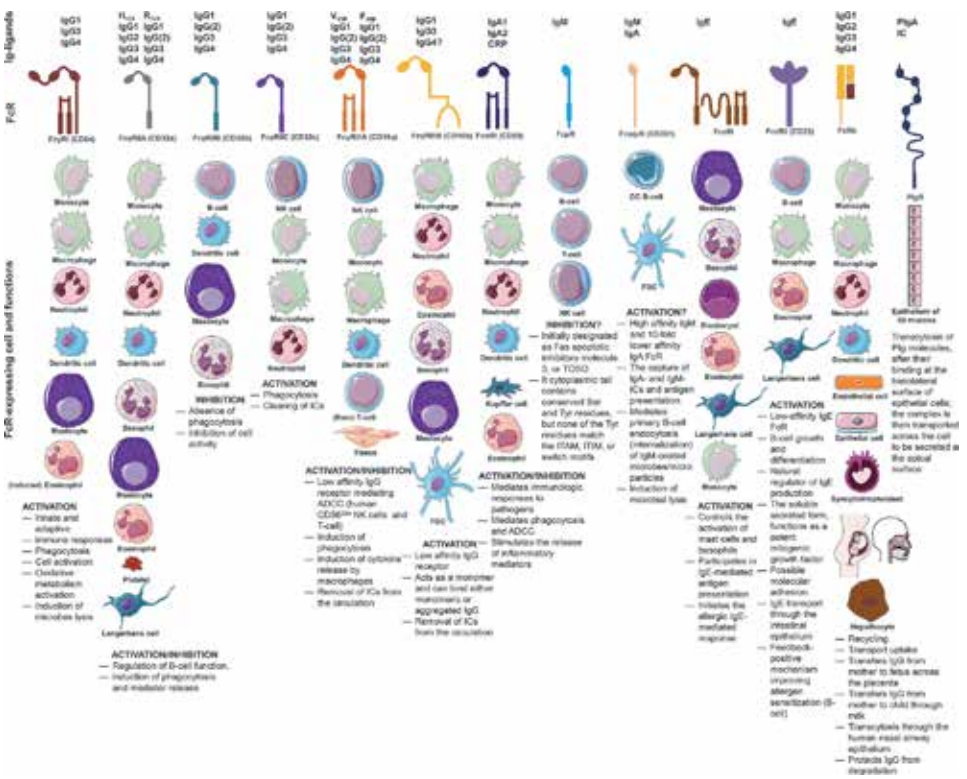


Figure 5.
Human FcRs and their cell localization and immune functions. DCs, dendritic cells; FcRn, neonatal fc receptor; FcRs, receptors of fragment crystallizable region; FDCs, follicular dendritic cells; F_{158}, phenylalanine at position 158; GC, germinal center; H_{131}, histidine at position 131; ICs, antigen-antibody immune complexes; PIgR, polymeric immunoglobulin receptor; R_{131}, arginine at position 131; V_{158}, valine at position 158.

3. B-cell ontogeny

The differentiation of B-cells from hematopoietic stem cells into pro-B, then pre-B, then immature B-cells, and finally into mature B-cells is characterized by several events, including (i) modification of membrane differentiation markers; (ii) Ig gene rearrangement, which takes place at the pro-B and pre-B stages, allowing the expression of BCR; and (iii) negative clonal selection.

3.1 B-cell ontogenesis and maturation

B-cell ontogenesis occurs in the fetal liver, then in the bone marrow, and continues throughout life. It starts from a hematopoietic stem cell and leads to the development of a so-called "immature" B-cell with the same and unique antigenic specificity. The immature B-cell migrates to the peripheral lymphoid organs, where the different stages of maturation will take place, leading to the Ig-producing plasma cells and memory B-cells [24]. Ultra-complex regulatory mechanisms are involved during all stages of B-cell development and lead to the generation of B-cell repertoire with a vast diversity of antigen recognition capacity.

3.1.1 Lymphoid progenitor cells and cell fate decisions

Knowing that B-cells, T-cells, and natural killer (NK) cells all develop from the early lymphoid progenitors that originate from totipotent hematopoietic stem cells, cell fate results from several lineage choices. The B-cell progenitors continue to develop in the bone marrow [21].

3.1.2 Positive and negative selection and B-cell receptor reformatting

During their development, B-cell and T-cell undergo a dual process of positive and negative selection in which cells that react with high affinity against self-antigens are eliminated because they constitute a significant danger of triggering autoimmune responses.

In the case of B-cells, outside the negative selection mechanisms, cell survival depends essentially on their ability to compete for survival factors such as the B-cell activating factor (BAFF, also known as B lymphocyte stimulator (BLyS), a subset of the tumor necrosis factor (TNF) tumor necrosis factor ligand superfamily member 13B), which is present in the circulation and produced by resident cells within secondary lymphoid organs [24]. Positive selection mainly corresponds to BCR functionality test and depends on a moderate response to the self-antigen, which stimulates cell maturation and survival. When self-reactivity exceeds a certain level, a process so-called *receptor reformatting* is triggered by inducing a new cycle of gene rearrangements, principally in the IG L chain loci (see Chapter 2). If the receptor reformatting fails, or if the developing B-cell leaves the bone marrow and encounters the self-antigen soon after it arrives in the spleen, this cell will be eliminated [21].

3.1.3 Immature B-cell generation

The differentiation of hematopoietic stem cell (HSC) into immature B-cell passes through four successive steps, which could be identified by the presence of certain markers, corresponding to the different stages of rearrangement of Ig genes:

a. **Early pro-B-cell.** These cells appear before the start of rearrangements. They are identified by CD45R, major histocompatibility complex class II (MHC II), and CDI9 and CD38 markers.

b. **Late pro-B-cell.** Variable (V), diversity (D), and joining (J) recombinations for Ig H chain gene segments and receptor expression for interleukin-7 (IL-7R) are induced at this stage.

c. **Pre-B-cell.** There is contact between adhesion molecules and, in particular, vascular cell adhesion molecule 1 (VCAM-1/CD106), on the surface of the stromal cells and its ligand, very late antigen-4 (VLA-4 or α4β1 integrin, a dimer composed of CD49d (α4) and CD29 (β1)), on the surface of the pre-B-cells. At this point, intact μH chains are produced and exported to the cell surface in association with proteins that simulate light chains: λ5, which is similar to a Cλ domain, and VpreB (CD179a), which has an Ig V domain-like structure. The μ chains associated to λ5 and VpreB form a complex which, together with Igα and Igβ proteins, can be expressed at the cell surface and transduce signals that inhibit other rearrangements of genes encoding the H chains but stimulate the genes encoding L chains and cell proliferation. Moreover, expression of the CD20 marker can be detected at this stage.

d. **Immature B-cell.** Thanks to a productive Ig L chain genes' rearrangement, these cells express IgM on their surface, which triggers a phenomenon termed *feedback inhibition*, which blocks any new rearrangement of the genes encoding the L chains, thus allowing the expression of a single and unique Ig specificity by each B-cell clone. At this point, the cells express the CD21 marker.

These stages are strictly dependent on the presence, in the fetal liver and bone marrow, of nonlymphoid stromal cells that come into contact with the B-cell precursors and provide the input of soluble factors in the cell microenvironment that are essential for differentiation, such as stem tell factor (SCF) and interleukin 7 (IL-7). It should be noted, moreover, that the probability of reaching productive/efficient rearrangements of Ig genes encoding the H and L chains and obtaining an intact Ig expressed on the cell surface (mIg/BCR) is low. So most of the time there are nonproductive rearrangements, which leads to the deletion of the resulting B-cells. In addition, the differentiation of B-cells also depends, according on the cell development stage, on the presence of intracellular enzymes. Thus, the enzymes encoded by the recombination-activating genes (RAGs), RAG-1 and RAG-2, are active in early and late pro-B-cells and in pre-B-cells. Finally, the activity of the enzyme terminal deoxynucleotidyl transferase (TdT), which is involved in the addition of N-nucleotides, stops at the pre-B-cell stage.

Key Point 2|Stromal cells

- Stromal cells constitute a support tissue of an organ, *i.e.*, a connective tissue cell that allows the multiplication of hematopoietic stem cells (HSCs) and their differentiation by creating a microenvironment adapted to the growth and differentiation of HSCs. They are also able to differentiate into many cell types.

- It has recently been suggested that stromal cells play a major role, not only in the functional regulation of many tissues and organs, but also more particularly in the immune responses. Of note, non-hematopoietic stromal cells play a key role in the development and function of the immune

system, but, paradoxically, they can also promote the persistence of many cancers and various immune-mediated diseases. The main non-hematopoietic stromal cells involved in immunity include fibroblasts, myofibroblasts, endothelial cells, pericytes, smooth muscle cells and mesenchymal stromal cells.

- Bone marrow stromal stem cells (BMSC) are almost all of mesenchymal origin and, therefore, are also known as skeletal or mesenchymal stem cells. They influence the microenvironment surrounding B-cell precursors, and thus exert local effects on their development through cytokines and chemokines.

HSCs are multipotent cells; *i.e.*, they are able to differentiate into each of the types of blood cells. After each division into two cells, one of them maintains the HSCs stock and the other one differentiates into one of three types of blood cell, *i.e.*, leukocyte, red blood cell, or platelet.

3.1.4 Transition from immature B-cell to mature B-cell

The immature B-cell leaves the bone marrow parenchyma and passes through an endothelial barrier and enters the blood sinusoids, where they are retained, before finally being released into the peripheral blood [29], which allows them to migrate to the spleen where they complete their development [30]. The transition from the immature B-cell to the mature B-cell takes place in a few days and leads, via alternative splicing of long primary mRNA transcripts from the IG heavy (IGH) locus [31], to the coexpression on naïve mature B-cells of membrane IgM and IgD that share the same antigenic specificity. Only about 5% of immature B-cells will sustainably give rise to peripheral B-cells; however, most newly formed ones disappear within a few days.

3.1.4.1 Tonic BCR signal and notch activation

A so-called BCR-mediated positive signaling *tonic signal* and activation of Notch pathway, more precisely of Notch2, which is induced following the interaction with delta-like canonical Notch ligand 1 (DLL-1) expressed on splenic endothelial niches and required for marginal zone (MZ) B-cell development [32], allow the triggering of a first differentiation by a lineage cell choice that does not involve the antigenic specificity.

3.1.4.2 Intensity of tonic signal and differentiation of lymphoid follicular B-cells or MZ B-cells

a. **Low tonic signal and Notch2 commitment.** These will lead to differentiation into MZ B-cells. The latter forms a ring around the follicle and has a more complex structure in humans, also surrounded by a perifollicular zone [PFZ] that surrounds the red pulp. It is a macrophage-rich area (MZ macrophages [MZMs] and metallophilic macrophages [MMMs]) that will efficiently retain antigens from the bloodstream. In mice, the MZ B-cells are CD21++CD23-CD11++CD11c++, IgM++IgD+/−. In humans, it is not yet known whether MZ B-cells are derived from the germinal center (CG), by an early exit at the IgM stage, or are produced by a lineage choice during early B-cell differentiation.

b. **Strong tonic signal, without Notch2 commitment.** These lead to differentiation of lymphoid follicular B-cells. Recall that these are structured by specialized stromal cells and follicular dendritic cells (FDCs), which play an important role in immune response by their ability to retain

antigen–antibody complexes as well as in the selection of memory B-cells during GC reactions (GCRs) [33]. They contain naive B-cells, and adjacent to which are T-cell zones that form the periarteriolar lymphoid sheath (PALS). Phenotypically, they are IgM+/-IgD++, CD21+/−, and CD23+, in mice.

3.2 Phases of B-cell ontogeny: antigen-dependent and antigen-independent

B-cell ontogeny can be separated into two main phases: earlier antigen-independent phase and later antigen-dependent phase (**Figure 6**). It should also be recalled that thymus-independent (TI) antigens have the ability to stimulate B-cells without T-cells' help and are traditionally divided into two categories, TI-1 antigens that can activate B-cells through coengagement of Toll-like receptors (TLR), such as LPS or other bacterial polysaccharides, and TI-2 antigens that lead to extensive cross-linking of the BCR, such as polymeric protein antigens or repeated structural motifs [33]. Some antigens fall outside these and form a third category. Finally, T-cell-independent responses should be discussed, knowing that T-cells could intervene at different levels of B-cell development in response to TI antigens (For review, see [34–36]).

3.2.1 Earlier antigen-independent phase

The first phase of B-cell differentiation and maturation would be antigen-independent. It takes place in the bone marrow and results in the generation of immature B-cells expressing pair L chains with μ chains to form cell surface monomeric IgM in association with transmembrane Igα and Igβ invariant chains, forming the BCR complex, which is able to recognize and capture antigens.

3.2.2 Later antigen-dependent phase

The second phase of B-cell activation and final differentiation is dependent on self-antigens, then on non-self-antigens within the secondary lymphoid organs (SLOs). It results in the formation of plasma cells and antigenic specific memory B-cells.

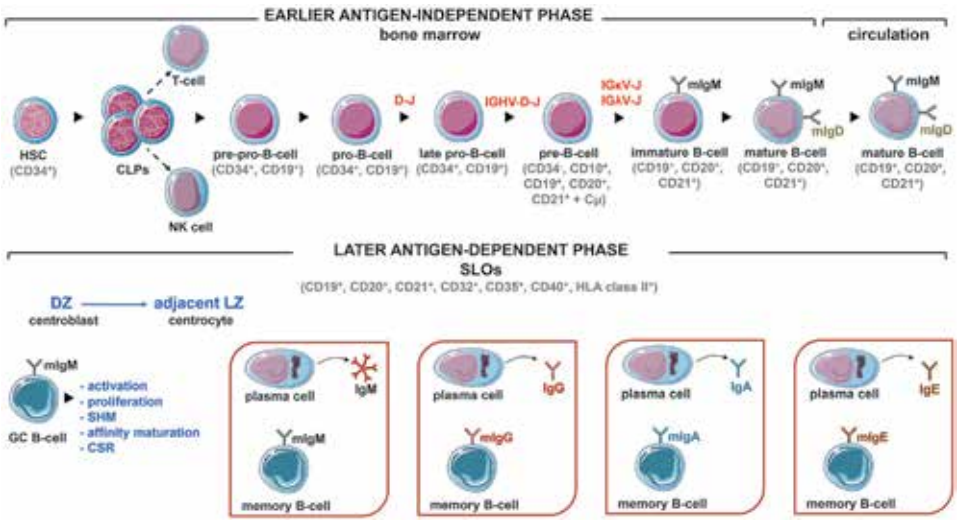


Figure 6.
Phases of B-cell ontogeny. CLPs, common lymphoid progenitors; mIgM, membrane immunoglobulin M; DZ, dark zone; LZ, light zone; HLA, human leucocyte antigen; GC, germinal center; CSR, class switch recombination.

4. B-cell markers: the main molecules of the B-lymphoid lineage

The majority of human peripheral blood B-cells express on their surface IgM and IgD that have the same antigenic specificity. In addition, in humans, a large population of circulating cells expresses membrane lgG or IgA (but very little IgE). In some tissues, including intestinal mucosa, B-cells selectively express membrane IgA. In addition to the Igα/Igβ heterodimer marker, which is part of the BCR complex, there are also other molecules on the B-cell surface that play notable roles in various cellular functions, especially in B-cell regulation, such as human complement receptor type 2 (CR2, C3d), designed as CD21, that regulates B-cell proliferative responses, and serving as a receptor for the C3d, C3dg, and iC3b proteins of complement. B-cells also express receptor for complement component C3b (CR1, CD35) as well as for Fc fragments (FcR) of certain Ig isotypes, such as IgG (FcγRII-B1, CD32). Of note, only FcγRIIB1 and FcγRIIB2 have an immunoreceptor tyrosine-based inhibitory motif (ITIM) sequence among the FcγR receptor family and are therefore inhibitory FcRs; they do not induce phagocytosis and represent an important receptor-mediated feedback circuit regulator by circulating antigen-specific IgG [24, 37]. CR2/CD21 has also been described as a receptor for the envelope glycoprotein gp350/220 of the Epstein–Barr virus (EBV) [38]. Other molecules are frequently used in routine practice as primary markers for identifying human B-cells, including CD19, CD20, and CD22 markers. CD19 is expressed at all stages of B-cell lineage, including normal plasma cells. CD20, a molecule restricted to the B-cell population, has been shown to be implicated to form calcium channels in cell membrane [39], and as an effective target for immunotherapy in treatment of B-cell lymphoma, as well as in a number of autoimmune diseases, such as type 1 diabetes [40]. It occurs at the early pre-B-cell stage of development and remains throughout all stages of B-cell maturation [41], ranging from pre-B-cells in the bone marrow to short-lived plasmablasts [42]. CD22, a B-cell-restricted surface molecule that regulates BCR signaling in mature B-cells [43], is an early marker and persists at all stages of B-cell differentiation, which has allowed it to be a useful pan marker for all mature B-cell subsets [44]. Recognized as a B-cell-specific sialic acid binding Ig-like lectin 2 (Siglec-2; B-lymphocyte cell adhesion molecule [BL-CAM]), CD22 has been exploited as a therapeutic target for humanized anti-CD22 monoclonal antibody to treat B-cell leukemia [45]. Both immature and mature mouse B-cells as well as subsets of T-cells and NK cells and subset of abnormal T-cells involved in the pathogenesis of systemic autoimmunity in MRL-Fas^{lpr} and MRL-Fas^{gld} mice express one of the five isoforms of the transmembrane tyrosine phosphatase CD45 (LCA) found on lymphocytes [46], *i.e.*, CD45R (B220, CD45RABC), which has been known to play a major role in lymphocyte signaling and activation. Other B-cell surface molecules allow them to cooperate with T-cells. They include essentially MHC II antigens (DR, DP, and DQ, in humans, and IA and IE, in mice) and CD40 (a member of the TNF receptor superfamily, also expressed on B-cell precursors in the bone marrow), which is capable of transducing extremely potent B-cell activation signals. Mutations in the gene coding for CD40 ligand (CD40L/CD154), predominantly expressed by activated $CD4^+$ T-cells, lead to X-linked hyper-IgM syndrome (HIGM1), a severe primary immune deficiency (PID) in humans, characterized by recurrent infections, associated with very low or absent IgG, IgA, and IgE levels, but normal or elevated serum levels of IgM [47]. B-cells can also be identified using the inhibitory co-receptor CD72 (Lyb-2 in mice), containing an ITIM in the cytoplasmic. CD72 specifically recognizes the RNA-containing endogenous TLR7 ligand Sm/ribonucleoprotein (RNP) by C-type lectin-like domain (CTLD) located in its extracellular region and specifically inhibits B-cell responses to Sm/RNP by recruiting SH2 domain-containing phosphatase 1 (SHP-1) to the

phosphorylated ITIM [48]. It has been reported to have a role in the regulation of systemic lupus erythematosus (SLE) development [49]. Its specific ligand is CD5 [50], which is usually expressed on most T-cells, but also on a subset of mature B-cells [51] and $CD5^+$ B1 cells, which emerge early in development and play a major role in autoimmunity [52]. Other markers, including CD10 and CD23, are fond at some stages of B-cell differentiation. CD10, a neutral endopeptidase, also referred to as common acute lymphoid leukemia antigen (CALLA), enkephalinase, and neprilysin, is expressed in early B-cell immature stages in bone marrow, but also in certain stages of immunopoiesis and is also present on antigen-activated cells in GC, in humans [53]. Defined initially as the low-affinity receptor for IgE (FcεRII) and as EBV cell surface (EBVCS) activation antigen or BLAST-2, CD23 exists in two distinct protein isoforms, CD23α (FcεRIIa), which is constitutively expressed only in normal B-cells and B-cell lines, and CD23β (FcεRIIb), which is an inducible molecule detectable on various cells [54], including most mature B-cells, follicular mantle (FM) B-cells, T-cell subsets (at low levels), NK cells, platelets, FDCs, monocytes, eosinophils, Langerhans cells, etc. CD23, a 45 kD type II transmembrane glycoprotein, can be cleaved and found in plasma as a freely soluble receptor (sCD23). In contrast to cross-linking of FcεRI that leads to degranulation of mast cells to release vasoactive mediators, engagement of membrane-bound CD23 suppresses the production of IgE by B-cells. Moreover, CD23 participates in many regulatory processes, including cytokine release, cell–cell contact, and

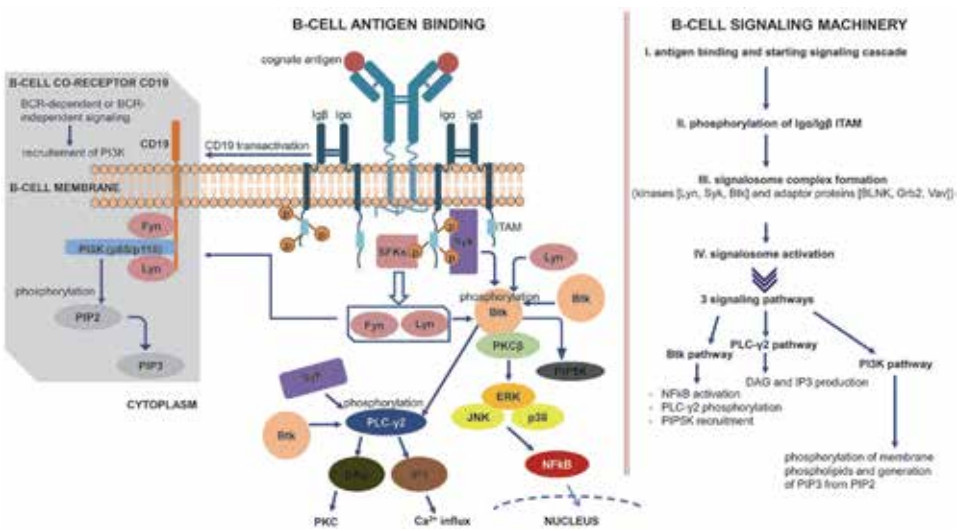


Figure 7.
BCR signaling. As mentioned above, the final activation of mature B-cell occurs in the SLOs, where they migrate through the blood. Within the SLOs, they receive a constant supply of antigen through the circulating lymph. The activation of B-cell is initiated after binding of an appropriate antigen to its BCR, leading to phosphorylation of the non-covalently associated Igα/Igβ transmembrane (CD79a and CD79b). The signaling mechanisms triggered during the B-cell activation can be summarized in four main steps: (i) antigen binding and starting signaling cascade, (ii) phosphorylation of Igα/Igβ ITAM, (iii) signalosome complex formation, and (iii) signalosome activation. Akt, protein kinase B/PKB; BCR, B-cell antigen receptor; BLNK, B-cell linker; Btk, Bruton's protein tyrosine kinase/non-receptor kinase; CD, cluster of differentiation; ERK, extracellular signal-regulated kinase; Fyn, proto-oncogene tyrosine-protein kinase Fyn/non-receptor Fyn proto-oncogene; Grb2, growth factor receptor-bound protein 2; ITAM, immunoreceptor tyrosine-based activation motifs; JNK, c-Jun N-terminal kinase; Lyn, Lck/yes novel tyrosine kinase; mIg, membrane-bound immunoglobulin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B-cells; PI3K, phosphoinositide-3 kinase; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PLCγ2, phospholipase C-γ2; PKCβ, protein kinase C β; SLOs, secondary lymphoid organs; SFKs, Src-family kinase/Src-protein-tyrosine kinase or Src family tyrosine kinase; Src, proto-oncogene tyrosine-protein kinase c-Src (cellular Src kinase); Syk, spleen tyrosine kinase/non-receptor tyrosine kinases; Vav, proteins acting as guanine nucleotide exchange factors (GEFs) for small G proteins of the rho family.

receptor-mediated positive and negative feedback circuits, either as a membrane-bound glycoprotein or as sCD23 [55]. Finally, a new type of immune cell, so-called X lymphocyte, that is a dual expresser (DE) of TCR and BCR and key lineage markers of both B- and T-cells, has just recently been discovered in patients with type 1 diabetes (For review see [56]).

5. Normal mature B-cell activation and signaling mechanisms

Antigen binding to mIg induces the BCR aggregation, which leads to the rapid transmembrane disulfide-linked heterodimer phosphoprotein Igα (CD79a)/Igβ (CD79b) ITAM phosphorylation through recruitment of Syk and SFKs (Fyn, Lyn). This process begins with the formation of a "signalosome" [57]. The signalosome activation leads to three main pathways [58], including Btk, PLC-γ2, and PI3K. BCR can transactivate the B-cell co-receptor CD19, which forms, on B-cell surface, a tetrameric co-receptor complex with CD21 and CD81 (target of anti-proliferative antibody 1 (TAPA-1), a tetraspanin family member tetraspanin 1 (Tspan1, NET-1), and Leu13 (CD225)) [59]. CD19 can also be BCR-independently activated but lacks intrinsic or associated tyrosine kinase activity [60]. As CD19 has a long cytoplasmic domain, it binds and amplifies the function of the SFKs and recruits a heterodimer p85/p110 class IA PI3K concurrently, which phosphorylates a membrane phospholipid, PIP2, leading to the production of a second messenger PIP3 [61], as well as promoting Btk and Akt, and a serine/threonine, kinase phosphorylation in B-cell [62] (**Figure 7**).

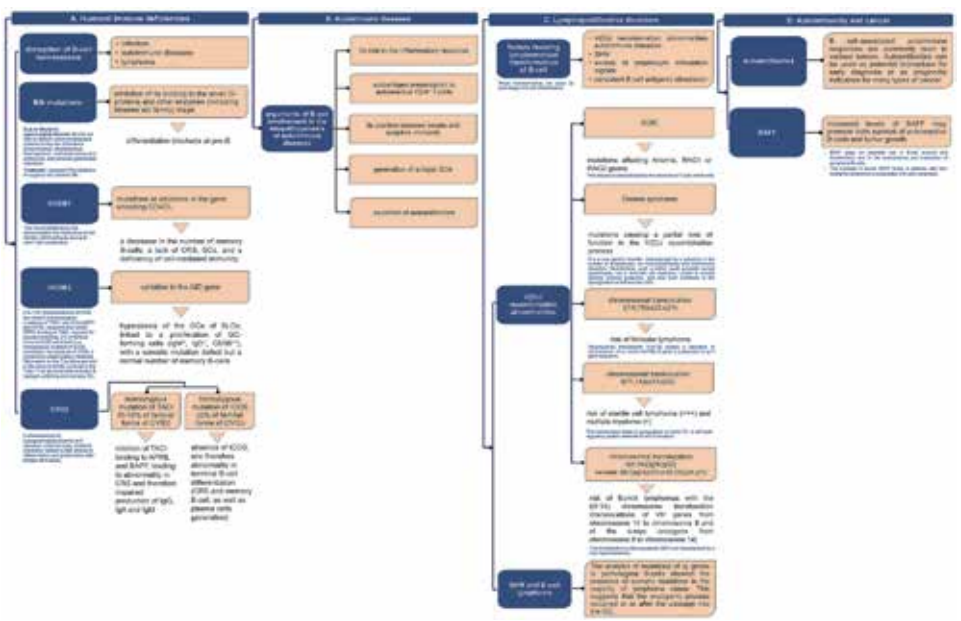


Figure 8.
Main types of diseases related to B-cell abnormalities. AID, activation-induced cytidine deaminase; Btk, Bruton's tyrosine kinase; HIGM1, X-linked hyper-IgM syndrome type 1; CD40L, CD40 ligand (CD154); CSR, class switch recombination; GCs, germinal centers; Ig H, immunoglobulin heavy chain; BAFF, B-cell activating factor also known as B-lymphocyte stimulator (BLyS) or tumor necrosis factor (TNF) ligand superfamily member 13B (TNF-like molecule BAFF); BCL-2, B-cell lymphoma 2; EBV, Epstein–barr virus; CVID, common variable immunodeficiency; IVIg, intravenous immunoglobulin; SCID, severe combined immunodeficiency; SHM, somatic hypermutation; SLOs, secondary lymphoid organs; TACI, transmembrane activator and calcium modulator and cytophilin ligand interactor; XLA, X-linked agammaglobulinemia (Bruton's agammaglobulinemia); HIGM2, hyper-IgM syndrome type 2 (autosomal recessive).

6. B-cell and pathologies

B-cells are threatened by various pathologies, including (i) immune deficiency, which results from a disruption in their homeostasis due to mutations or deletions, abnormalities in mechanisms of gene repair, Ig class generation, Ig affinity modulation, etc.; (ii) autoimmune disorders, in which potentially dangerous self-reactive clone B-cells escape from mechanisms of tolerance; (iii) cancerous tumors, which result from a disorder in the succession of phases of proliferation and cell death or in the processes of generation of cell diversity; and (iv) allergies that are particularly related to regulatory abnormalities involving Br1 (IL-10^{+}), Br3 (TGF-β^{+}), and regulatory B-cells (Breg, Foxp3^{+}) [52]. The main B-cell-associated diseases are illustrated in **Figure 8**.

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Chapter 2

Immunogenetic Aspect of B-Cell Antigen Receptor Diversity Generation

Mourad Aribi

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: μ → δ → γ3 → γ1 → α1 → γ2 → γ4 → ε → α2 in humans (**Figure 1**), or μ → δ → γ3 → γ1 → γ2b → γ2a → ε → α in mice.

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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

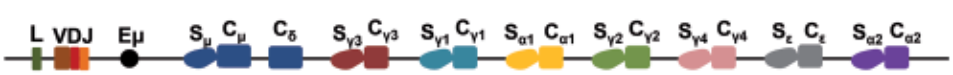

Figure 1.
The organization of the IGH chain C-region genes in humans. Eμ: intronic enhancer, S: switch region, IGH: immunoglobulin heavy chain.

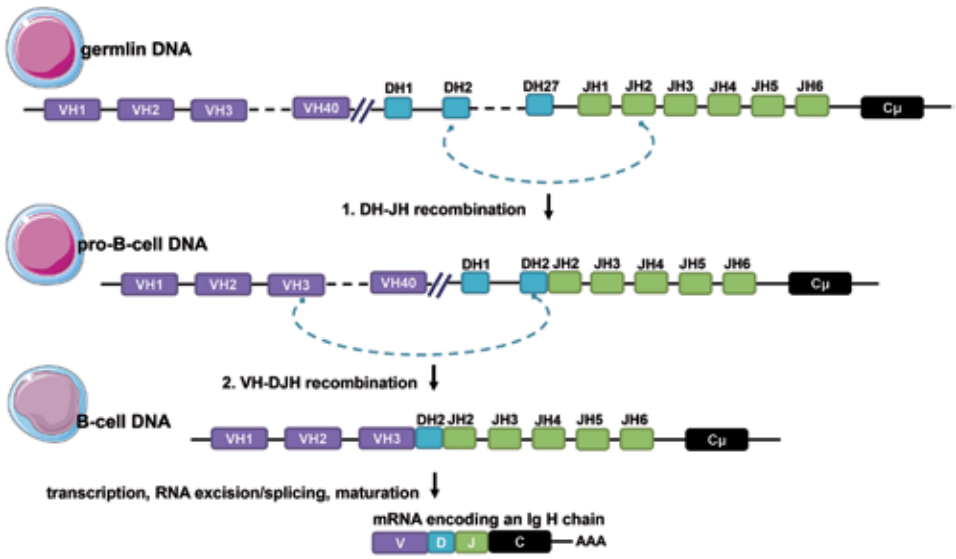

Figure 2.
Ordered rearrangement of gene segments (adapted from [3]). In the ordered rearrangement model, rearrangements of gene segments at the H chain locus precede those at the L chain locus. Sequential rearrangements lead first to the recombination of one of the DH segments with one of the JH segments, 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 × 27 × 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.

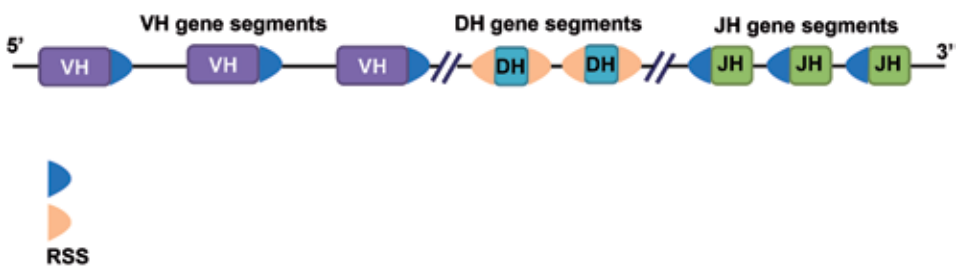

Figure 3.
Cleavage site of RAG proteins at the V, D, and J gene segments of the IGH locus: RSS positions. RAG: recombination-activating gene, RSS: recombination sequence signal, IGH: immunoglobulin heavy chain locus.

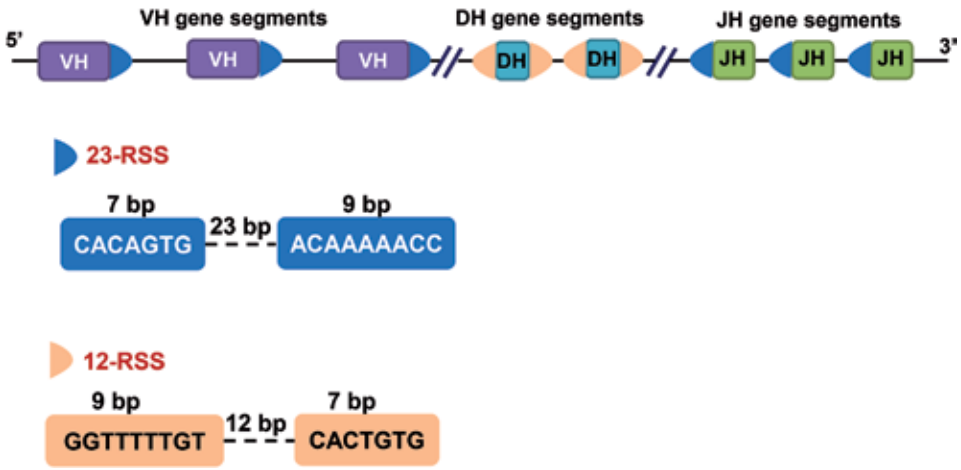

Figure 4.
RSS motifs. There are two types of RSS, one includes a 12-nucleotide spacer (12-RSS), and the other includes a 23-nucleotide spacer (23-RSS). Both 12-RSS and 23-RSS include a highly conserved palindromic heptamer and nonamer sequences. Bp: base pairs, RSS: recombination sequence signal.

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 (nongermline/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

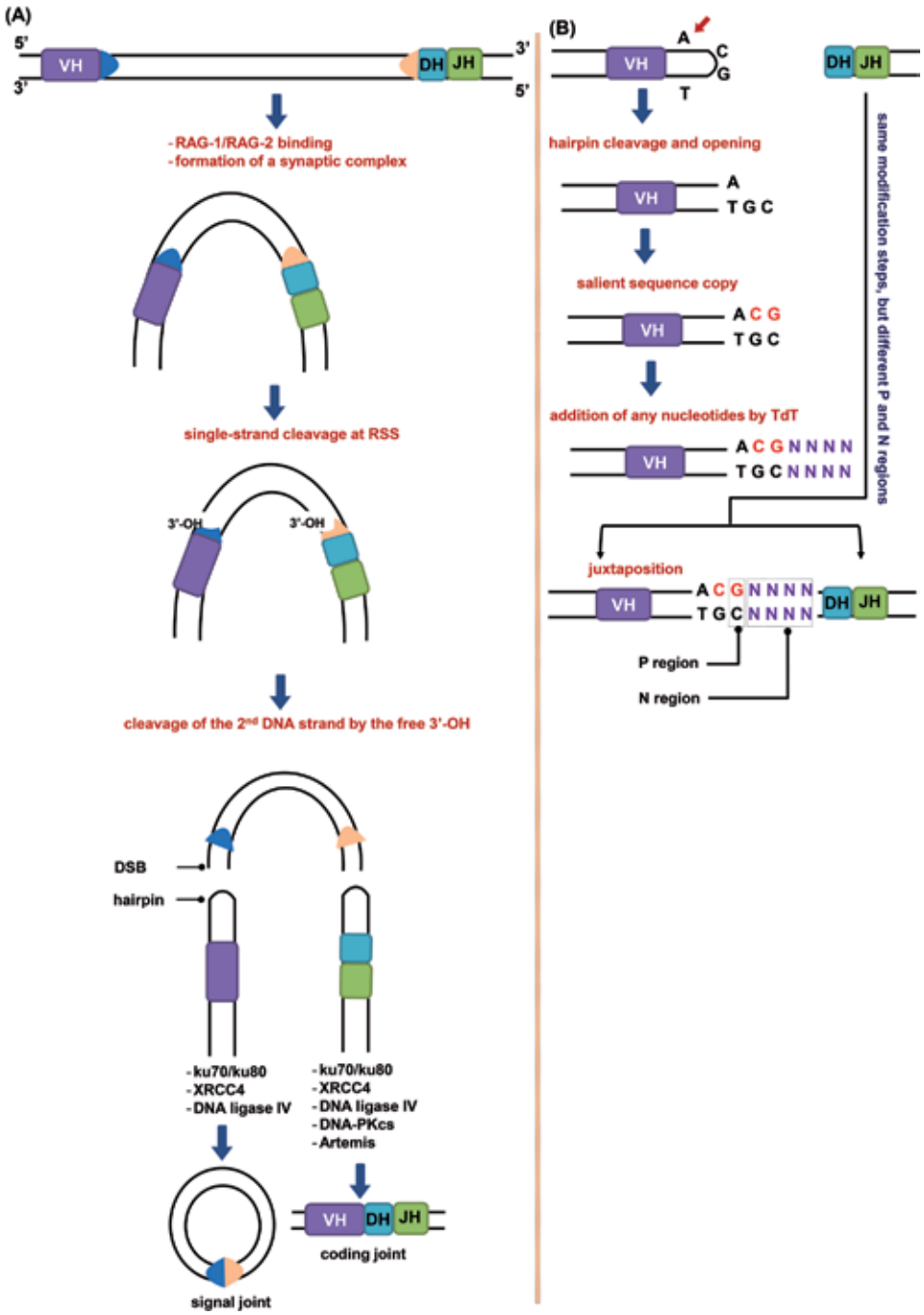

Figure 5.
Molecular mechanisms of V(D)J recombination and junctional diversity generation (adapted from [3]). (A) Main steps of V(D)J recombination. (B) Edge modification of coding regions and junctional diversity. DNA-PKcs: DNA-dependent serine/threonine protein kinase complex, DSB: double strand-breaks, N: nongermline/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 Eμ 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 μ0 transcripts, and at downstream of intronic IGH enhancer Eμ, generating Iμ transcripts. Both μ0 and transcripts Iμ 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′ Dβ1 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 Lκ and Lλ 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 λ5 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 Lκ 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).

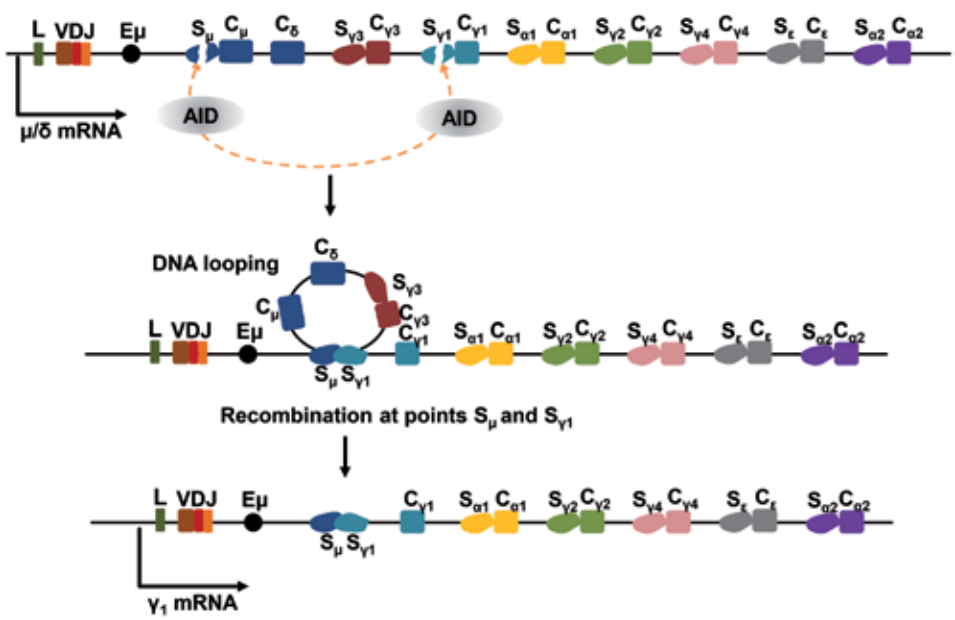

Figure 6.
CSR process. Here is an example of switching to the IgG1 isotype. First, AID induces DSB formation after deamination of S_{μ} and $S_{\gamma 1}$ regions. Subsequently, these two regions recombine by an intrachromosomal deletional recombination, and the expressed VDJ segment associates with the $C_{\gamma 1}$ gene. AID: activation-induced cytidine deaminase, CSR: class switch recombination (also called immunoglobulin heavy chain class switching/ isotype switching), DSB: double strand-breaks, Eμ: 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 though 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 (Sμ) 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**).

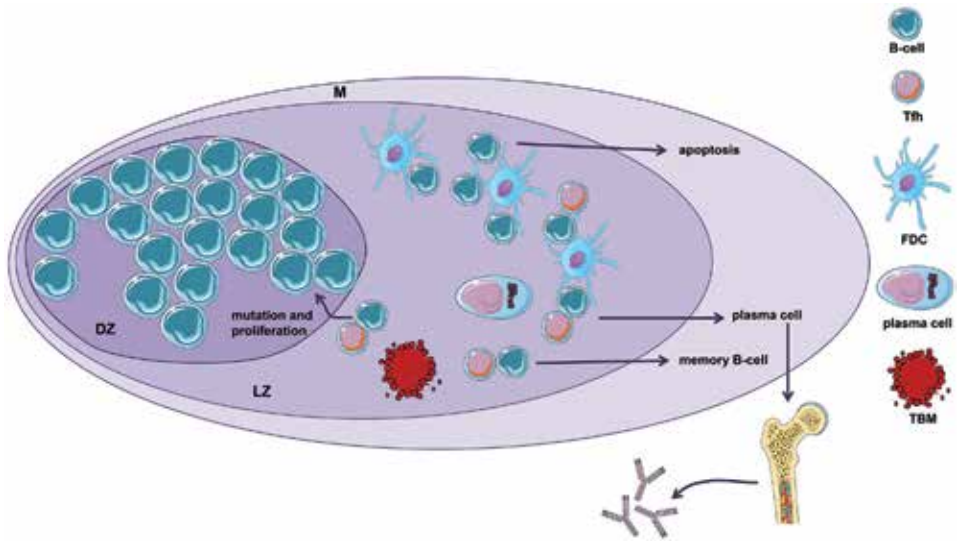

Figure 7.
Schematic representation of the organization of the germinal center. The dark zone is occupied mainly by proliferating centroblasts. These are cells in which SHM would take place. After proliferation, B-cells, now called centrocytes, are in the adjacent LZ, which also contains Tfh cells and 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 naive $CD4^+$ 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/WRCYmotifs (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.

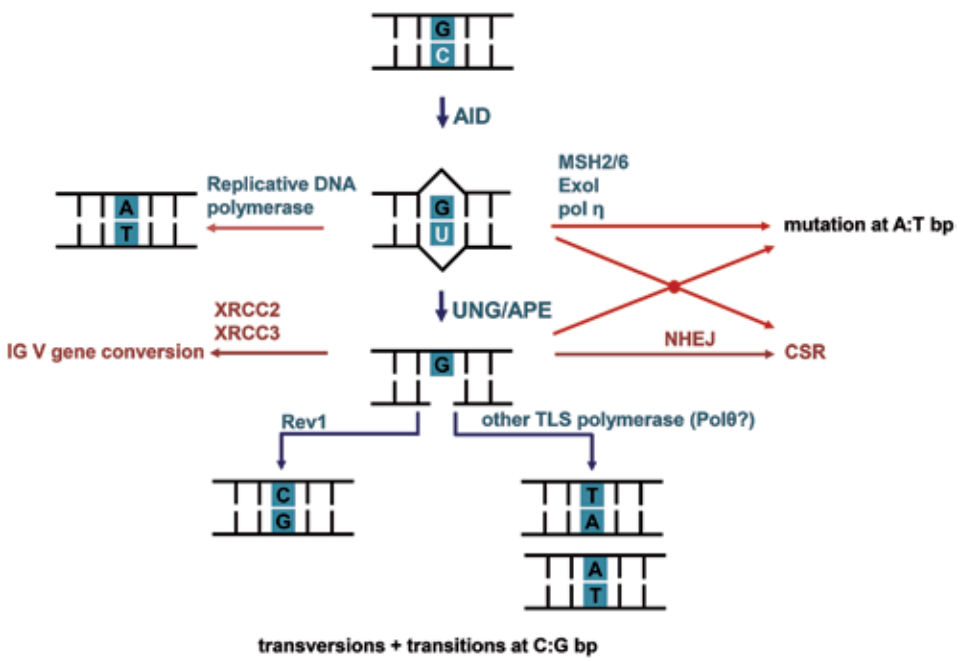

Figure 8.
DNA deamination model of IG gene diversification by AID during SHM (adapted from [51]). AID creates mutations in DNA by deamination of cytosine base, which turns it into uracil, which is then targeted by BER or MMR mechanisms or simply replicated producing mutations. AID: activation-induced 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 eta (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 ¾ 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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Section 2

B-Cell Lymphomagenesis

Chapter 3

B-Cell Lymphomas

Subramanian Kalaivani Selvi, B.H. Srinivas and Sadhanandham Shrinuvasan

Abstract

B-cell lymphomas arise from different stages of differentiation of B-cells and constitute a broad spectrum, extending from small- to large-cell types, and from low to high grades of clinical behavior. It has undergone several terminologies and classifications. Because of the diverse terminology that is used in the multiple classifications of lymphomas, there have been attempts to develop uniform pathologic descriptions with clinical usefulness. The current WHO classification uses morphologic, immunophenotypic, genotypic, and clinical features to classify the lymphoid neoplasms into five broad categories as precursor B-cell neoplasms, peripheral B-cell neoplasms, precursor T-cell neoplasms, peripheral T-cell neoplasms and Hodgkin lymphoma. Hodgkin lymphoma though originates from B-cell has distinctive pathologic features and is treated as a separate entity. This chapter discusses about the etiology and pathogenesis, clinical features, recent WHO classification of B-cell lymphoma (2016), the highlights of modifications brought in, the morphology, immunophenotype, staging, treatment and prognosis of various B-cell lymphomas.

Keywords: B-cell lymphomas, recent WHO classification, pathogenesis, morphology, immunophenotype, treatment, prognosis

1. Introduction

B-cell lymphomas are malignant neoplasms that arise from different stages of differentiation of B-cells and constitute a broad spectrum, extending from small- to large-cell types, and from low to high grades of clinical behavior. It constitutes the major type of non-Hodgkin's lymphoma (NHL) [1–3].

The recognition of lymphoma evolved from Thomas Hodgkin's 1832 paper entitled "On Some Morbid Appearances of the Absorbent Glands and Spleen" [4], followed by various nomenclatures and classifications that classified lymphomas into Hodgkins and non-Hodgkin's lymphomas [5–11]. In 1974, Lennert et al. [12] and Lukes and Collins [13] classified NHL on the basis of the cell of origin as B cell lymphomas and T cell lymphomas which was later confirmed at the molecular level with the identification of specific Ig gene and T-cell receptor (TCR) gene rearrangements [14–17]. In 1982, a Working Formulation (WF) classified NHL according to histologic grade as low, intermediate, and high [18]. In 1994, a Revised European American Lymphoma (REAL) Classification was proposed which diagnosed lymphomas by identifying clinical features, morphology, immunophenotype, and genetic data [19]. The World Health Organization (WHO) then adopted the diagnostic principles of the REAL Classification, and is used as the schema for the diagnosis of all hematopoietic neoplasms [20, 21].

The 2001 and 2008 World Health Organization classification of hematopoietic and lymphoid tumors established guidelines for the diagnosis of malignant lymphomas; however, subsequently, there have been major advances with significant clinical and biologic implications which necessitated further revisions [22, 23]. Hence further revision was done in 2016 which reflects a consensus among hematopathologists, geneticists, and clinicians regarding both updates to current entities as well as the addition of a limited number of new provisional entities leading to more targeted therapeutic strategies. The current WHO classification uses morphologic, immunophenotypic, genotypic, and clinical features to classify the lymphoid neoplasms into five broad categories as precursor B-cell neoplasms, peripheral B-cell neoplasms, precursor T-cell neoplasms, peripheral T-cell neoplasms and Hodgkin lymphoma. Hodgkin lymphoma though originates from B-cell has distinctive pathologic features and is treated as a separate entity. This chapter discusses about the etiology and pathogenesis, clinical features, recent WHO classification of B-cell lymphomas (2017), the highlights of modifications brought in, the morphology, immunophenotype, staging, treatment and prognosis of various B-cell lymphomas [24–26].

2. B-cell lymphomas: origin and development

The differentiation of the B-cell lineage starts from stem cells the common lymphoid precursor to plasma cells that occurs successively in the fetal liver, bone marrow, and lymph nodes. The characteristic marker of B cells is the presence of immunoglobulins, which act as the cell surface antigen receptor. The genes that code for antibody are rearranged in the course of differentiation from stem cell to pre-B cell [20]. Understanding the stages of B-cell maturation has facilitated the recognition of the interrelationships between the various types of B-cell lymphomas and leukemia.

The WHO classification distinguishes two major categories within B-cell lymphomas: precursor and mature. The precursor B-cell lymphomas comprise those lymphoblastic lymphomas and leukemia that derive from progenitor cells that have not yet been activated by antigen and are still in an undifferentiated stage. All other lymphomas representing different stages of differentiation are included in the category of mature B-cell lymphomas. Naïve B-cell can give rise to mantle cell lymphoma, small lymphocytic lymphoma/ chronic lymphocytic lymphoma. Lymphomas originating in the cells of the germinal centres are follicular lymphomas, Burkitt lymphoma and diffuse large B cell lymphoma. Post germinal center cells can give rise to marginal zone lymphoma, small lymphocytic lymphoma/ chronic lymphocytic lymphoma, diffuse large B cell lymphoma, plasma cell myeloma, lymphoplasmacytic lymphoma [1, 20, 22–25].

B-cell lymphomas constitute a broad spectrum, extending from small- to large-cell types, and from low to high grades of clinical behavior. The term "grade" is defined by the size and shape of cells and nuclei; density of chromatin; the number of mitoses (proliferation index), which may indicate the aggressiveness of a tumor; and by its clinical behavior. However, aggressiveness and tumor grade cannot be used synonymously because their correlation is not consistent; some lymphomas of highly aggressive behavior, such as mantle cell lymphoma, may exhibit low histologic grades.

Hodgkin lymphoma (HL) encompasses another group of lymphoid neoplasms that are characterized by neoplastic Reed-Sternberg cells derived from the germinal center or post-germinal center B cells and differ from NHL in several respects. Hence it is treated as a separate category though it is derived from B-cells.

3. Etiology and pathogenesis

Chromosomal Translocations and Other Acquired Mutations are present in the majority of lymphomas. Many specific rearrangements are associated with particular neoplasms, suggesting a critical role in their genesis. The mutation can produce a "dominant negative" protein that interferes with a normal function (a loss of function) or inappropriate increase in some normal activity (a gain of function). Oncoproteins created by genomic aberrations often block normal maturation, turn on pro-growth signaling pathways that enhance the self-renewal of tumors cells, or protect cells from apoptotic cell death. Pro-growth signaling mutations produce a constitutively active tyrosine kinase; oncogenic tyrosine kinases activate RAS and its two downstream signaling arms, the phosphoinositide-3-kinase/AKT8 virus oncogene cellular homolog (PI3K/AKT) and mitogen-activated protein kinases (MAPK) pathways and thereby drive cell growth. Oncogenic mutations most frequently occur in germinal center B cells during antibody diversification. B cells that enter germinal centres after antigen stimulation upregulate the expression of activation-induced cytosine deaminase (AID), a specialized DNA-modifying enzyme which is essential for immunoglobulin (Ig) gene modifications: (1) class switching, an intragenic recombination event in which the IgM heavy-chain constant gene segment is replaced with a different constant segment leading to a switch in the class (isotype) of antibody produced; and (2) somatic hypermutation, which creates point mutations within Ig genes that may increase antibody affinity for antigen. Certain protooncogenes, such as myelocytomatosis oncogene cellular homolog (MYC), are activated in germinal center B-cell lymphomas by translocations to the transcriptionally active Ig locus. Other proto-oncogenes, such as BCL6, a transcription factor that has an important role in many B cell malignancies, are frequently activated in germinal center B-cell lymphomas by point mutations that also seem to stem from "mistargeted" DNA breaks induced by AID. A different type of regulated genomic instability is unique to precursor B cells, which express a V (D)J recombinase that cuts DNA at specific sites within the Ig [1].

Other factors include immunosuppression; infectious agents like Epstein-Barr virus, Human T-cell lymphotropic virus type 1, *Helicobacter pylori*, Hepatitis C virus, Human herpesvirus 8 (Kaposi sarcoma), Human herpesvirus 6, Human T-cell lymphotropic virus type 2 are known to be associated with lymphomas [27–31]. Male gender, increasing age, family history of non-Hodgkin's lymphoma, prior cancer history, drug history, immunosuppressive agents like phenytoin, methotrexate; occupational history like exposure to herbicides, pesticides, wood dust, epoxy glue, solvents; jobs in farming, forestry, painting, carpentry, tanning, hair dye use, sunlight exposure, nutritional factors, blood transfusion are the other possible etiologic factors [1, 32–37].

4. Clinical features

The clinical presentation of the various lymphoid neoplasms is most often determined by the anatomic distribution of disease. Two-thirds of non-Hodgkin's Lymphomas present as enlarged nontender lymph nodes. The remaining one-third of NHLs present with symptoms related to the involvement of extranodal sites leading to mass effect, obstructive and compressive signs and symptoms. The lymphocytic leukemia most often come to attention because of signs and symptoms related to the suppression of normal hematopoiesis by tumor cells in the bone marrow. Plasma cell neoplasm, multiple myeloma, causes bony destruction of the skeleton and often presents with pain due to pathologic fractures. Other symptoms related to

lymphoid tumors are frequently caused by proteins secreted from the tumor cells or from immune cells that are responding to the tumor. Specific examples include the plasma cell tumors, in which much of the pathophysiology is related to the secretion of whole antibodies or Ig fragments; Significant cytopenias are rare, unless marrow involvement is extensive, or there are associated immune-mediated cytopenias, hypersplenism, or, rarely, hemophagocytosis. Hepatosplenomegaly is a common feature of advanced low-grade B-cell lymphoma. Bleeding manifestations are common in lymphoblastic lymphomas/leukemia with marrow involvement [1, 2].

5. Investigations

Complete history and physical examination; inquiry about B symptoms, human immunodeficiency virus risk, infections, autoimmune diseases, immunosuppressive therapy.

Complete blood cell count and peripheral smear examination including hemoglobin, total leukocyte count with differential and platelet count.

Biochemistry profile—lactate dehydrogenase; alkaline phosphatase, uric acid, creatinine, calcium, and albumin.

Imaging—computed tomography of chest, abdomen, pelvis, and neck, Selected radiologic procedures as clinically appropriate (e.g., gallium, positron emission tomography scan, magnetic resonance imaging, ultrasound, bone scan).

Bone marrow aspiration and biopsy—morphology, immunophenotyping, cytogenetics, molecular tests, and gene rearrangement studies.

Lumbar puncture with cytology in patients with CNS involvement.

Gastrointestinal endoscopy for patients with Waldeyer ring involvement or abdominal symptoms.

Cytologic assessment of third space fluids (pleura, peritoneum) in case of effusions.

Other blood evaluations: levels of ß 2-microglobulin and cytokines (interleukin-2 receptor, tumor necrosis factor).

6. 2016 WHO classification of B lymphoid neoplasms

The current WHO classification uses morphologic, immunophenotypic, genotypic, and clinical features to classify the lymphoid neoplasms into five broad categories as precursor B-cell neoplasms, peripheral B-cell neoplasms, precursor T-cell neoplasms, peripheral T-cell neoplasms and Hodgkin lymphoma. Hodgkin lymphoma though originates from B-cell has distinctive pathologic features and is treated as a separate entity (**Table 1**).

The WHO classification distinguishes two major categories within B-cell lymphomas:

a. Precursor B-cell neoplasms

b. Mature B-cell neoplasms

6.1 Precursor B-cell neoplasms

Acute lymphoblastic leukemia/lymphoma (ALLs) are neoplasms composed of immature B (pre-B) which are referred to as lymphoblasts. About 85% are B-ALLs, which typically manifest as childhood acute "leukemia." Many of the chromosomal aberrations seen in ALL dysregulate the expression and function of transcription factors required for normal B development. B-ALLs have loss-of-function mutations

in genes that are required for B-cell development, such as Paired Box 5 (PAX5), E2-alpha (E2A), and early B-cell factor (EBF), or a balanced t(12;21) involving the genes translocation-Ets-leukemia virus (ETV6) and Runt Related Transcription Factor 1 (RUNX1), two genes that are needed in very early hematopoietic precursors. All of these varied mutations disturb the differentiation of lymphoid precursors and promote maturation arrest, and in doing they induce increased self-renewal, a stem cell-like phenotype. Approximately 90% of ALLs have numerical or structural chromosomal changes. Most common is hyperploidy (>50 chromosomes), but hypoploidy and a variety of balanced chromosomal translocations are also seen [1, 25, 38].

Patients present with fatigue due to anemia; fever due to infections secondary to neutropenia; and bleeding due to thrombocytopenia. They can so present with mass effects caused by neoplastic infiltration, including bone pain, generalized lymphadenopathy, splenomegaly, hepatomegaly; testicular enlargement; headache, vomiting, and nerve palsies due to central nervous system involvement [1, 25].

6.1.1 Morphology and immunophenotype

In leukemic presentations, the marrow is hypercellular and packed with lymphoblasts, which replace the normal marrow elements, lymphoblasts have more condensed chromatin, less conspicuous nucleoli, and smaller amounts of cytoplasm that usually lacks granules. Histochemical stains show periodic acid-Schiff–positive cytoplasmic material. Immunostaining for terminal deoxynucleotidyl transferase (TdT), is positive in more than 95% of cases in addition to B-cell markers. B-ALLs are arrested at various stages of development. The lymphoblasts usually express the pan B-cell marker CD19/CD22/CD79a and the transcription factor PAX5. CD10 is expressed in common ALL and pre-B ALLs express in addition cytoplasmic IgM heavy chain (μ chain) and surface immunoglobulins in mature forms (**Figure 1**).

6.1.2 New provisional entities in recent WHO classification

- B-ALL with intrachromosomal amplification of chromosome 21 [24, 39, 40].
- B-ALL with translocations involving tyrosine kinases or cytokine receptors ("BCR-ABL1—like ALL").

6.1.3 Treatment and prognosis

Treatment includes chemotherapy, steroids, radiation therapy and marrow transplantation. About 95% of children obtain a complete remission, and 75–85% are cured

WHO classification of B lymphoid neoplasms
B-lymphoblastic leukemia/lymphoma
B-lymphoblastic leukemia/lymphoma, NOS B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities B-lymphoblastic leukemia/lymphoma with t(9;22)(q34.1;q11.2);BCR-ABL1 B-lymphoblastic leukemia/lymphoma with t(v;11q23.3);KMT2A rearranged B-lymphoblastic leukemia/lymphoma with t(12;21)(p13.2;q22.1); ETV6-RUNX1 B-lymphoblastic leukemia/lymphoma with hyperdiploidy B-lymphoblastic leukemia/lymphoma with hypodiploidy B-lymphoblastic leukemia/lymphoma with t(5;14)(q31.1;q32.3) IL3-IGH B-lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3);TCF3-PBX1 Provisional entity: B-lymphoblastic leukemia/lymphoma, BCR-ABL1–like Provisional entity: B-lymphoblastic leukemia/lymphoma with iAMP21
Mature B-cell neoplasms

WHO classification of B lymphoid neoplasms
Monoclonal B-cell lymphocytosis*
B-cell prolymphocytic leukemia
Splenic marginal zone lymphoma
Hairy cell leukemia
Splenic B-cell lymphoma/leukemia, unclassifiable
Splenic diffuse red pulp small B-cell lymphoma
Hairy cell leukemia-variant
Lymphoplasmacytic lymphoma
Waldenstro¨m macroglobulinemia
Monoclonal gammopathy of undetermined significance (MGUS), IgM*
m heavy-chain disease
g heavy-chain disease
a heavy-chain disease
Monoclonal gammopathy of undetermined significance (MGUS), IgG/A*
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Monoclonal immunoglobulin deposition diseases*
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) Nodal marginal zone lymphoma
Pediatric nodal marginal zone lymphoma
Follicular lymphoma
In situ follicular neoplasia*
Duodenal-type follicular lymphoma*
Pediatric-type follicular lymphoma*
Large B-cell lymphoma with IRF4 rearrangement*
Primary cutaneous follicle center lymphoma
Mantle cell lymphoma
In situ mantle cell neoplasia*
Diffuse large B-cell lymphoma (DLBCL), NOS
Germinal center B-cell type*
Activated B-cell type*
T-cell/histiocyte-rich large B-cell lymphoma
Primary DLBCL of the central nervous system (CNS)
Primary cutaneous DLBCL, leg type
EBV1 DLBCL, NOS*
EBV1 mucocutaneous ulcer*
DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK1 large B-cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
HHV81 DLBCL, NOS*
Burkitt lymphoma
Burkitt-like lymphoma with 11q aberration*
High-grade B-cell lymphoma, with MYC and BCL2 and/or BCL6 rearrangements*
High-grade B-cell lymphoma, NOS*
B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma

Changes from the 2008 classification.

Table 1.
WHO classification of B lymphoid neoplasms

with chemotherapy, whereas only 35–40% of adults are cured. Treatment of t(9;22)-positive ALLs with BCR-ABL kinase inhibitors in combination with conventional chemotherapy is highly effective and has greatly improved the outcome in children. The outlook for adults with ALL remains more guarded, in part because of differences

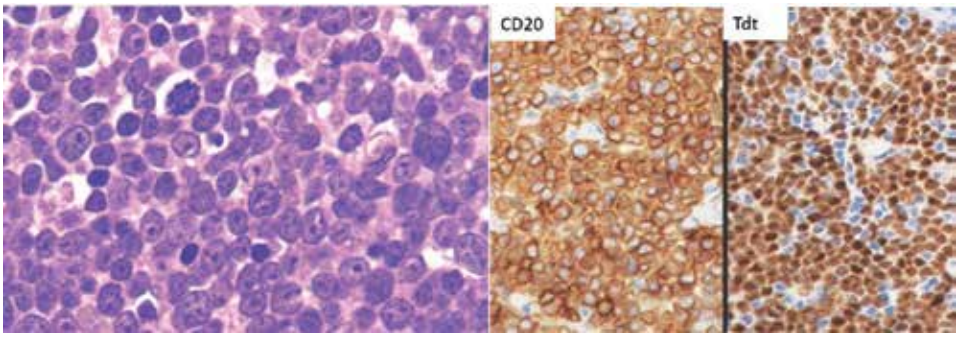

Figure 1.
B-lymphoblastic lymphoma showing CD20, Tdt positivity.

in the molecular pathogenesis of adult and childhood ALL, but also because older adults cannot tolerate the very intensive chemotherapy regimens that are curative in children. Factors associated with worse prognosis includes: (1) age younger than 2 years, (2) presentation in adolescence or adulthood; and (3) peripheral blood blast counts greater than 100,000. Factors associated with favorable prognosis include (1) age between 2 and 10 years, (2) a low white cell count, (3) hyperdiploidy, (4) trisomy of chromosomes 4, 7, and 10, and (5) the presence of a t(12;21) [1, 25, 38].

6.2 Chronic lymphocytic leukemia/small lymphocytic lymphoma

Chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) are used interchangeably by the degree of peripheral blood lymphocytosis. CLL is the most common leukemia of adults with absolute lymphocyte count >5000 per mm^3. The median age at diagnosis is 60 years. SLL constitutes only 4% of NHLs and has lymph node involvement The common genetic anomalies associated are deletions of 13q14.3, 11q, and 17p, and trisomy 12q [1, 25]. Patients are usually asymptomatic at diagnosis and can present with easy fatigability, weight loss, and anorexia. Generalized lymphadenopathy and hepatosplenomegaly are present in 50–60% of patients. The leukocyte count is high in most cases; leukopenia can be seen in individuals with SLL and marrow involvement.

6.2.1 Morphology and immunophenotype

Peripheral blood contains large numbers of small round lymphocytes with scant cytoplasm. Some of these cells are usually disrupted in the process of making smears, producing smudge cells. Prolymphocytes can also be circulating based on which it is classified as CLL or CLL/PLL or PLL (Prolymphocytic leukemia). The bone marrow is almost always involved by interstitial infiltrates or aggregates of tumor cells. Infiltrates are also virtually always seen in the splenic white and red pulp and the hepatic portal tracts. Lymph nodes are diffusely effaced by predominantly small lymphocytes with mild irregular nucleus, condensed chromatin, and scant cytoplasm along with proliferation centers, which contain aggregates of mitotically active larger activated lymphocytes. (**Figure 2**).CLL/SLL has a distinctive immunophenotype. The tumor cells express the pan B-cell markers CD19 and CD20, CD23, CD5, LEF1 (Lymphoid Enhancer Binding Factor 1), CD200. Negative for SOX11 (SRY (sex determining region) - Box 11), CD10.

6.2.2 Highlights in recent WHO classification

Cytopenias or disease-related symptoms are now insufficient to make a diagnosis of CLL with $<5 \times 10^9$/L PB CLL cells.

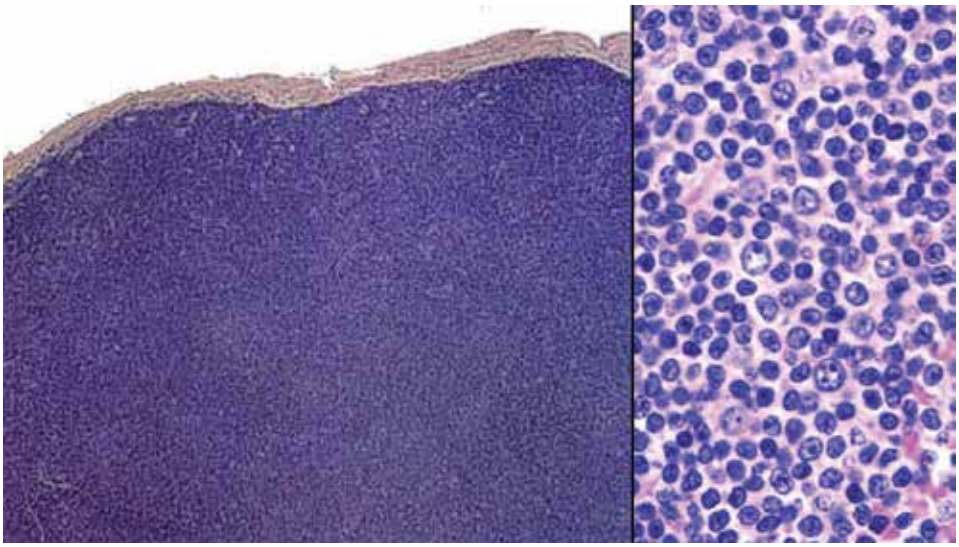

Figure 2.
Small lymphocytic lymphoma showing diffuse effacement by small lymphoid cells.

Large/confluent and/or highly proliferative proliferation centres are adverse prognostic indicators [24, 41–44].

Mutations of potential clinical relevance, such as TP53 (tumor protein 53), NOTCH1 (*Notch* homolog *1*), SF3B1 (Splicing Factor 3b Subunit 1), ATM (Ataxia telangiectasia mutated), and BIRC3 (Baculoviral IAP repeat-containing protein 3), have been recognized

6.2.3 Treatment and prognosis

The treatment, course and prognosis depend primarily on the clinical stage. Median survival is 4–6 years but can be more than 10 years in those with minimal tumor burdens. Factors associated with worse outcome include (1) deletions of 11q and 17p, (2) lack of somatic hypermutation, (3) expression of ZAP-70(z-chain-associated protein kinase), and (4) presence of NOTCH1 mutations. CLL also have the risk of transformation into diffuse large B -cell lymphoma [1, 25]. Symptomatic patients are generally treated with "gentle" chemotherapy and immunotherapy with antibodies against proteins found on the surface of CLL/SLL cells, particularly CD20. Hematopoietic stem cell transplantation is being offered to the relatively young. The most promising new therapy is Bruton's tyrosine kinase (BTK) inhibitors [1, 25].

6.3 Follicular lymphoma

Follicular lymphoma (FL) is the most common form of indolent NHL. It usually presents in middle age and afflicts males and females equally. Follicular lymphoma likely arises from germinal center B cells and is strongly associated with chromosomal translocations involving BCL2 (B-cell lymphoma 2). Its hallmark is a (14; 18) translocation that juxtaposes the IGH locus on chromosome 14 and the BCL2 locus on chromosome 18. The t (14; 18) is seen in up to 90% of follicular lymphomas and leads to overexpression of BCL2. Follicular lymphoma tends to present with painless, generalized lymphadenopathy. Involvement of extranodal sites, such as the gastrointestinal tract, central nervous system, or testis, is relatively uncommon [1, 25, 45, 46].

6.3.1 Morphology and immunophenotype

Follicular lymphoma usually shows effacement of nodal architecture by follicular nodules that occupy both cortex and the medulla with two cell types in varying proportions: (1) Centrocytes which are small cells with irregular or cleaved nucleus and scant cytoplasm (2) Centroblasts which are larger cells with open nuclear chromatin, several prominent nucleoli, and modest amounts of cytoplasm. Based on the number of these centroblasts FL is classified into Grade 1, 2, and 3a,b. Grade 1 follicular lymphomas have 0–5 centroblasts per HPF; 6–15 centroblasts per HPF as grade 2, and greater than 15 per HPF as grade 3 follicular lymphomas. Low-grade (grades 1 and 2) follicular lymphomas are composed of a relatively homogeneous population of small cleaved lymphocytes. Grade 3 follicular lymphomas, which by definition have an increased number of large noncleaved cells and have been provisionally subcategorized into 3a and 3b, with the former having a mix of cleaved and large noncleaved cells, and the latter having sheets or large clusters of large noncleaved cells Peripheral blood involvement is seen in about 10% of cases. Bone marrow involvement occurs in 85% of cases and characteristically takes the form of paratrabecular lymphoid aggregates. The neoplastic cells express CD19, CD20, CD10, surface Ig, and BCL6 (B-cell lymphoma 6). BCL2 is expressed in more than 90% of cases. CD5 and cyclin D1 are negative. Ki67 is low [25] (**Figure 3**).

6.3.2 Highlights in recent WHO classification

- Mutational landscape better understood but the clinical impact remains to be determined [24, 47–51].

In situ follicular neoplasia.

- New name for in situ follicular lymphoma reflects low risk of progression to lymphoma.

6.3.3 Pediatric-type FL

- A localized clonal proliferation with excellent prognosis; a conservative therapeutic approach may be sufficient.

- Occurs in children and young adults, rarely in older individuals.

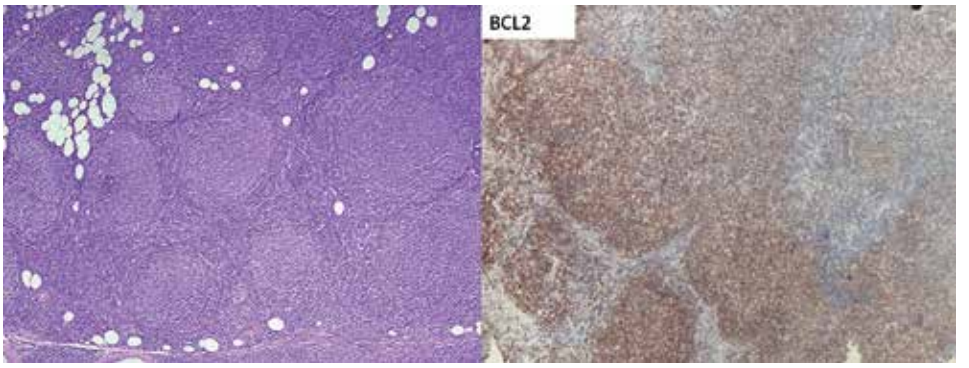

Figure 3.
Follicular lymphoma showing nodular pattern with neoplastic follicles occupying cortex and medulla and showing BCL2 positivity.

6.3.4 Duodenal-type FL

- Localized process with low risk for dissemination. Predominantly diffuse FL with 1p36 deletion.

- Accounts for some cases of diffuse FL lacks BCL2 rearrangement; presents as a localized mass, often inguinal.

6.3.5 Treatment and prognosis

Histological grade correlates with prognosis with grade 1–2 follicular lymphoma cases being indolent and usually not curable by aggressive therapy; and needs to palliate patients with low-dose chemotherapy or immunotherapy (e.g., anti-CD20 antibody). Median survival is 7–9 years. Histologic transformation occurs in 30–50% of follicular lymphomas, most commonly to diffuse large B-cell lymphoma [1, 25].

6.4 Mantle cell lymphoma

Mantle cell lymphoma (MCL) is an uncommon lymphoid neoplasm. It usually presents in the fifth to sixth decades of life and shows a male predominance. Virtually all mantle cell lymphomas have an (11; 14) translocation involving the IgH locus on chromosome 14 and the cyclin D1 locus on chromosome 11 that leads to overexpression of cyclin D1. The most common presentation is painless lymphadenopathy. Symptoms related to the involvement of the spleen (present in ~50% of cases) and extranodal sites are also common. In GIT present as lymphomatous polyposis of the lower gastrointestinal tract [1, 25, 52].

6.4.1 Morphology and immunophenotype

MCL consists of a homogeneous population of small lymphocytes with irregular to occasionally deeply clefted (cleaved) nuclear contours. In most cases the nuclear chromatin is condensed, nucleoli are inconspicuous, and the cytoplasm is scant. It usually has a diffuse growth pattern or surrounds reactive germinal centers in a mantle zone pattern. Extension of the lymphoma into the capsule and perinodal fat is common Occasionally, tumors composed of intermediate-sized cells with more open chromatin and a brisk mitotic rate are observed; immunophenotyping is necessary to distinguish these "blastoid" variants.

Mantle cell lymphomas express high levels of cyclin D1. Most tumors are also express CD19, CD20. It is usually CD5+, SOX11 +, FMC7+ (Flinders Medical Centre), BCL1+ and CD23−, CD10−, CD200−, LEF1−, which help to distinguish it from CLL/SLL. The IgH genes lack somatic hypermutation, supporting an origin from a naive B cell [25] (**Figure 4**).

6.4.2 Highlights in recent WHO classification

- Two MCL subtypes recognized with different clinicopathological manifestations and molecular pathogenetic pathways [24, 52–54]

    1. unmutated/minimally mutated immunoglobulin heavy chain gene (IGHV) and mostly SOX11 positive

    2. mutated IGHV and mostly SOX11 negative (indolent leukemic non-nodal MCL with PB), bone marrow (BM), (splenic involvement, may become

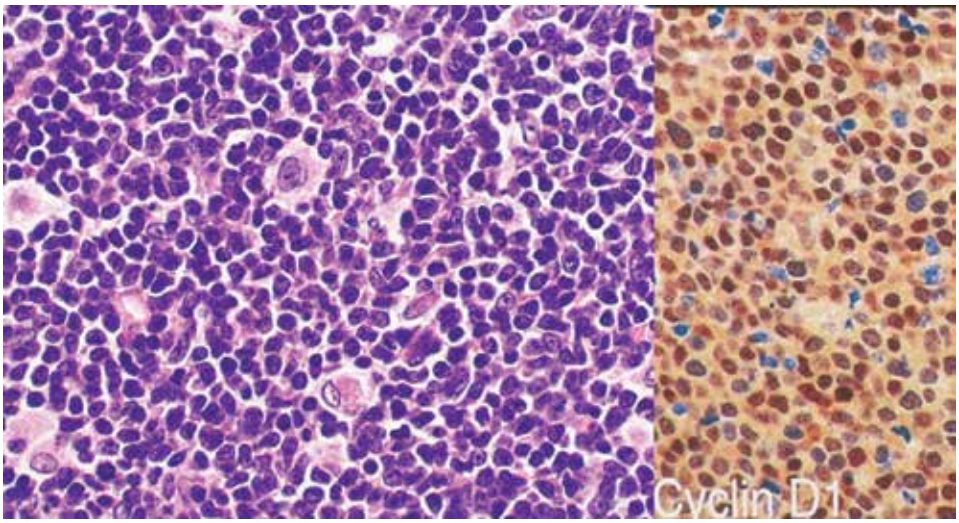

Figure 4.
Mantle cell lymphoma showing cyclin D1 positivity.

more aggressive).Mutations of potential clinical importance, such as TP53, NOTCH 1/2, recognized in a small proportion of cases.

- CCND2 (Cyclin D2) rearrangements in approximately half of cyclin D1 negative MCL.

  In situ mantle cell neoplasia—New name for in situ MCL, reflecting low clinical risk.

6.4.3 Treatment and prognosis

MCL is usually not curable with conventional chemotherapy, and most patients succumb to organ dysfunction caused by tumor infiltration. The prognosis is poor with median survival of only 3–4 years. The blastoid variant is associated with even shorter survivals. Hematopoietic stem cell transplantation and proteasome inhibitors are newer therapeutic approaches that show some promise [1, 25].

6.5 Marginal zone lymphomas

Marginal zone lymphoma (MZL) is a group of B-cell tumors that arise from lymph nodes, spleen, or extranodal tissues. In most cases, there is evidence of somatic hypermutation of memory B-cell origin. The disease begins as a polyclonal immune reaction. With time, however, tumors may acquire additional mutations that render their growth and survival antigen-independent, such as the (11; 18), (14; 18), or (1; 14) chromosomal translocations, which are relatively specific for extranodal marginal zone lymphomas [1, 25].

6.5.1 Nodal marginal zone B-cell lymphoma

Nodal marginal zone B-cell lymphomas are uncommon lymphomas in which the tumor cells resemble the cytology of those in splenic and extranodal marginal zone B-cell lymphomas of MALT, but there is no evidence for splenic or extranodal disease. Clinically, these lymphomas appear more extensive at presentation than

MALT lymphomas. They have a tendency to early relapse, and a small minority transform to large cell lymphoma.

6.5.2 Splenic marginal zone B-cell lymphoma

Splenic marginal zone B-cell lymphoma (SMZL) is a small B-cell lymphoma of the white pulp of the spleen that often involves the splenic hilar lymph nodes, bone marrow, and peripheral blood. Patients with splenic marginal zone B-cell lymphoma characteristically present with splenomegaly and many have B-symptoms (fever, weight loss, and night sweats). Peripheral blood shows circulating neoplastic lymphocytes that have a villous appearance. These cases were previously termed 'splenic lymphoma with villous lymphocytes'.

6.5.3 Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue

Extranodal marginal zone B-cell lymphomas arises in normal sites for mucosal immunity (MALT), such as intestinal Peyer patches, or in sites of inflammation triggered by autoimmune disorders, such as Hashimoto thyroiditis or Sjögren syndrome, or by infection (*Helicobacter pylori*-associated chronic gastritis. They may regress if the inciting agent (e.g., *Helicobacter pylori*) is eradicated suggesting that extranodal marginal zone lymphomas arise in chronically inflamed tissues that lie on a continuum between reactive lymphoid hyperplasia and full-blown lymphoma. Transformation to large cell lymphoma may occur. The t(11; 18) chromosomal abnormality is more specific and involves fusion of the API2 gene (an apoptosis inhibitor) on chromosome 11q21 and the MLT1 gene (a caspase-like protease) on chromosome 18q21. It is found in 40% of patients with MALT lymphomas. Two, less common, translocations are t(1; 14) (p22; q32) and t(14; 18) (q32; 21). These three translocations are not found in marginal zone B-cell lymphomas of spleen and lymph node.

6.5.4 Morphology and immunophenotype

These lymphomas are composed of small- to medium-sized lymphocytes that exhibit variable cytological features. In some cases, lymphocytes with irregular nuclear contours resembling follicular small cleaved cells or centrocytes may predominate. Other cases may be composed primarily of cells with abundant pale cytoplasm resembling monocytoid B cells. Cases with an abundance of small lymphocytes or plasma cells also may be seen. Regardless of the neoplastic cells' appearance, they produce a diffuse infiltrate that invades epithelial structures, producing lymphoepithelial lesions (**Figure 5**).

The lymphoid component expresses B-cell markers such as CD20, CD19 and CD43 and monotypic surface Ig (usually IgM without IgD). Negative for CD5, CD10, CYCLIN D1, and CD23.

6.5.5 Treatment and prognosis

Management of gastric MALToma with antibiotic therapy for *H. pylori* resulted in regression of lymphoma. Cases with the t(11;18)(q21;q21) appear to be resistant to *H. pylori* eradication therapy, Radiation therapy or single-agent chemotherapy is also effective in low-grade MALToma. MALT lymphomas have an indolent natural course and are slow to disseminate. Recurrences that can occur after many years. Median survival has been variable. SMZL tends to respond favorably to splenectomy alone in contrast to its poor response to chemotherapy [1, 25].

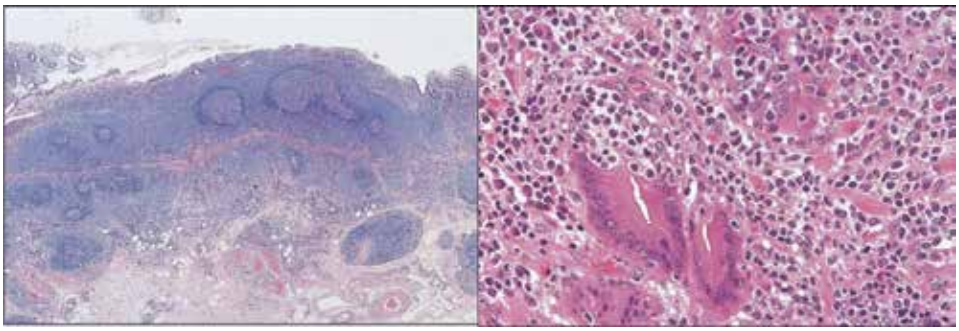

Figure 5.
Extranodal marginal zone lymphoma of stomach showing lymphoepithelial lesions.

6.6 Lymphoplasmacytic lymphoma

Lymphoplasmacytic lymphoma is a B-cell neoplasm of older adults that usually presents in the sixth or seventh decade of life and is characterized by tumor cells that undergo terminal differentiation to plasma cells. Most commonly, the plasma cell component secretes monoclonal IgM, often in amounts sufficient to cause a hyperviscosity syndrome known as Waldenström macroglobulinemia. Unlike multiple myeloma, complications stemming from the secretion of free light chains (e.g., renal failure and amyloidosis) are relatively rare and bone destruction does not occur. About 90% of Lymphoplasmacytic lymphoma are associated with acquired mutations in Myeloid differentiation factor 88 (MYD88) [55, 56]. The dominant presenting complaints are nonspecific and include weakness, fatigue, and weight loss. Approximately half the patients have lymphadenopathy, hepatomegaly, and splenomegaly. Anemia caused by marrow infiltration is common. About 10% of patients have autoimmune hemolysis caused by cold agglutinins. Cryoglobulinemia resulting from the precipitation of macroglobulins at low temperatures, which produces symptoms such as Raynaud phenomenon and cold urticaria [1, 25].

6.6.1 Morphology and immunophenotype

The neoplasm is composed of infiltration of lymphocytes, plasma cells, and plasmacytoid lymphocytes in varying proportions, often accompanied by mast cell hyperplasia. The lymphoid cells expresses CD20 and surface Ig, the plasma cells secretes the same Ig that is expressed on the surface of the lymphoid cells. This is usually IgM but can also be IgG or IgA (**Figure 6**).

6.6.2 Highlights in recent WHO classification

MYD88 L265P mutation in the vast majority of cases impacting diagnostic criteria even though finding is not specific for LPL [24, 55, 56].

IgM monoclonal gammopathy of undetermined significance (MGUS) is more closely related to LPL and other B-cell lymphomas than to myeloma.

6.6.3 Treatment and prognosis

LPL is an incurable progressive disease, however the tumor growth can be controlled with low doses of chemotherapy and immunotherapy with anti-CD20 antibody. Symptoms caused by the high IgM levels like hyperviscosity and hemolysis can be alleviated by plasmapheresis. The clinical course is typically indolent,

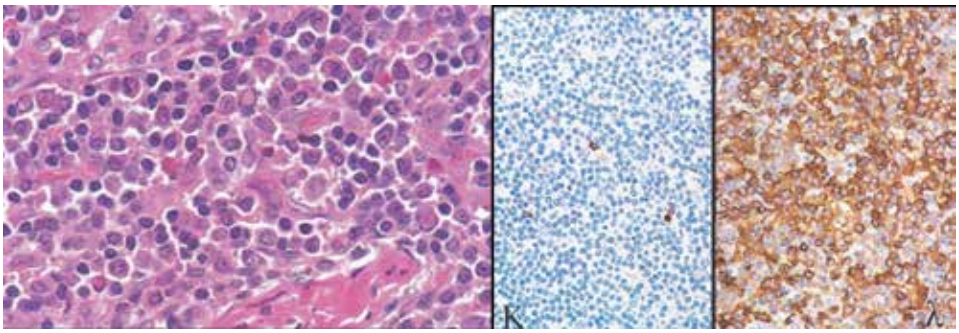

Figure 6.
Lymphoplasmacytic lymphoma showing lambda restriction.

with median survivals of 5–10 years. Transformation to large-cell lymphoma is uncommon [1, 25].

6.7 Burkitt lymphoma

Burkitt Lymphoma is a high-grade B-cell lymphoma composed of medium-sized, rapidly dividing lymphocytes. Less commonly, they have a leukemic phase. It is classified into (1) African (endemic) Burkitt lymphoma, (2) sporadic (nonendemic) Burkitt lymphoma, and (3) a subset of aggressive lymphomas occurring in individuals infected with HIV. Translocations of the MYC gene on chromosome 8 lead to increased MYC protein levels. The translocation partner for MYC is usually the IgH locus [t (8; 14)] but may also be the Ig κ [t (2; 8)] or λ [t (8; 22)] light chain loci. The translocated MYC allele often harbors additional point mutations. Endemic Burkitt lymphomas are seen to be latently infected with Epstein bar virus (EBV) (100%) and 20 to 40% in sporadic and immunodeficiency-associated Burkitt lymphomas. Both endemic and sporadic Burkitt lymphomas are found mainly in children or young adults. Most tumors involve extranodal sites. Endemic Burkitt lymphoma usually presents as mandibular mass and unusually involves abdominal viscera. In contrast, sporadic Burkitt lymphoma often involves the ileocecum and peritoneum [1, 25, 57–59].

6.7.1 Morphology and immunophenotype

Burkitt Lymphoma shows diffuse infiltrate of intermediate-sized lymphoid cells with round or oval nuclei, coarse chromatin, several nucleoli, and a moderate amount of cytoplasm with high mitotic index and numerous apoptotic, cells the nuclear remnants of which are phagocytosed by interspersed benign macrophages. These phagocytes have abundant clear cytoplasm, creating a characteristic "starry sky" pattern. These are tumors of mature B cells that express surface IgM, CD19, CD20, CD10, and BCL6, and negative for BCL2 and Tdt. Ki 67 approaches nearly 100% [1, 25] (**Figure** 7).

6.7.2 Highlights in recent WHO classification

- TCF3 (Transcription Factor 3) or ID3 (Inhibitor of DNA Binding 3), mutations in up to; 70% of cases [24, 57–61].

6.7.3 Burkitt-like lymphoma with 11q aberration

- A new provisional entity that closely resembles Burkitt lymphoma but lacks MYC rearrangement and has some other distinctive features.

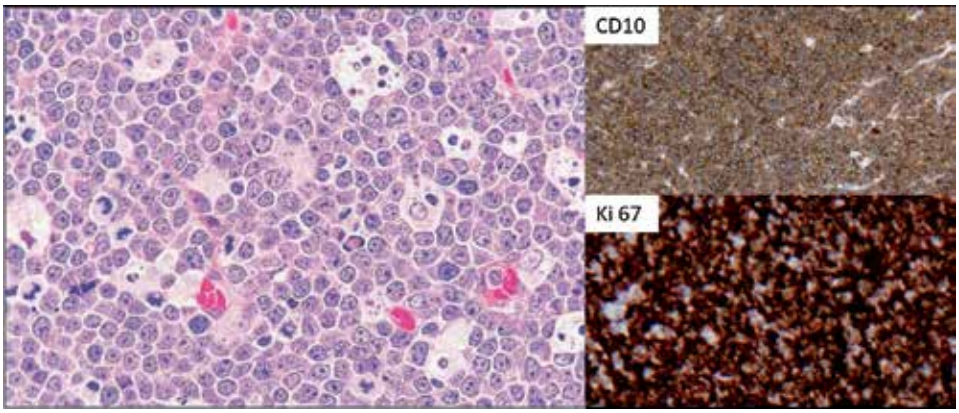

Figure 7.
Burkitt lymphoma showing starry sky pattern with CD10 positivity and high Ki67.

6.7.4 Treatment and prognosis

Burkitt lymphoma is very aggressive but responds well to intensive chemotherapy. Most children and young adults can be cured. The outcome is more guarded in older adults.

6.8 Diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma (DLBCL) is the most common form of NHL. There is a slight male predominance. The median patient age is about 60 years, but can also occur in young adults and children.

DLBCL is molecularly heterogeneous. One frequent pathogenic event is dysregulation of BCL6. About 30% of DLBCLs contain various translocations that have in common a breakpoint in BCL6 at chromosome 3q27. Mutations are also seen in multiple other oncogenes including MYC and overexpression of the antiapoptotic protein BCL2. Tumors with BCL2 rearrangements usually lack BCL6 rearrangements. Co-expression of MYC with BCL2 and or BCL6 is now considered as double hit or triple hit lymphomas [1, 25, 63]. DLBCL typically presents as a rapidly enlarging mass at a nodal or extranodal site. Extranodal sites include the gastrointestinal tract, skin, bone, brain, and other tissues. Bone marrow involvement is relatively uncommon and usually occurs late in the course. Rarely, a leukemic picture emerges.

6.8.1 Morphology and immunophenotype

DLBCL usually shows a diffuse pattern of growth by a monotonous population of large cells that have a round or oval nucleus that appears vesicular due to margination of chromatin to the nuclear membrane, but large multilobated or cleaved nuclei are prominent in some cases. The cytoplasm is usually moderately abundant and may be pale or basophilic (**Figure 8**). More anaplastic tumors may even contain multinucleated cells with large inclusion-like nucleoli.

DLBCL express CD19 and CD20 and show variable expression of germinal center B-cell markers such as CD10 and BCL6 and for markers IRF4 (interferon regulatory factor4) and MUM1 (melanoma associated antigen (mutated) 1) which classifies DLBCL into germinal center type and non-germinal center type. Cases with CD10 expression by >30% of cells are regarded as GC type as well as cases that are CD10−, BCL6+, IRF4/MUM1−. All other cases are regarded as of non-GC type [25, 62].

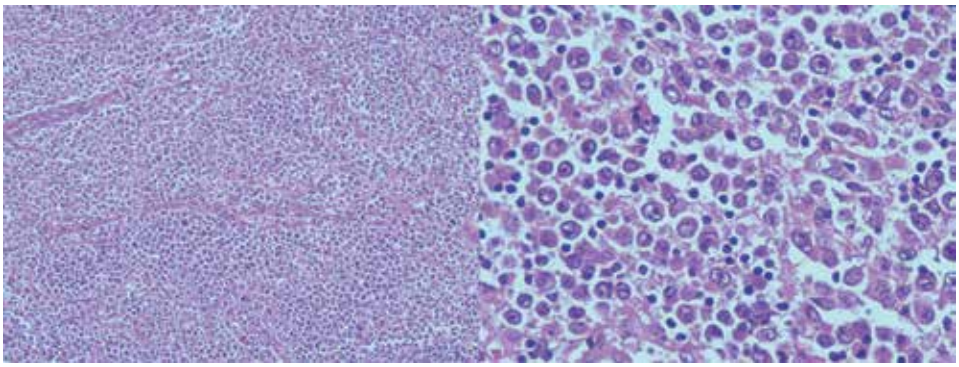

Figure 8
Diffused large cell lymphoma showing diffuse effacement by large cells.

6.8.2 Highlights in recent WHO classification

- The distinction of germinal center B-cell-like (GCB) vs. activated B-cell-like (ABC)/non-GC type required with the use of immunohistochemical algorithm may affect therapy [24, 62–65].
- Coexpression of MYC and BCL2 considered new prognostic marker (double-expressor lymphoma).
- Mutational landscape better understood but the clinical impact remains to be determined. EBV+ DLBCL, NOS.
- This term replaces EBV+ DLBCL of the elderly because it may occur in younger patients.
- Does not include EBV+ B-cell lymphomas that can be given a more specific diagnosis.

6.8.3 Treatment and prognosis

DLBCLs are aggressive tumors that are rapidly fatal without treatment. With intensive combination chemotherapy, 60–80% of patients achieve a complete remission, and 40–50% are cured. Adjuvant therapy with antiCD20 antibody improves both the initial response and the overall outcome. DLBCLs with MYC translocations have a worse prognosis than those without and may be better treated with chemotherapy regimens for Burkitt lymphoma [1, 25].

6.9 Hairy cell leukemia (HCL)

Hairy cell leukemia (HCL) is a rare but distinctive B-cell neoplasm constitutes about 2% of all leukemia. It is predominantly a disease of middle-aged white males, with a median age of 55 and a male-to-female ratio of 5:1. Hairy cell leukemia are associated in more than 90% of cases with activating point mutations in the serine/threonine kinase BRAF (B-Raf) [66]. Patients usually presents with massive splenomegaly. Hepatomegaly is less common and lymphadenopathy is rare. Pancytopenia is seen in more than half the cases [1, 25].

6.9.1 Morphology and immunophenotype

On routine peripheral blood smears, the cells have round, oblong, or reniform nuclei and moderate amounts of pale blue cytoplasm with thread-like or bleb-like extensions. On bone marrow aspiration the cells cannot be aspirated and gives "dry tap", Bone marrow biopsy shows diffuse interstitial infiltrate of tumor cells enmeshed in an extracellular matrix composed of reticulin fibrils giving fried egg appearance. Spleen appears beefy red on gross appearance. Microscopy shows heavily infiltrated splenic red pulp leading to obliteration of white pulp.

The classic immunophenotypic profile of HCL consists of bright monotypic surface immunoglobulin, bright coexpression of CD20, CD22 and CD11c, and expression of CD103, CD25, CD123, T-bet, Annexin A1 (ANXA1), DBA.44 (CD72), FMC-7 and cyclin D1 (usually weak) [1, 25].

6.9.2 Treatment and prognosis

Hairy cell leukemia follows an indolent course and is sensitive to "gentle" chemotherapeutic regimens, which produce longlasting remissions. BRAF inhibitors appear to produce excellent responses in tumors that have failed conventional chemotherapy. The overall prognosis is excellent [1, 25].

6.10 Plasma cell neoplasms and related disorders

These B-cell proliferations contain neoplastic plasma cells that virtually always secrete a monoclonal Ig or Ig fragment. These plasma cell neoplasms are also termed as plasma cell dyscrasias. Multiple myeloma is associated with frequent rearrangements involving the IgH locus and various proto-oncogenes. Included among the loci that are recurrently involved in translocations with the Ig heavy-chain gene on chromosome 14q32 are the cell cycle-regulatory genes cyclin D1 on chromosome 11q13 and cyclin D3 on chromosome 6p21. Deletions of chromosome 17p that involve the TP53 tumor suppressor locus also occur and are associated with a poor outcome. Late-stage, highly aggressive forms of the disease such as plasma cell leukemia are associated with the acquisition of rearrangements involving MYC. More recent deep sequencing of myeloma genomes has identified frequent mutations involving components of the nuclear factor κ B (NF-κB) pathway, which supports B-cell survival. Based on these studies, it is evident that myeloma is molecularly heterogeneous [1, 67].

The various spectrum of plasma cell dyscrasias are [68]:

Non-IgM monoclonal gammopathy of undetermined significance

Serum monoclonal protein (non-IgM type) <30 g/dl.
Clonal bone marrow plasma cells <10%.

The absence of end-organ damage such as hypercalcemia, renal insufficiency, anemia, and bone lesions (CRAB) or amyloidosis that can be attributed to the plasma cell proliferative disorder.

IgM monoclonal gammopathy of undetermined significance

Serum IgM monoclonal protein <30 g/dl.
Bone marrow lymphoplasmacytic infiltration <10%.

No evidence of anemia, constitutional symptoms, hyperviscosity, lymphadenopathy, hepatosplenomegaly, or other end-organ damage that can be attributed to the underlying lymphoproliferative disorder.

Light-chain monoclonal gammopathy of undetermined significance

Abnormal free light chain (FLC) ratio (<0.26 or >1.65).

Increased level of the appropriate involved light chain (increased κ FLC in patients with a ratio >1.65 and increased λ FLC in patients with a ratio).

No immunoglobulin heavy chain expression on immunofixation.

The absence of end-organ damage such as hypercalcemia, renal insufficiency, anemia, and bone lesions (CRAB) or amyloidosis that can be attributed to the plasma cell proliferative disorder Clonal bone marrow plasma cells<10%.

Urinary monoclonal protein <500 mg/24 h.

Solitary plasmacytoma

Biopsy-proven solitary lesion of bone or soft tissue with evidence of clonal plasma cells.

Normal bone marrow with no evidence of clonal plasma cells.

Normal skeletal survey and *magnetic resonance imaging* (MRI) or Computed tomography (CT) of spine and pelvis (except for the primary solitary lesion) Absence of end-organ damage such as hypercalcaemia, renal insufficiency, anemia, or bone lesions (CRAB) that can be attributed to a lymphoplasma cell proliferative disorder.

Solitary plasmacytoma with minimal marrow involvement

Biopsy-proven solitary lesion of bone or soft tissue with evidence of clonal plasma cells.

Clonal bone marrow plasma cells <10%.

Normal skeletal survey and MRI (or CT) of spine and pelvis (except for the primary solitary lesion) Absence of end-organ damage such as hypercalcemia, renal insufficiency, anemia, or bone lesions that can be attributed to a lymphoplasma cell proliferative disorder.

Heavy-chain disease is a rare monoclonal gammopathy characterized by synthesis and secretion of free heavy-chain fragments and is seen associated with diseases including lymphoplasmacytic lymphoma and an unusual small bowel marginal zone lymphoma that occurs in malnourished populations (so-called Mediterranean lymphoma).

Smoldering multiple myeloma—both criteria must be met.

- Serum monoclonal protein (IgG or IgA) ≥30 g/L or urinary monoclonal protein ≥500 mg per 24 h and/or clonal bone marrow plasma cells 10–60%.

- Absence of myeloma defining events or amyloidosis.

Multiple myeloma is a plasma cell neoplasm commonly associated increased plasma cells in the marrow with accompanying features like lytic bone lesions, hypercalcemia, renal failure, and acquired immune abnormalities. It is commonly seen in older adults, with a peak age of incidence of 65–70 years.

Revised International Myeloma Working Group diagnostic criteria for multiple myeloma and smoldering multiple myeloma [68].

Definition of multiple myeloma

- Clonal bone marrow plasma cells ≥10% or biopsy-proven bony or extramedullary plasmacytoma* and any one or more of the following myeloma defining events:

Myeloma defining events:

- Evidence of end-organ damage that can be attributed to the underlying plasma cell proliferative disorder, specifically:
- Hypercalcaemia: serum calcium >0.25 mmol/L (>1 mg/dL) higher than the upper limit of normal or >2.75 mmol/L (>11 mg/dL)
- Renal insufficiency: creatinine clearance 177 μmol/L (>2 mg/dL)
- Anemia: hemoglobin value of >20 g/L below the lower limit of normal, or a hemoglobin value <100 g/l
- Bone lesions: one or more osteolytic lesions on skeletal radiography, CT, or positron emission tomography-computed tomography (PET-CT)

Anyone or more of the following biomarkers of malignancy

- Clonal bone marrow plasma cell percentage* ≥60%
- Involved: uninvolved serum free light chain ratio§ ≥100
- 1 focal lesion on MRI studies

6.10.1 Morphology and immunophenotype.

Multiple myeloma is characterized by infiltration of marrow by plasma cells in the interstitium, nodules or in diffuse sheets that completely replace normal elements (**Figure 9**). Plasma cells can be relatively normal-appearing plasma cells, plasmablasts or binucleated and multinucleated cells. Other cytologic

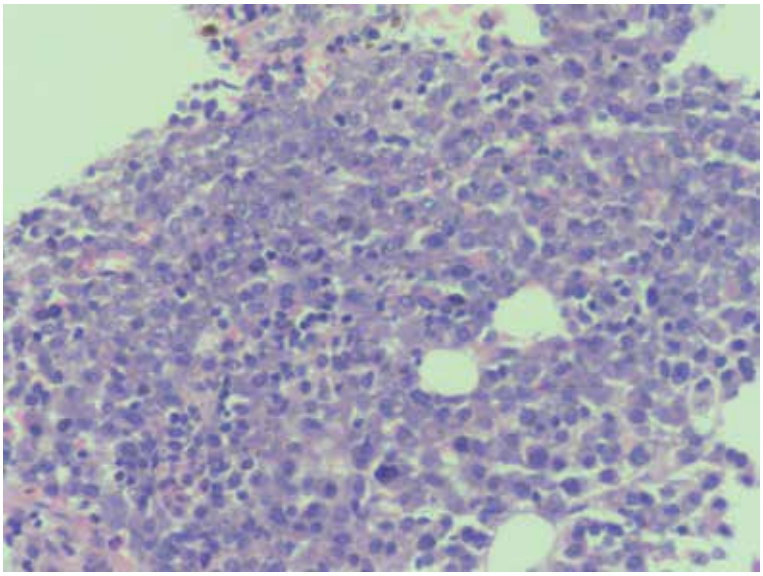

Figure 9.
Bone marrow biopsy showing sheets of plasma cells replacing normal hematopoietic elements.

variants include flame cells with fiery red cytoplasm, Mott cells with multiple grapelike cytoplasmic droplets, and cells containing a variety of other inclusions—Russell bodies (cytoplasmic) or Dutcher bodies (nuclear), including fibrils, crystalline rods, and globules. Peripheral blood smears shows rouleaux formation. Rarely, tumor cells enters the peripheral blood, giving rise to plasma cell leukemia. Plasma cell tumors are positive for CD 38, CD138, and often express CD56, a feature that can be helpful in identifying small populations of neoplastic cells.

6.10.2 Treatment and prognosis

The prognosis is variable but has improved in recent years with new therapeutic approaches. The median survival is 4–7 years. Translocations involving cyclin D1 are associated with a good outcome, whereas deletions of 13q, deletions of 17p, and the t(4;14) are associated with more aggressive course. Hematopoietic stem cell transplantation prolongs life but has not yet proven to be curative. Solitary osseous plasmacytoma almost inevitably progresses to multiple myeloma but can take 10–20 years or longer. In contrast, extraosseous plasmacytomas, particularly those involving the upper respiratory tract, are frequently cured by local resection. About 75% of patients of smoldering myeloma progress to multiple myeloma over a 15-year period. Approximately 1% of patients with MGUS develop asymptomatic plasma cell neoplasm, usually multiple myeloma, per year, a rate of conversion that remains roughly constant over time [1, 25, 68].

7. Staging, prognosis, and treatment

The Ann Arbor staging classification (**Table 2**) which was developed for HD in 1971, has been the standard scheme for NHL [69, 70]; however, it does not account for tumor burden and does not correlate well with prognosis. The Prognosis and

Stage I	Involvement in a single lymph node region or single extralymphatic site
Stage II	Involvement of two or more lymph node regions on the same side of the diaphragm; localized contiguous involvement of only one extralymphatic site and lymph node region (stage IIE)
Stage III	Involvement of lymph node regions on both sides of the diaphragm; may include spleen
Stage IV	Disseminated involvement of one or more extralymphatic organs with or without lymph node involvement

Table 2.
The Ann Arbor staging classification.

International Prognostic Index	Adverse factors	Risk group	No. of factors
	Performance status = 2	Low	0, 1
	Lactate dehydrogenase > normal	Low-intermediate	2
	Extranodal sites = 2	High-intermediate	3
	Stage III and IV disease	High	4, 5
	Age >60 years		

Table 3.
International Prognostic Index.

treatment depends not only on the stage but also on a variety of clinical parameters that reflect tumor bulk and kinetics including the size of the mass, lactate dehydrogenase (LDH) level, number of extranodal sites, etc.

The international prognostic index (IPI) (**Table 3**) was developed to correlate clinical parameters with prognosis and appears to be more useful than the Ann Arbor staging system in predicting survival. Studies have indicated that the IPI correlates with prognosis for all histologies [71–73]. Additional biologic and genetic parameters, particularly genomic profiling, further subdivide prognostic groups in NHL.

Therapy follows the assessment of the patient, the pathology, and the stage of the disease. The ability of the patient to tolerate therapy is dependent on age, performance status, and, if present, immunodeficiency due to a prelymphomatous condition. Various treatment modalities include observation, radiotherapy for localized disease, chemotherapy, immunotherapy, and stem cell transplantations.

8. Conclusion

B-cells though plays a major role in humoral immunity can also lead to various pathologic diseases. B-cell lymphomas are malignant neoplasms that arise from various stages of differentiation of B-cells which are classified based on the cell of origin and behavior. Historically, there has been much controversy in the classification of lymphoid neoplasms, but consensus has been reached through the use of objective molecular diagnostic tools. The current WHO classification uses morphologic, immunophenotypic, genotypic, and clinical features to classify the lymphoid neoplasms into five broad categories as precursor B-cell neoplasms, peripheral B-cell neoplasms, precursor T-cell neoplasms, peripheral T-cell neoplasms and Hodgkin lymphoma. B-cell lymphomas include precursor B-cell neoplasms and peripheral B-cell neoplasms. Hodgkin lymphoma though originates from B-cell has distinctive pathologic features and is treated as a separate entity. B-cell lymphomas range in their clinical behavior from low grade to high grade and also have histological or clinical progression during a patient's clinical course. For these reasons, the WHO classification does not attempt to stratify lymphoid malignancies in terms of grade. Also both morphology and immunophenotype often change over time with the acquisition of additional genetic changes. Hence B-Cell lymphomas are expanding with emerging new entities due to its wide molecular/genetic landscape and are being revised frequently for better understanding and diagnosis which helps in managing with targeted therapies.

Conflict of interest

Authors declare no conflict of interest.

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Chapter 4

B Cell Lymphomagenesis

Željka Škunca

Abstract

Lymphoid neoplasms are a heterogeneous group of malignancies whose diagnosis depends on a very good analysis of hematopathology and morphology, immunophenotype, cytogenetic, molecular, and clinical characteristics. B-cell lymphomas begin from different developmental stages of B cells in germinal centers of secondary lymphoid tissue. The evolution of B-cell lymphomagenesis depends on different numbers of signal pathways. Proteins that play key point of signaling networks are changed by aberrant chromosomal expression, translocation, and/or accumulation, and those events determine the fate of the affected B cells. Many chemokines and cytokines have been implicated in providing the line for the cellular surviving and interaction in lymphoid organogenesis. Specific chromosomal alterations were associated with significant changes in gene-expression signatures that reflect various aspects of lymphoma cell biology as well as the host response to the lymphoma. The goal of this study was to find out a correlation between tumor markers and survival in patients with subgroups of DLBCL. The goal is to find out chronic autoimmune or pathogen-induced immune reactions resulting in lymphoid neogenesis. So we address (i) chemokines and adhesion molecules involved in lymphoid neogenesis, (ii) the autoimmune diseases and pathogens which are associated with the development of B-cell lymphomas, and (iii) the molecular mechanisms involved in the initiation and progression of DLBCL.

Keywords: DLBCL, lymphomagenesis, GCB, ABC

1. Germinal center B-cell dynamics

Characteristic chromosome translocation is associated with certain entities of the disease that is known to play a key role in the development of lymphoma. Chromosomal translocation alone, however, is not sufficient for the appearance of a tumor. Various factors, including microenvironment, epigenetic, and genetic changes, play a role in the development of lymphomas. Follicular dendritic cells (FDC) produce high-affinity antibody needed for the development of B-cell memory. These cells can be detected in B-cell non-Hodgkin lymphomas (NHL). Gene-expression profiling revealed that the FDC network could affect the clinical outcome in DLBCL [1–3].

Transgenic mouse model with different genes linked to chromosomal translocations has shown that chromosomal translocation alone is not sufficient for the development of lymphoma [1]. Transgenic mice with the c-*MYC* (myelomatosis viral oncogene homolog) genome develop lymphoma after a long time. The *c-MYC* gene is located at 8q24 and is associated with the translocation t (8; 14) (q24; q32). This was the first recurring chromosome abnormality ever reported in lymphoproliferative disorders. *C-MYC* in place next to immunoglobulin heavy locus (IGH) results in the expression of c-Myc protein, which is a key transcriptional factor that promotes cell cycle and

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tumor proliferation. 8q24/*c-MYC* restenosis is found in 7–15% of the activated B-cell-like (ABC) subgroup of diffuse large B-cell lymphoma (DLBCL), and the reports are predominantly associated with extranodal localization [4–8]. B-cell lymphomas include follicular lymphoma (FL), mantle cell lymphoma (MCL), and marginal zone lymphoma (MZL). Tumor cells show a strict topographical and functional relationship with FDCs, included with reactive lymphoid and stromal cells [3, 9]. The FDC patterns, described for FL and MCL, are reminiscent of the distribution pattern of FCD meshwork seen in the GC or the mantle zone of the secondary lymphoid follicle, respectively [3, 9]. T cells migrate to B-cell-enriched follicles and germinal centers within secondary lymphoid organs to provide help to B cells. Correlation T and B cells within germinal centers are essential for B-cell differentiation into germinal center B cells and selection of mutated cells into memory B cells or memory plasma cells. Different stages of maturation lead to B cell becoming a helper T cells and B follicular helper T (T_{FH}) cells [10].

C-MYC translocation is characteristic of Burkitt's lymphoma, where it is found in 80–90% of cases. In B-cell lymphoma 2 (*BCL2*) transgenic mice, it has been shown that polyclonal B-cell proliferation causes the growth of lymphoid follicles, but only 20% of mice develop monoclonal diffuse lymphomas after long latency. Long-term exposure to oncogene, progression from polyclonal to monoclonal disease, and appearance of the *BCL2* transgenic mouse model suggests that lymphoma development depends on the secondary genetic events [8].

C-MYC is a strong transcription factor and promotes cell proliferation and growth, DNA replication, and protein biosynthesis [11]. Dysregulation of c-MYC is essential in the pathogenesis of a number of B-cell lymphomas but is rarely reported in T-cell lymphomas. *C-MYC* rearrangement is found in multiple B-cell lymphomas including Burkitt lymphoma, diffuse large B-cell lymphoma (DLBCL), and B-cell lymphoma unclassifiable, with features intermedia between DLBCL and Burkitt lymphoma (BCLU). In normal cells *c-MYC* is a highly regulated transcription factor that is involved in cell cycle regulation, cell metabolism, mitochondrial biogenesis, nucleic acid synthesis, and apoptosis [12]. *C-MYC* expression is detected in a much higher proportion of DLBCL (30–50%) and is associated with concomitant expression of *BCL2* in 20 to 35% of cases [8]. Most of these tumors do not carry *c-MYC/BCL2* chromosomal alterations and have been named "double-hit lymphoma." Most studies use a cutoff of 40% *c-MYC*-expressing cells to define these cases; the cutoff for *BCL2* expression has varied considerably in the literature, but a figure of 50% is recommended [8, 13–16].

Germinal centers (GCs) form in the center of the B-cell follicles of secondary lymphoid organs, interspersed within a network of stromal cells known as follicular dendritic cells (FDCs) [17, 18]. In follicles that do not contain GCs (primary follicles), FDCs play an important role, helping B cells to cluster into compact, well-defined follicle [18–20]. FDC has a long-term retention of antigen within complement immune complexes in a form that can support affinity-dependent "testing" of immunoglobulin somatic hypermutation (SHM)-modified B-cell Ag receptors (BCRs) that occurs during GC selection [21, 22]. Adaptive immunity involves the development of antibodies in GCs through SHM in GC dark zones (DZs) and clonal selection in light zones (LZs) [22, 23].

The spleen, lymph nodes, and mucosal-associated lymphoid tissues constitute secondary lymphoid tissues that are located strategically to efficiently trap foreign antigens entering peripheral tissues and mucosal sites. In secondary lymphoid tissues, activation of B cells and T cells by native and processed antigenic determinants represents the beginning of the GC reaction [24]. The B cells bind membrane-bound immunoglobulins (B-cell receptors (BCR)) and express Ag-derived peptides in major histocompatibility complex (MHC-II) molecules on their surface [6]. Secondary lymphoid tissues facilitate interactions between antigen-bearing dendritic cells (DCs), B cells, and T cells to initiate adaptive immune responses. B cells express

B-cell receptors (BCRs) on their cell membrane, and BCRs allow the cell to bind to a specific antigen and initiate an antibody response [18, 23, 25]. Despite these common features, differences in routes of antigen transport, lymphocyte trafficking, and unique cell populations determine the role of a specific secondary lymphoid tissue in immune responses to various foreign antigens. In the GCs, naive B cells can have clonal expansion, somatic hypermutation, and maturation [26, 27].

Germinal centers (GCs) are organized into two major zones: dark and light zones. The dark zone contains large centroblasts that are rapidly proliferating and undergoing somatic mutation. These cells grow to small nonproliferating centrocytes in the light zone, and binding antigen on follicular dendritic cells (FDCs) and development further depend on receiving signals from helper T cells [18, 22, 23, 28].

The activated B cells have two possibilities: (1) turnover into extrafollicular areas followed by proliferation and terminal differentiation into short-lived plasma cells that secrete antibody and (2) turnover into B-cell follicles followed by proliferation of GCs [27]. The mechanisms responsible for this remain poorly understood, although various studies suggest that the affinity of the BCR for the foreign antigen, the amount of antigen-receptor engagement, and the costimulatory signals received from T cells may all be involved. These observations suggest that the affinity maturation of the antibody response occurs in GCs, through the processes of clonal proliferation, somatic hypermutation, and selection [27].

T cells play a key role to induce B-cell proliferation and somatic hypermutation and to limit GC reactions. T cells in the germinal center activate helper T cells (CD4+CD57+CD25−) migrating in the germinal center to activate the B-cell zone-homing C-X-C chemokine receptor type 5 (CXCR5) [29, 30]. B cells primarily excrete interleukin 4 (IL-4). They begin in the paracortical T-cell area of IgD follicle expression, but T-cell maturation results in a characteristic follicular CD10+ immunophenotype that has been acquired. Although the germinal center formation is intact in the presence of the FDC network, FDC plays a key role in the formation of memory B cell, and interaction is dependent on the expression of the tumor necrosis factor (TNF) family. The germinal center is not only created from different stages of B-cell maturation but also through the division of immunophenotypically different and functionally specialized T cells, dendritic cells, and subpopulations of stromal cells and their interactions. The antigen-stimulated B cells with the germinal center are highly mobile within the zone as well as between the dark and light zone [18, 23, 31].

In general, naive B cells in the central lymphatic system mature through the transformation and proliferation of B cells toward plasma cells and B cells that produce antibodies. Malignant diseases may develop during such processes, and B-cellular neoplasms tend to imitate different stages of normal B-cell differentiation [1].

In the primary phase of differentiation, the progenitor of the B cell passes through renaming, and further development is followed by immunoglobulin genes (Ig) and occurs at naive B cells in peripheral lymphoid tissue or passes through apoptosis. Naive B cells can enter the primary follicle, forming a germinal center (GC), where mature CD10- and B-cell lymphoma 6 (*BCL6)* centroblasts expression mature [2]. Centroblasts continue maturing in centrocytes, which later co-express *BCL2 gene*. Naive B cells can be directly developed in plasma cells. The germinal center is partially monitored by Bcl6 protein, which in humans is encoded by the *BCL6* [32] and IRF4/MUM1 proteins, which is essential for further lymphocyte differentiation into plasma cells or memory B cell. On the other hand, antigen stimulation of lymphocyte can go toward apoptosis [2]. MUM1 is a lymphocyte-specific transcriptional factor of the interferon regulatory factor (IRF) family, included in the regulation of gene expression in response to interferon and other cytokines. MUM1 provides immunoglobulin gene expression in the final step of B-cell differentiation within germinal center light zones. At this stage Bcl-6 is downregulated [2].

Secondary follicles are important for B-cell differentiation and maturation. B-cell differentiation begins via gene resection for the DJ region of the IgH gene. Each immunoglobulin molecule consists of two identical heavy chains and two identical light chains. This region represents the organization of heavy-chain locus. The locus includes variable (V), diversity (D), joining (J), and constant (C) segments. Within B-cell development, DNA recombination level joins a single D segment with a J segment; the fused DJ segment of a DNA molecule containing information coding for a protein or peptide sequence of this partially rearranged DJ region is then joined to the V segment. The introduction of region V into DJ results in VDJ coupling, and then the conversion successfully generates IGH protein chain. After the successful generation of the IgH gene, Ig light chains (IgL) are formed. The removal of IgL starts with light chain kappa. When the IgH and IgL genes are productive, B cells are called naive B cells because they are not exposed to the appropriate antigens and reside in the marginal zone of the secondary follicle [4]. The cells that have not been exposed to antigens are known as naïve or virgin B cells and remain at the mantle zone until they are exposed to antigens [22]. According to the B-cell differentiation stage, B-cell precursor includes B lymphoblastic leukemia/lymphoma, while pre-GC neoplasms include cell lymphoma. GC B-cell tumors are follicular lymphoma, Burkitt's lymphoma, Hodgkin's lymphoma, and diffuse large B-cell lymphoma (DLBCL). In the last stage of B-cell differentiation, post-GC neoplasms, including marginal zone lymphoma, mucosa-associated lymphoma tissue (MALT), lymphoplasmic lymphoma, chronic lymphocytic leukemia (CLL), plasma cell myeloma, and some cases of DLBCL, are developed [1, 22] (**Figure 1**).

The majority of B-cell lymphomas appear from germinal center (GC) B cells, but it is unclear to what extent B cells in lymphoma keep the functional dynamics of GC B cells or are blocked at a special stage of the GC reaction [20].

The germinal center is probably the source of many lymphomas. The germinal center reaction begins when antigens, with T-cell signals, activate centroblasts that migrate from the dark zone to the light zone and begin to express their antibodies

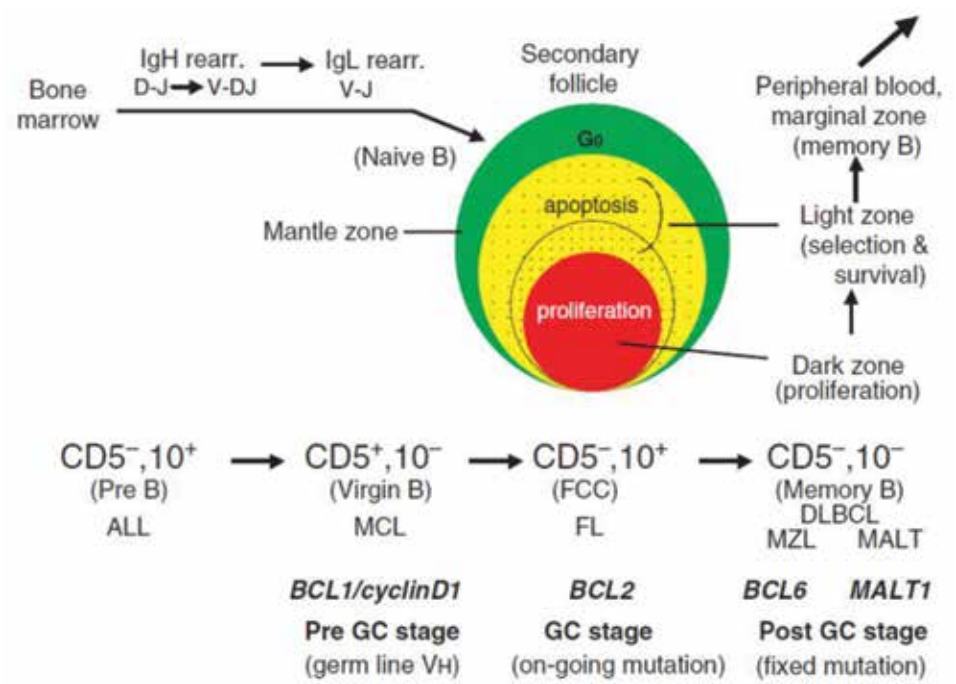

Figure 1.
B-cell differentiation and association of B-cell neoplasms [1].

on the cell surface known as centrocytes. Then apoptosis begins and competes for survival signals derived from FDCs and T_{FH} cells. Germinal center selection is dependent on the tendency of their surface antibody to the antigen and can enter into the light zone of germinal centers, which are morphologically centrocytes and which are indivisible B cells with a notched core. The action or process of reentering into the dark zone as centroblasts gives a chance for nonselected B- cell mutants to gain more mutations in order to improve affinity toward antigen. Interactions with T cells are to prevent the generation of autoreactive germinal center B cells [18, 23, 33].

During the germinal center reaction, two different modifications of the B-cell DNA change B-cell receptor: somatic hypermutation and switch-off recombination. Both require activation-induced cytidine deaminase (AID). IgM, IgA, or IgE recurrence of IgM, IgG heavy-chain recombination changes occur, while somatic hypermutations imply mutations in the immunoglobulin-variable region, region that produces a population of B cells with an increase (or decrease) affinity for a particular antigen. These genetic modifications are essential for normal immune response but also a source of DNA damage that can become pathological with lymphoma [8].

Mature B-cell neoplasms involve about 30 different entities, while DLBCL is the most common type of non-Hodgkin's lymphoma (NHL), which accounts for about 40% of all non-Hodgkin's lymphomas (NHLs). B-cell lymphomas are identified by a combination of morphological features and recognizable immunophenotype [20]. A list of transcription factors that modulate the B-cell germinal center phenotype and where cell selectively express BCL6, and includes interferon-regulatory factor 4 (IRF4). IRF4 co-bound with the transcription factors PU.1 (plays a critical role in the development of white cells), and B-cell-activating transcription factor (BATF) to Ets or activator protein 1 (AP-1) composite motifs, united with genes involved in B cell activation and the GC response [18, 20, 23].

CD5 is a transmembrane glycoprotein with expression on the surface of T cells and a subset of B cells. The absence of CD5 performs thymocytes hyperresponsive to stimulation through the T-cell antigen receptor (TCR) in vitro. CD5 can influence the developing thymocytes by acting as a negative regulator of TCR-mediated signal transduction [34]. The CD5 signature included downregulated extracellular matrix genes such as *POSTN*, *SPARC*, *COL1A1*, *COL3A1*, *CTSK*, *MMP9*, and *LAMB3* and comprised upregulated genes including *TRPM4* [35–38] (**Figure 2**).

Bcl6 protein suppresses many genes involved in plasma cell differentiation, cell cycle progression, and responses to DNA damage and cell death. One of these genes is the transcription factor Blimp-1, the main regulator of plasma cell differentiation that reduces gene expression of mature B cells. The transcription factors Blimp-1 and IRF4 are essential for plasma cell generation. However, their function in mature plasma cells has remained elusive. B cells germinate center begins to differentiate plasma cells, monitored by interferon regulatory factor 4 (IRF4)/MUM1, whose transcription factor is needed for conversion to plasma cells. IRF4 increases the expression of Blimp-1 [39], and this growth is suppressed by *BCL6*, so differentiation takes place in favor of plasma cells [25, 39].

The transcriptional repressor Blimp-1 originally cloned as a silencer of type I interferon (IFN)-β gene expression controls cell fate decisions in multiple tissue contexts. Blimp-1 functional loss in 3D cultures of mammary epithelial cells (MECs) results in the accumulation of dsRNA and expression of type III IFN-λ [39] (**Figure 3**).

Chromosomal translocation in B-cell neoplasms is divided into three functional categories: proliferation, inhibition of differentiation, and antiapoptotic activity. These three categories are essential for understanding the mechanism of lymphomagenesis. For DLBCL with *BCL6* gene translocation, Bcl6 protein expression is a side for post-GC staging cells, but it is not yet clear what signal is needed. It is clear that the antiapoptotic function must be present for *BCL2*, which is expressed at the post-GC

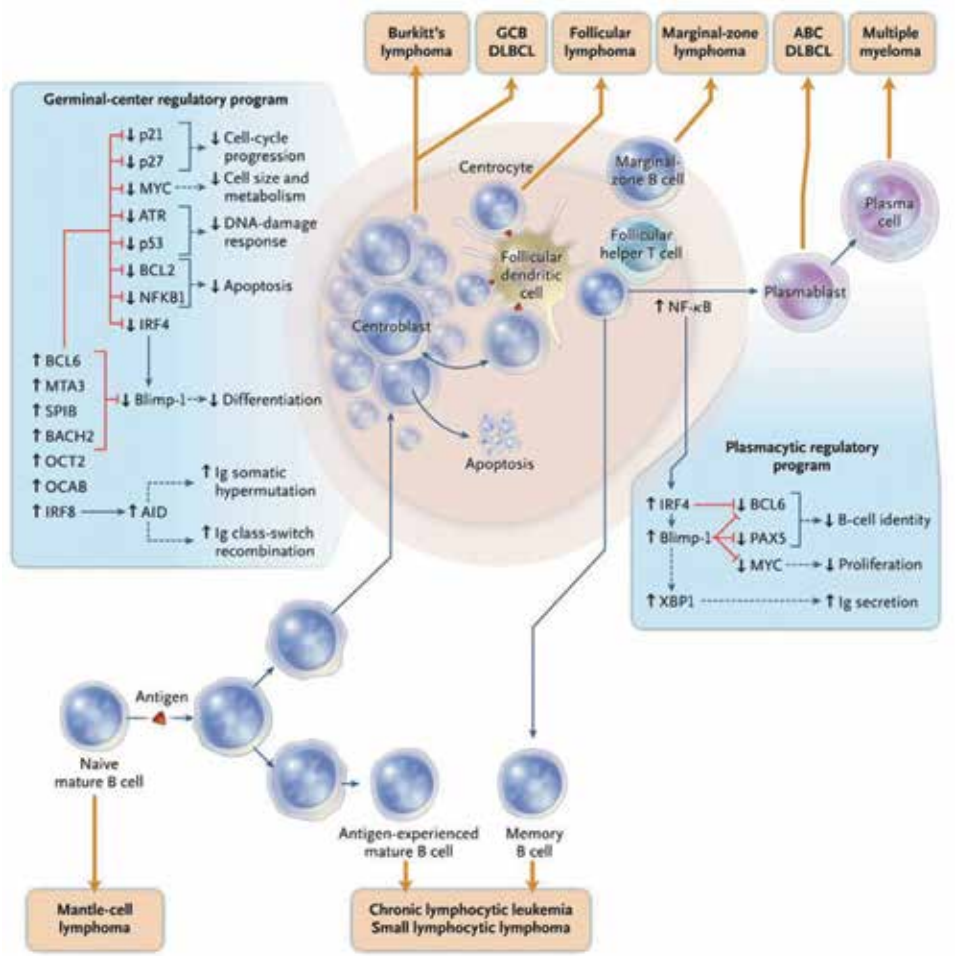

Figure 2.
B-cellular differentiation and lymphomagenesis ([25], p. 1418).

centers. There are indications that *BCL6* has the function of protecting cells from terminal differentiation and shows epigenetic changes that are conserved from human hematopoietic stem/progenitor cells (HSPCs) to mature B cells. These results suggest that *BCL6* may function in a "hit-and-run" role in lymphomagenesis [8].

Lymphomagenesis is a complex process that takes place in many steps, in which lymphocyte signal distortion, transcriptional regulation, and apoptosis lead to transformation. Experiments suggest that distorted expression of casein kinase II subunit alpha (CK2alpha) creates predispositions for lymphoma development. Further experiments suggest that CK2alpha lymphomas monitor the panel of lymphocyte transcription factors such as, for example, activator protein 1 (AP-1) and nuclear factor-kappaB (NF-κB) [40, 41]. NF-κB represents a group of related homodimeric and heterodimeric transcription factors that can activate different target genes involved in proliferation, differentiation, immune response, cell growth, and survival. Among the molecules induced by NF-κB are the cytokines, chemokines, and effectors of the immune response. NF-κB participates in response to inflammatory signals through T receptors, TNF receptors, and IL-1 receptors [40–42]. CREB is one of the best understood phosphorylation-dependent transcription factors (CREB) [16]. In effector T and B cells, immune receptor signals induce within minutes a rise of intracellular Ca++, the activation of the phosphatase calcineurin, and the

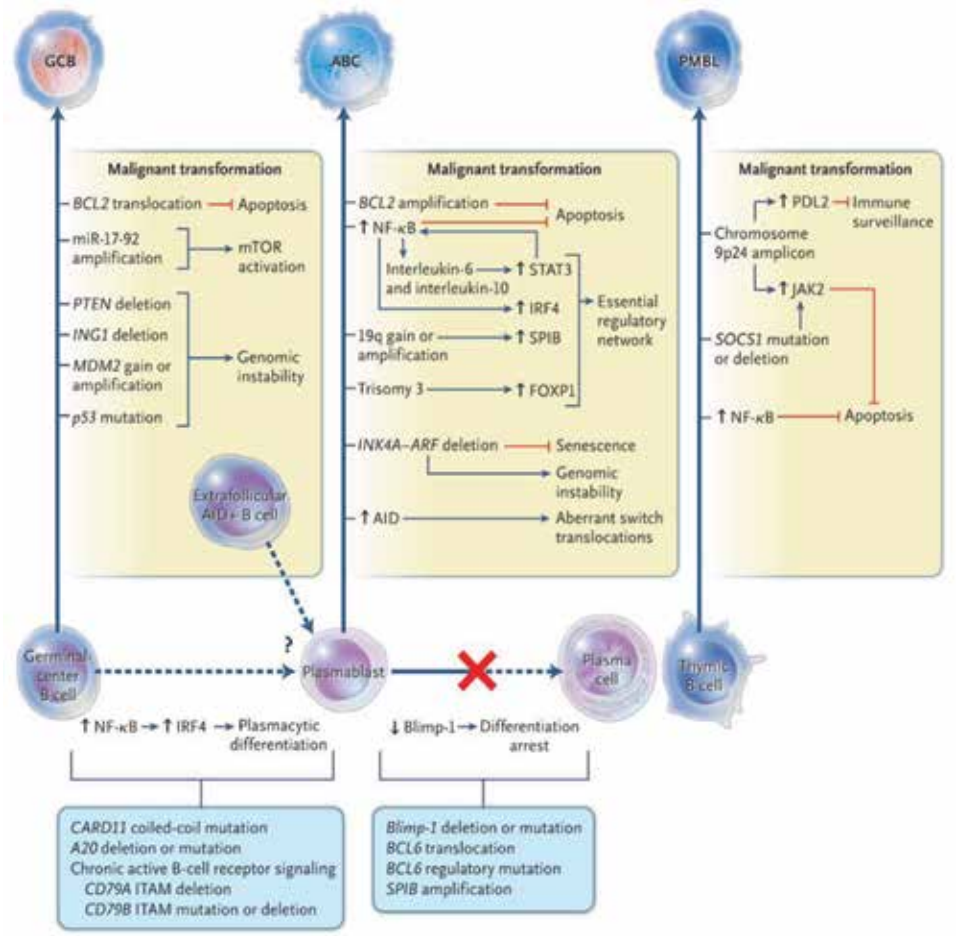

Figure 3.
Oncogene path for three subtypes of DLBCL ([25], p. 1421).

translocation of NFAT transcription factors from cytosol to nucleus; the induction of NFATc1/αA takes place during the G1 phase of cell cycle [43, 44]. The NFAT signaling pathway oversees different aspects of cellular functions; NFAT has a function like calcium sensor, including calcium signaling with other pathways involved in development and growth, immune response, and inflammatory response. The NFAT family of transcription factors regulates different cellular functions such as cell survival, proliferation, migration, invasion, and angiogenesis [44, 45].

TCF/LEFs are multifunctional proteins that use their sequence-specific DNA-binding and context-dependent interactions to specify which genes will be regulated by Wnts. The TCF/LEF families have been shown to regulate the expression of specific genes, such as c-Myc and Cyclin D1 [46]. These transcription factors are interdependent and closely linked with each other to form a network in the above signaling pathways, which regulate the entire process of osteoblast proliferation and differentiation [14]. Normally, these factors appear to be involved in the physiological activation response of lymphocytes before controlling the growth of malignant cells [47, 48].

Proliferation, differentiation, and apoptosis are associated with different types of lymphoma and chromosomal translocations. Each lymphoma has a separate proliferation of the site, which is most clearly described in the follicular lymphoma that proliferates in the follicle. Special chromosomal translocations are associated with

each type of lymphoma. *BCL1* translocation results in disturbed cell cycle regulation. *BCL2* and API2-MALT1 have an antiapoptotic function [49]. API2-apoptosis inhibitor 2-MALT1 (mucosa-associated lymphoid tissue lymphoma translocation gene 1)-induced NF-kappaB activation may contribute to antiapoptotic action probably through NF-kappaB-mediated upregulation of apoptotic inhibitor genes [49].

2. Classification and pathogenesis of DLBCL

Non-Hodgkin's lymphoma (NHL) classification is based on pathohistological findings. The history of classification of lymphoid neoplasms begins in the 1940s. Based on the advancement in knowledge, the work classification and Kiel have replaced the revised European-American Classification of Lymphomas, and it is a step ahead of the classification given by the WHO [8].

Classifications are an essential part of modern medicine, offering a consensus on the terminology and disease definitions to be used interdisciplinary both in research and clinical practice. Evolution of lymphoma classification includes numerous attempts from descriptive schemes, relying on morphology to strictly clinically oriented stratifications proposed by hematologists, usually without significant international acceptance. The classification of the WHO (Swerdlow et al. 2008) differs from the previous classifications in defining the real entities of the disease through multiple divisions. The new entities defined in this release include extended immunophenotypes and some gene information relevant to the classification. Panel antibody was selected based on morphological differential diagnosis and tumor location. DLBCL is a diffuse proliferation of large neoplastic B lymphoid cells of the same size or up to two times than the normal lymphocyte or extremely large as in macrophages. The origin of these cells are B cells of the germinating center and postgerminant cells [8]. DLBCL can occur in the form of nodal and extranodal diseases. Approximately 40% of patients are present with extranodal disease. Any tissue organ can be the primary site of DLBCL, but the gastrointestinal tract is the most common site [33].

Tumor cells are B large transformed lymphocytes, but the morphological picture of DLBCL is varied, and the predominant morphological cell type can distinguish six subtypes of DLBCL: centroblastic, immunoblastic, T lymphocyte/histiocyte rich, anaplastic, plasmablastic, and DLBCL with cells similar to Reed-Sternberg cells and expression of anaplastic lymphoma kinase (ALK) protein. Tumor cells are positive, as defined by the disease, on pan-B markers CD19, CD20, CD22, and CD79a, and the positivity of intracytoplasmic or surface immunoglobulin (Ig) markers is distinctly expressed as CD5, CD10, CD30, and CD45 markers. Numerous chromosomal abnormalities have been detected with DLBCL, but no specific diagnostic changes have been isolated. The most frequent changes are breaks of regions 14q32, 3p21, 3q27, 22q12, 1q25, and 18q21 and additional chromosomes 7,9,12 and 4q31-35,13p13-14 and 17p11-13,8,9. In 11–23% of cases, DLBCL with advanced clinical stage of the disease revealed nine regions of chromosomal amplification and isolated amplified genes with a potential role in the formation of lymphoma. The clinical significance of each individual change has not been established, but some changes are associated with advanced clinical stage or poorer response to therapy. The abovementioned chromosomal abnormalities are generally not found as individual changes. The accumulation of a greater number of abnormalities indicates the progression of genetic changes, which play an important role in the pathogenesis of DLBCL [8, 33].

3. The WHO classification which was revised in 2016

See **Table 1**.

Diffuse large B-cell lymphoma (DLBCL), not otherwise specified (NOS)
Germinal center B cell-like (GCB)
Activated B-cell-like (ACB)
T-cell/histiocyte-rich large B-cell lymphoma
Primary DLBCL of the central nervous system
Primary cutaneous DLBCL, leg type
Epstein–Barr virus (EBV) positive DLBCL, not otherwise specified (NOS)
EBV mucocutataneous ulcer
DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK+ large B-cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
Human herpesvirus 8 (HHV8)+ DLBCL, not otherwise specified (NOS)
Burkitt lymphoma
Burkitt-like lymphoma with 11q aberration
High-grade B-cell lymphoma, with MYC and BCL2 and/or BCL6 translocations
High-grade B-cell lymphoma, NOS
B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical
Hodgkin lymphoma

Table 1.
The 2016 update of WHO classification of DLBCL: subtypes and related entities [8].

4. Prognostic markers in DLBCL

4.1 Gene expression profile (GEP)

Clinical and morphological differences in DLBCL suggest the biological significance of subtypes, which may help in adapting the therapy to be defined. The development of DNA microarray techniques provided the ability to identify gene expression of the tumor with a new molecular entity and a molecular predictor essential for survival. DNA microarray can analyze thousands of sites of previously identified genes. DLBCL has been identified by several tumor markers associated with unfavorable outcome after therapy and which can be linked to tumor cells and normal B lymphocytes. Phenotypic cells resemble normal B lymphocytes, but the clinical course is completely different [50–52].

Gene expression profile has shown that DLBCL can be divided into two molecular entities that differ in the gene expression profile. Hans et al. have identified two subgroups of DLBCL [34, 52]. One subgroup was called germinal center B-cell-like (GCB) and has a characteristic gene expression of normal GC B cells, in about 50% of cases, and the second group was called activated B-cell-like (ABC). Type-3 group is defined as nonclassified cases. According to the GEP classification, the observed prognosis distinguishes two major subtypes after the chemotherapy. Patients with GCB lymphoma have a more favorable outcome in 5-year survival than patients in the ABC group (68% vs. 24%) [52]. The distinction between GCB and ABC

subgroups and their effect on survival are independent of any clinical features of the disease, and these findings are further confirmed in other retrospective series with patients treated with chemotherapy [34, 42, 45–49].

In DLBCL, the ratio of *BCL2*-positive cases is highly variable, ranging from 24 to 80% among the study population in the previous studies that used immunohistochemistry (IHC); therefore, its prognostic relevance is controversial [16, 53–55].

Overexpression of the *BCL2* is associated with poor prognosis [55]. ABC-DLBCL subgroup is characterized by transcription expression of the *BCL2* gene [55], trisomy 3, and 18q21-q22 and loss of 6q21-q22 [56]. Activation of the antiapoptotic NF-κB signal pathway occurs at ABC but not at GCB DLBCL [56].

GCB DLBCL is characterized by frequent *REL* amplifications, *BCL2* translocations, and ongoing somatic hypermutation of the immunoglobulin genes [56].

Immunohistochemistry studies have a significant role, although complexity in the expression and performance of the interpretation of findings is very complex. They are used in the prognosis of DLBCL and in the therapeutic advantage of using rituximab [52].

DLBCL is a common type of aggressive non-Hodgkin's lymphoma with clear biological and clinical characteristics. Many studies have focused on the significance of internal tumor characteristics, such as the presence of proteins or the presence of gene expression and chromosomal translocation. Significant component of cell biology is inherited from non-transformed stem cells. Each of the B-cellular lymphoma inherits the traces of B-cell differentiation, although the extent to which this malignancy is maintained and the maintenance of molecular and physiological properties of normal B cells are not yet clear. Redistribution of immunoglobulin genes in DLBCL and other lymphomas carries mutations having somatic hypermutation characteristics, and the diversity of antibodies normally occurs only with the germinal center of the secondary lymphoid organ. This data suggest that DLBCL is generated from each of the germinal centers of the B cell or from the B cell at a later stage of differentiation [1, 25, 50, 52, 56].

4.1.1 Germinal center B-cell-like (GCB)

CB DLBCL appears to be generated from the normal germinal B cell, the translocation t (14, 18) involving *BCL2* [54], and the amplification of *c-REL* on the chromosome 2p was detected in 45% GCB DLBCL, whereas it was never detected at ABC subtype [56]. This finding suggests that different subgroups represent different diseases and the consequence is a different mechanism of malignant transformation and significantly different survival after chemotherapy. CD10 and *BCL-6* are considered as germinal center markers, while IRF4/MUM1 is expressed on plasma cells in atypical light zones of the germinal center. By morphological characteristics, the center is uniform form monomorphic. Expressions CD10, BCL-6, and MUM1 are used to classify GCB and ABC lymphoma subtype [1, 25, 46, 50, 56].

Many factors of B-cell differentiation in the germinal center affect the expression of transcription factors. The key B-cell transcription factor is PAX5 (PAX5 is a nuclear protein that has the ability to bind to wild-type PAX5 target loci) [53] and is required for regulating many B-cell-specific transcription factors and for B-cellular development from the B stage to mature B cells. The transcription factor PU.1 is an E26 transformation-specific family transcription factor that is required for the development of the immune system. PU.1 functions at both early and late stages of lymphoid and myeloid differentiation [57], and the IRF involved with Blim1/PRDM1 was isolated from the family in further regulation of the transcriptional factor *BCL6*, which is essential for the formation and maintenance of the germinal center. Translocation t (3q27) is included in *BCL6* gene and is found in 20–40% diffuse lymphomas. IRF4/MUM1 expression is required for activation-induced cytidine deaminase (AID) expression, which is important for two significant functions

of the germinal center, namely, somatic hypermutation (SHM) and AID-mediated class switch recombination (CSR) [58]. They depend on hierarchical expression and the common regulation of transcription factors. For example, *BCL6* and IRF4/MUM1 are expressed mutually exclusively in germinal center cells, but with DLBCL *BCL6* and IRF4 / MUM1, they have co-expression [8, 59].

The Epstein–Barr virus (EBV) is the second factor associated with disturbed GCB regulation. EBV is associated with numerous lymphoid neoplasms including Burkitt's lymphoma and classical Hodgkin's lymphoma and posttransplant lymphoproliferative disease. The transformation that leads through EBV is capable of major negative selection in the germinal center. EBV/LMP2A induces escape of the defective B cells of the germinal center of the apoptosis and does not only offer an intriguing model of lymphomagenesis but also confirms the significant role of exogenous factors such as viruses involved in forming the response of the germinal center [60]. EBV latent membrane protein 2A (LMP2A) is expressed on the membranes of B lymphocytes and blocks B-cell receptor (BCR) signaling in EBV-transformed B lymphocytes in vitro. In vivo studies indicate that LMP2A allows B-cell survival in the absence of normal BCR signals [60].

CD10 expression is associated with a more favorable prognosis, and MUM1 is normally found on plasma cells and in a small part of the atypical spot zone in the germinal center and represents a marker for a non-germinal center [61].

Patients in this group have significantly better 5-year overall survival and survival without signs of disease activity after chemotherapy with combined drugs (68% for GCB vs. 24.7% for non-GCB with use of gene-expression profiling) [8, 52, 56].

4.1.2 Activated B-cell-like (ABC)

ABC-DLBCL is generated from the postgerminal center of B cells during plasmacytic differentiation. ABC is characterized by frequent changes in chromosome arm 3p but not 3q [62]. ABC lymphoma has a subtype gene expression that has the properties of normal B cells that are activated over B-cell receptors.

Trisomy 3 is a common chromosomal aberration and is found in 26% of respondents. The forkhead transcription factor (FOXP1) is the third most potentially monitored gene in trisomy 3. High FOXP1 expression can contribute to B-cell lymphomagenesis by promoting B-cell survival inhibiting plasma cell differentiation. The small FOXP1 isoform (FOXP1-iso) is predominantly expressed in ABC-DLBCL, and studying its oncogenic potential and transcriptional activity is directly compared to FOXP1-FL in DLBCL cell lines and primary human B cells [61–64].

ABC lymphomas have expression of the plasma cell genome, including transcriptional factor XBP1, which is the regulator of immunoglobulin secretion. XPB1 is a protein which in humans is encoded by the *XPB1* gene located on chromosome 22. The XBP1 protein is a transcription factor that regulates the expression of genes important of the immune system and in the cellular stress response. *C-MYC* is necessary and sufficient for activation of the IRE1/XBP1 pathway [65, 66].

The constituent part of the NF-κB activation causes ABC lymphoma with expressing the IRF4 transcription factor, and this may push them toward differentiation in the plasma cells [42].

ABC lymphomas can obtain genetic changes that interfere with Blimp-1, thereby blocking full differentiation into plasma cells. The block in differentiation represents an early step in the pathogenesis of ABC subtypes, but the nature of the cell precursor is unclear. These lymphomas contain high activation-induced cytidine deaminase (AID) values, and their IgH genes have severe mutations. However, most ABC lymphomas did not undergo a recombinant trigger, and they have IgM expression, unlike most of the normal B cells of the germinal center and other GCB lymphomas [39].

NF-κB is present as a latent, inactive, IκB-bound complex in the cytoplasm. When a cell accepts any of a large number of extracellular signals, NF-κB quickly enters the nucleus and activates gene expression. Key step for controlling NF-κB activity is the regulation of the I kappa B kinase (IKK) [42, 55, 62].

Various genetic abnormalities activate CARD11 in ABC subtype lymphoma. In normal B cells, antigen-receptor-induced NF-kB activation requires CARD11, a cytoplasmic scaffolding protein. To determine whether CARD11 contributes to tumorigenesis, we sequenced the CARD11 gene in human DLBCL tumors [67]. In about 10% of patients, CARD11 is an oncogene with acquired mutations that activate NF-κB and can prolong cell survival. Mutant CARD11 generates large protein aggregates in the cytoplasm, which represent possible NF-κB activation sites. The survival of lymphoma ABC with the wild-type CARD11 depends on B-cell receptors and kinases [67].

ABC lymphomas have expression of the plasma cell genome, including transcriptional factor XBP1, which is the main regulator of immunoglobulin secretion. ABC lymphoma has presented expression of genes characteristic of mitogen-activated B cells in the blood and is associated with a worse prognosis [66]. ABC-DLBCL is characterized by gain of 3q, 18q, and 19q and loss of 6q and 9p21, and GCB DLBCL is characterized by gain of 1q, 2p, 7q, and 12q; the genomic imbalances characteristic of the CD5(+) and CD5(−)CD10(+) groups were similar to those of the ABC and GCB types [68].

Lymphoma ABC does not have one expression of the germinal center gene, but has gene expression that is normally present in B cells after B-cell receptor stimulation. MUM1 is found on plasma cells at later stage B-cellular differentiation and is associated with the gene profile of activated B-cellular DLBCL. The presence of *BCL2* and Cyklin D2 is adverse prognostic parameters. Polymorphic centroblasts and immunoblasts are more commonly present in activated B-cell DLBCL. This group is characterized by a group of genes that are characteristic of plasma cells, in part, added to the endoplasmic reticulum and Golgi's proteins involved in secretion. There is a significantly higher percentage of patients in this group [69].

5. Conclusion

The contribution of GC selection to phenomena such as immunodominance and original antigenic guilt has also not been established and is likely to be of considerable interest. Answering these questions will be crucial to our quest to produce more broad and new medications and will undoubtedly bring novel insights into the general functioning of GC, as well as of the immune system in general.

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Chapter 5

Diffuse Large B-Cell Lymphoma

Patrascu Ana Maria, Ionela Rotaru, Valeriu Surlin and Stefan Patrascu

Abstract

Diffuse large B-cell lymphoma (DLBCL) is a heterogenous class of aggressive lymphoma and is considered as the most common subtype of non-Hodgkin lymphomas (NHL). Several genetic anomalies such as point mutations, numerical alterations, and, more rarely, translocations and gene amplifications play a role in the pathogenesis of this class of B-cell lymphoma and have been related to specific histological and immunophenotypic subtypes. On the other hand, the treatment protocol in DLBCL did not witness significant changes during the last two decades. The widespread adoption of rituximab as an important adjuvant to standard chemotherapy protocol in CD20+ cases was a notable exception, which provided significant improvement in disease-free survival and overall survival, with limited toxicity. However, no less than 20% of patients diagnosed with DLBCL exhibit relapse after the initial response to R-CHOP regimen, while more than 15% of the patients exhibit primary refractory disease. This is the reason why a review of all the morphological, clinical, and therapeutic particularities of DLBCL is required.

Keywords: diffuse large B-cell lymphoma, immunohistochemistry, lymphoma, molecular diagnostics, rituximab-CHOP

1. Introduction

Non-Hodgkin lymphomas (NHL) are the most common type of lymphoma, accounting for roughly 85% of this category of lymphoid neoplasia. NHL are most commonly classified as B-cell, T-cell, and natural killer (NK) cell lymphomas, according to their cell of origin. Diffuse large B-cell lymphoma (DLBCL) is a heterogenous class of aggressive lymphoma and is considered as the most common subtype of NHL. Several genetic anomalies such as point mutations, numerical alterations, and, more rarely, translocations and gene amplifications play a role in the pathogenesis of this class of B-cell lymphoma and have been related to specific histological and immunophenotypic subtypes [1].

2. Etiopathogeny of diffuse large B-cell lymphoma

2.1 Epidemiology

The incidence of diffuse large B-cell lymphoma (DLBCL) is increasing with age, from 0.3 cases/100,000/year in those between the ages of 35 and 39 years and

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26.6/100,000/year for the 80- to 84-year-old age group [2, 3]. Mean age of diagnosis is 65 years for DLBCL and between 20 and 30 years of age for primary mediastinal subtype [4].

This type of lymphoma is more common in Caucasians than African and Asian populations, while the extranodal involvement is more frequent in European and the Far East population than the American patients. The sex ratio ranges from 1.5 to 3.5/1 in favor of men [5].

2.2 Pathogeny

DLBCL originates in mature B cells in certain stages of differentiation. Various changes occur in the B cell toward its malignant transformation, which are induced by genetic mutations. During their ontogenesis, the B cells are passing in the secondary lymphoid tissues where the antigen-dependent activation occurs. The activation and amassment of B cells in the secondary lymphoid tissue lead to the creation of the germinal center, which is critically dependent on BCL-6. If certain genetic alterations occur during lymphocyte development, then the determinatives of the neoplastic changes are settled. The type of lymphoma is determined by the stage of maturation of the B cell and by the type of anomalies that are interfering with their development and differentiation [6]. More specifically, the upregulation of B-cell lymphoma-2 (Bcl-2) protein expression and the inactivation of BCL6 which in turn blocks the apoptosis have both been observed in DLBCL.

Moreover, the elevation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) and the upregulation of the avian myelocytomatosis virus oncogene cellular homolog (c-Myc) expression, leading to an increase in B cell proliferation, have also been cited [7]. The signal pathways for the B-cell receptor (BCR) and the activation of NFkB and downregulation of the B-cell lymphoma-6 (Bcl-6) pathways have a special pathogenic significance in DLBCL [7].

3. Classification and standardization

3.1 Classification

DLBCL can originate from three types of cells and can be classified accordingly as germinal center DLBCL (GCB DLBCL), from the centroblasts of the germinal center; activated B-cell DLBCL (ABC-DLBCL), from the plasmablasts that are involved in the terminal differentiation of the B cells; and primary mediastinal large B-cell lymphoma (PMBCL), which derives from thymic B cells. This classification has a prognostic utility, as the activated B-cell subtype has an unfavorable evolution [8].

Apart from the cell-of-origin classification, several other classifications for non-Hodgkin lymphomas have been suggested during the last half-century, each with its own advantages and drawbacks: *Rappaport*, *Kiel*, *International Working Formulation (IWF)*, and *the Revised European-American Lymphoma classification (REAL).* The last classification that is currently in use has been offered by the World Health Organization (WHO) in 2001 and was last revised in 2016. According to the maturation stage in which the B cell is in, and to the type of anomalies that occur during differentiation and maturation, DLBCL presents several variants and subtypes.

The World Health Organization classification of the lymphoid neoplasms has systemized DLBCL into several subtypes, each with its own morphological, clinical, and immunohistochemical particularities. The 2016 revision of the WHO classification brought several updates concerning diagnostic and prognostic factors

for DLBCL, such as the acknowledgment of the prognostic role of double-hit DLBCL-NOS, which involves the double expression of MYC and BCL2, or the newly introduced designation of DLBCL EBV+ (NOS) which is replacing the old term of "DLBCL EBV+ of the elderly" (**Table 1**) [9].

Chronic lymphocytic leukemia/small lymphocytic lymphoma
Monoclonal B-cell lymphocytosis
B-Cell prolymphocytic leukemia
Splenic marginal zone lymphoma
Hairy cell leukemia
Splenic B-cell lymphoma/leukemia, unclassifiable
Splenic diffuse red pulp small B-cell lymphoma
Hairy cell leukemia variant
Lymphoplasmacytic lymphoma
Waldenstrom macroglobulinemia
Monoclonal gammopathy of undetermined significance (MGUS), IgM
m heavy-chain disease
g heavy-chain disease
a heavy-chain disease
Monoclonal gammopathy of undetermined significance (MGUS), IgG/A
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Monoclonal immunoglobulin deposition diseases
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
Nodal marginal zone lymphoma
Pediatric nodal marginal zone lymphoma
Follicular lymphoma
In situ follicular neoplasia
Duodenal-type follicular lymphoma
Pediatric-type follicular lymphoma
Large B-cell lymphoma with IRF4 rearrangement
Primary cutaneous follicle center lymphoma
Mantle cell lymphoma
In situ mantle cell neoplasia
Diffuse large B-cell lymphoma (DLBCL), NOS
Germinal center B-cell type
Activated B-cell type
T-cell/histiocyte-rich large B-cell lymphoma
Primary DLBCL of the central nervous system (CNS)
Primary cutaneous DLBCL, leg type
EBV1 DLBCL, NOS
EBV1 mucocutaneous ulcer

DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK1 large B-cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
HHV81 DLBCL, NOS
Burkitt lymphoma
Burkitt-like lymphoma with 11q aberration
High-grade B-cell lymphoma, with MYC and BCL2 and/or BCL6 rearrangements
High-grade B-cell lymphoma, NOS
B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma

Table 1.
2016 WHO classification of mature B-cell neoplasms [9].

3.2 Variants of DLBCL and related pathologic entities

Over time, several variants and subtypes of DLBCL have been identified, according to their clinical features, localization, and morphology, which are all included in the recent REAL and WHO classifications. Extranodal DLBCL are relatively rare, accounting for 1–5% of NHL [10]. Less than 40% of DLBCL cases are originating in extranodal sites, especially in the gastrointestinal tract but also the mediastinum, bones, central nervous system (CNS), testes, and breast [11].

3.2.1 Primary mediastinal large B-cell lymphoma (PMBCL)

Roughly 10% of the total number of DLBCL is represented by PMBCL, a subtype which originates in medullary thymic B cells and affects young women, around the age of 30 and 40 years. They clinically appear as large mediastinal tumors sometimes involving the lungs and pericardium, which can lead to local compression with superior vena cava syndrome and airway obstruction. PMBCL is positive for pan-B markers, CD23 and CD30, and is negative to CD15 and sIg. BCL6, CD10, and interferon regulatory factor 4 (IRF4) can sometimes be positive [12]. Several chromosomal abnormalities can be found, such as gains in chromosomal arm 9p and 2p corresponding to JAK2 and cREL loci [13, 14]. Because of the diagnosis confusions between PMBCL and DLBCL with secondary mediastinal involvement, the reports concerning the survival rates of the two pathologic entities have been similar [15].

More recently the improvements in diagnosis of PMBCL have allowed to observe a higher survival rate of this type of lymphoma compared to DLBCL [16]. Some variants of PMBCL, which own morphologic, immunophenotypical, and molecular resemblance with the nodular sclerosis subtype of Hodgkin lymphoma, are included in the category of B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma [9, 10, 17].

3.2.2 Primary DLBCL of the central nervous system (CNS)

Only a limited number of cases (2–3%) of cerebral neoplasms are lymphomas, the most frequent of which is DLBCL. Primary DLBCL of CNS can be both

germinal center and non-germinal center, while some genetic anomalies suggest the implication of the microenvironment as a pathogenic factor [18].

The clinical manifestations usually range from neurological deficits to psychiatric symptoms, but ocular involvement, seizures, and symptoms of increased intracranial pressure can also be found [18].

3.2.3 Primary testicular lymphomas (PTL)

PTL are usually DLBCL (>80%) but can also classified as follicular, plasmablastic, Burkitt, mantle cell, or plasmablastic lymphomas in a minority of cases. Usually this type of lymphoma is diagnosed in stages 1–2, while in advanced stages, it can also affect the central nervous system, the liver, the skin, the Waldeyer ring, and the lungs [19]. PTL displays a modified expression of adhesion molecules that can be correlated with extranodal involvement. In most cases (60–96%) the cell of origin analysis indicates the activated B-cell (ABC) pattern. Several mutations have been observed, such as MYD88 mutations in 70% of cases, rearrangements of forkhead box protein P1 (FOXP1), programmed death-ligand 1 (PDL 1), and class II MHC transactivator (CIITA), as well as inactivation mutations of beta-2 microglobulin (B2M) gene [10].

3.2.4 Primary breast lymphoma

Primary breast lymphoma is a rare form of breast cancer, with cell of origin studies usually indicating an activated B-cell pattern. The cytogenetic analysis shows multiple chromosomal anomalies, such as trisomies 3 and 18 and translocations of chromosome 18 involving IGH/MALT1 [20].

3.2.5 Primary bone lymphoma (PBoL)

PBoL is a rare type of lymphoma that is confined to the bone tissue, preponderantly affecting the middle-aged male patients. It should be differentiated from primary medullar lymphoma which, unlike PBoL, does not display cortical involvement [21]. The germinal center subtype is more common, frequently displaying BCL2, BCL6, and MYC rearrangements [10].

4. Clinical, morphologic, and cytogenetic characteristics

4.1 Clinical aspects

DLBCL patients have a wide spectrum of clinical manifestations, a frequent nodal involvement, but also, in up to 40% of cases, extranodal symptoms (skin, digestive and central nervous system, etc.). All these clinical manifestations, together with the general signs and symptoms (such as fever, weight loss, sweating) usually indicate a more aggressive phenotype. On rare occasions, the diagnosis is incidentally established when the clinical examination detects enlarged lymph nodes with no other signs or symptoms. Medullary involvement is uncommon in the early phases of the disease, as it is only found in less than 30% of cases [22].

As for the paraclinical investigations, usual blood tests consist in complete blood count, lactate dehydrogenase and uric acid determination, serology, osteomedular biopsy, and cytologic exam of the cerebrospinal fluid. Computed tomography (CT) scan or, even better, positron emission tomography–computed tomography (PET-CT) is considered mandatory to assess the extension of the disease.

PET-CT is of great diagnostic and prognostic importance, but it is also necessary for the post-chemotherapy assessment, such that in case of negative scans at the end of chemotherapy regimen, the overall prognosis is favorable [23, 24]. The drawbacks of this technique consist of the possibility of false-positive results due to reversible thymic hyperplasia, infections, and sarcoidosis or as a consequence of hematopoietic growth factor therapy [23].

4.2 Morphologic and cytogenetic characteristics

The search for an exclusively cytologic classification of DLBCL is mostly abandoned, due to the wide observer-dependent variations in morphologic analysis and nosologic framing. The cytologic exam usually differentiates between centroblastic, immunoblastic, anaplastic, and T-cell/histiocyte-rich B-cell lymphoma.

The subtype of DLBCL is decided by the genetic anomalies that occur during B-cell differentiation and maturation process [6, 25].

Even though gene expression profiling has the best accuracy for the identification of the cell of origin in DLBCL, its numerous drawbacks—such as the diverse cellular regulating pathways, some financial limitations, etc.—make its widespread clinical use still impractical. Consequently, several surface molecules have been evaluated for the differentiation between GCB and ABC DLBCL, which prove to be consistent with gene expression profiling.

The Hans algorithm has structured DLBCL in these two main subtypes, by analyzing three essential markers: multiple myeloma 1 protein (MUM1), CD10, and bcl-6 (**Figure 1**). The same classification can be achieved by using the germinal center B cell-expressed transcript 1 (GCET1), CD10, BCL-6, MUM1, and FOXP1 biomarkers (the Tally and Choi algorithms) with a slightly better accuracy (**Figure 2**). All these algorithms are important, as the cell of origin classification tends to display a prognostic role, due to the fact that DLBCL-GCB subtype usually has a favorable prognosis compared to non-GCB subtype. To further add to the complexity of this classification, GC and ABC DLBCL subtypes also express several particularities in cell marker expression, activation pathways, and outcomes [26].

However, the detection of other cell marker expression has become a fundamental component both in establishing an accurate prognosis and in the development of an optimal therapeutic algorithm.

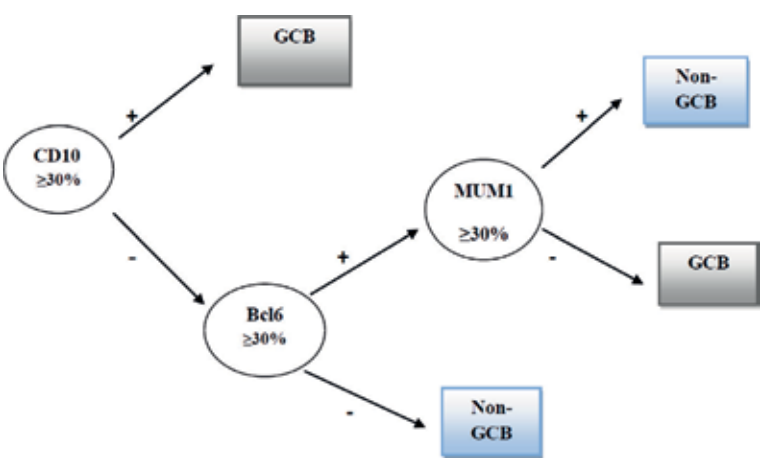

Figure 1.
Hans algorithm for DLBCL subtyping.

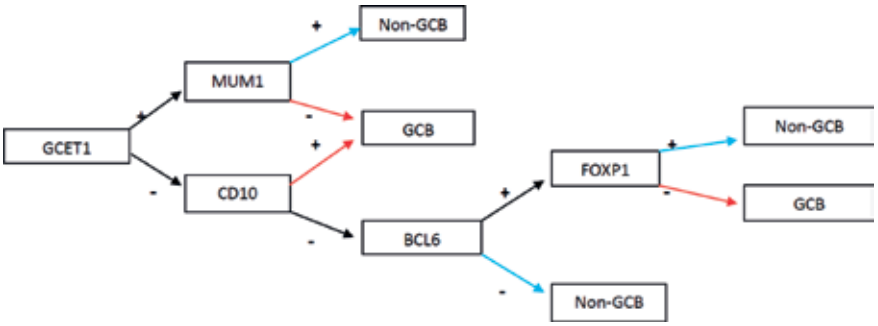

Figure 2.
Choi algorithm for DLBCL subtyping.

5. Treatment of diffuse large B-cell lymphoma

The treatment protocol in DLBCL did not witness significant changes during the last two decades. The widespread adoption of rituximab as an important adjuvant to standard chemotherapy protocol in CD20+ cases was a notable exception, which provided significant improvement in disease-free survival, overall survival, and complete remission rates, with limited toxicity. However, no less than 20% of patients diagnosed with DLBCL exhibit relapse after the initial response to R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) regimen, while more than 15% of the patients exhibit primary refractory disease [27]. All these factors required the search for alternative therapeutic regimens, such as R-CHOEP (rituximab, cyclophosphamide, doxorubicin, vincristine, etoposide, and prednisone), R-miniCHOP (adjusted short-term R-CHOP therapy), R-ACVBP (adriamycin-cytoxan-vindesine-bleomycin-prednisone-rituximab), etc., that are strictly codified by several therapeutic protocols, such as the 2015 ESMO guidelines [10].

The choice for any of these regimens should be adapted to the specific clinical status of each patient, as the exact therapy depends on a number of factors, such as the stage of the disease, age, and biological status. However, despite numerous attempts, no clear recommendation has been published thus far.

The association of radiation therapy to chemotherapy offered conflicting results. While some studies showed some benefits for the combined therapy, most of the authors did not find any real advantages, especially in the case of localized disease without bulky mass [28]. There were some suggestions of a combined therapeutic algorithm, involving four R-CHOP cycles followed by either local radiation therapy or two more cycles of R-CHOP, in the case of complete remission. Armitage et al. pleaded for mandatory radiation therapy after six cycles of R-CHOP in the case of bulky disease [29]. More recently MInT (MabThera International Trial) study recommended the same therapeutic algorithm for localized bulky disease [30].

In the case of localized disease, the therapeutic protocol varies with the specific organ involvement. For primary DLBCL of the testis, R-CHOP should be associated with radiotherapy and methotrexate or intrathecal cytarabine, due to the potential risk of CNS involvement. In case of documented CNS involvement before the onset of therapy, then high-dose intravenous and intrathecal methotrexate is advised. Conversely, cytarabine should be used instead of methotrexate for lymphomatous meningitis [31]. R-CEOP can be advised for patients with an altered cardiac function.

In the case of advanced stage DLBCL, the therapeutic options varied greatly since the early 1980s. The third-generation regimens, ProMACE/CytaBOM

(cyclophosphamide, doxorubicin, etoposid, Cytosar, bleomycin, vincristine, methotrexate, prednisone) and M-BACOD (methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, and dexamethasone), showed no benefits for complete remission or disease-free survival when compared to CHOP. However, a lower toxicity for CHOP was observed [32].

The R-ACVBP regimen, consisting of rituximab, doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone, for induction of remission, and consolidation with methotrexate, doxorubicin, and cyclophosphamide, was tested by the GELA (Groupe d'Etude des Lymphomes de l'Adulte) study on 379 patients. This regimen showed some advantages over the R-CHOP-21 but at the price of significantly higher toxicity [33].

Given these circumstances, there is a growing need for new therapeutic alternatives, fitted for the morphologic, immunohistochemical, and genetic profile of each patient selected for individualized treatment protocol.

The role of immunophenotype variability for the therapeutic outcome has long been the cornerstone for DLBCL management strategy, largely because the treatment of lymphomas evolves toward new therapies (immunotherapy, targeted therapy), which is made possible by analyzing the biology and the signaling pathways of this disease [34, 35]. Furthermore, a growing number of biological agents are available, varying from interferon to rituximab or radiolabeled antibodies, but also the more recent acquisitions in targeted therapy, such as ibrutinib, acalabrutinib, and daratumumab [36–38].

6. Conclusions

Despite the numerous advances and better understanding of the clinical, immunophenotypic, and genetic characteristics of DLBCL, but also in face of several breakthroughs in its treatment, the prognosis of this type of NHL has witnessed only modest improvements. The search for new biomarkers and efficient therapeutic agents in the context of future individualized treatment will be crucial in our quest for improved results.

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Conflict of interest

None.

Abbreviations

ABC DLBCL	activated B-cell DLBCL
B2M	beta-2 microglobulin
Bcl-2	B-cell receptor 2
Bcl-6	B-cell receptor 6
CIITA	class II MHC transactivator
c-Myc	avian myelocytomatosis virus oncogene cellular homolog
CNS	central nervous system

DLBCL	diffuse large B-cell lymphomas
FOXP1	forkhead box protein P1
GCB DLBCL	germinal center DLBCL
GCET1	germinal center B cell-expressed transcript 1
IRF4	interferon regulatory factor 4
MUM1	multiple myeloma 1 protein
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	non-Hodgkin lymphoma
PDL 1	programmed death-ligand 1
PET-CT	positron emission tomography-computed tomography
PMBCL	primary mediastinal large B-cell lymphoma
PTL	primary testicular lymphomas
R-ACVBP	adriamycin-cytoxan-vindesine-bleomycin-prednisone-rituximab
R-CHOP	rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone
R-CHOEP	rituximab, cyclophosphamide, doxorubicin, vincristine, etoposide, and prednisone
WHO	World Health Organization

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Section 3

Minimal Residual Disease and Acute Leukemia

Chapter 6

B-Cell Precursors: Immunophenotypic Features in the Detection of Minimal Residual Disease in Acute Leukemia

Olga Chernysheva, Lyudmila Yuryevna Grivtsova, Alexander Popa and Nikolay Nikolayevich Tupitsyn

Abstract

Minimal residual disease (MRD) as a tool to monitor response to therapy is both a criterion for detailed risk stratification and an independent prognostic factor in childhood acute lymphoblastic leukemia (ALL). Immunological assays particularly flow cytometry (FC) are priority methods in MRD monitoring. Multicolor flow cytometry makes it possible to most fully characterize the immunophenotype of tumor B lymphoblasts and reveal leukemia-associated immunophenotypes not only according to the CD58 and CD38 antigens but also as an additional criterion of aberrancy. This allows you to identify and select individual criteria for further monitoring of minimal residual disease for each patient with ALL. The aim of this chapter is to compare immunophenotyping features of normal B-cell precursors and B-lymphoblasts in acute leukemia and to show possibilities of use of a leukemia-associated immunophenotype in monitoring of the MRD.

Keywords: bone marrow, B-cell precursors, acute leukemia, leukemia-associated immunophenotype, flow cytometry, minimal residual disease

1. Introduction

Control of response to chemotherapy is an intrinsic part of current treatment protocols [1–5]. Assessment of response in key points of chemotherapy programs helps both to stratify patients by risk groups more accurately and to avoid serious chronic side effects and patient overtreatment [6–12]. In case of acute leukemia, the number of tumor blasts undetectable in bone marrow (BM) morphologically at different therapy stages or minimal residual disease (MRD) is a criterion for such assessment.

In adult patients, problems of most significant points of immunological detection and the role of MRD levels are a matter of discussion [2]. The MRD significance is demonstrated in full in pediatric oncology. The MRD is of importance both in prognosis of acute lymphoblastic leukemia (ALL) and in prediction of recurrence [13]. Key points for MRD assessment are determined as well as their clinical significance and MRD levels that help in detailed risk stratification of patients [1].

Immunological quantification of MRD cells solves different problems depending on chemotherapy stages. In the middle of induction therapy (day 15), it evaluates primary response [14]. While at the end of induction therapy (day 33), the purpose is final risk stratification of patients with respect to clinical and immunological prognostic factors [1, 14, 15]. MRD assessment at the end of induction consolidation (day 78) identifies a patient group with so called slow response. These patients remain MRD-positive by days 15 and 33 and reach MRD-negativity by day 78 only. This group is characterized by good prognosis [3].

Many international research groups have developed flow cytometry (FC) protocols for MRD diagnosis; however, there is not a common approach yet. The St Jude Children's Research Hospital (Memphis, USA) has proposed a simplified 3-color FC assay to detect MRD cells on day 15 of induction therapy [16] that is based on elimination of normal B-cell precursors (BCP) under the effect of corticosteroids [17] that are a basis for therapy at the given stage. The BFM international study group has developed an MRD monitoring protocol basing on the detection of B-LP with aberrant (leukemia-associated) immunophenotype (LAIP). Antigens CD58 and CD38 are most common markers to characterize the aberrant immunophenotype [18, 19]. However, tumor lymphoblasts show no aberrance by these antigens [20]. There are no clearly identified alternative combinations of aberrant markers.

In ALL from T-cell precursors (T-ALL), there are no clearly cut criteria for MRD assessment [21] and research in this field is ongoing.

The aim of this chapter is to compare immunophenotyping features of normal B-cell precursors and B-lymphoblasts in acute leukemia and to show possibilities of use of a leukemia-associated immunophenotype in monitoring of the MRD.

2. Materials and methods

The study involved 191 ALL cases (160 B-ALL [142—primary diagnosis, 18—diagnosis in relapse] and 31 T-ALL). The diagnosis was made basing on a combination of morphocytochemical and immunophenotyping assays of BM puncture biopsies.

In most cases (88.8% of B-ALL and 87.1% of T-ALL), the immunophenotyping at diagnosis was performed by 3-color FC (at least 20 markers was analyzed). The immunophenotyping using EuroFlow 8-color standardized panels was made in 11.2% of B-ALL and 12.9% of T-ALL cases. Lineage of blasts was determined using ALOT (acute leukemia orientation tube) (**Table 1**).

A more accurate B-ALL (**Table 2**) or T-ALL (**Table 3**) 8-color standardized panel was used depending upon the identified blast lineage. The BCP aberrance was assessed basing on expression of the following antigens: CD58, CD38, CD81, CD9, CD123, CD66c, CD13, CD33, CD20, CD21, and CD24; T-cell precursors (TCP) were characterized with respect to CD99, CD56 expression.

MRD quantification was made in 397 BM specimens from ALL patients at different therapy stages (days 15, 33, and 78, **Table 4**).

No./ fluorochrome	Markers							
	FITC	PE	PerCP-cy5.5	PE-cy7	APC	APC-117	V450	V500
1	cy MPO	cy CD79a	CD34	CD19	CD7	sm CD3	cy CD3	CD45

Table 1.
Acute leukemia orientation tube.

No./ fluorochrome	Markers							
	FITC	**PE**	**PerCP-cy5.5**	**PE-cy7**	**APC**	**APC-117**	**V450**	**V500**
1	CD58	CD66c	CD34	CD19	CD10	CD38	CD20	CD45
2	cyIgM	CD33	CD34	CD19	CD117 + sIgM	sIg-λ	sIg-κ	CD45
3	nuTdT	CD13	CD34	CD19	CD22	CD24	CD9	CD45
4	CD15 + CD65	NG2	CD34	CD19	CD123	CD81	CD21	CD45

Table 2.
EuroFlow consortium 8-color standardized panel for B-ALL diagnosis.

No./fluorochrome	Markers							
	FITC	**PE**	**PerCP-cy5.5**	**PE-cy7**	**APC**	**APC-117**	**V450**	**V500**
1	nuTdT	CD99	CD5	CD10	CD1a	smCD3	cyCD3	CD45
2	CD2	CD117	CD4	CD8	CD7	smCD3	cyCD3	CD45
3	TCRγδ	TCRαβ	CD33	CD56	cyTCRαβ	smCD3	cyCD3	CD45
4	CD44	CD13	HLA-DR	CD45RA	CD123	smCD3	cyCD3	CD45

Table 3.
EuroFlow consortium 8-color standardized panel for T-ALL diagnosis.

ALL immuno-subtype FC color number		MRD monitoring points								
		Day 15			Day 33			Day 78		
		3 col.	**4–6 col.**	**8 col.**	**3 col.**	**4–6 col.**	**8 col.**	**3 col.**	**4–6 col.**	**8 col.**
B-ALL	Total		139			148			54	
	No.	59	68	12	51	79	18	3	38	13
	Percentage	42.4	49.0	8.6	34.4	53.4	12.2	5.6	70.4	24.0
T-ALL	Total		30			26			—	
	No.	9	19	2	6	18	2	—	—	—
	Percentage	30.0	63.3	6.7	23.1	69.2	7.7	—	—	—

Table 4.
The number of analyses at each MRD monitoring point.

At diagnosis, every BM specimen was characterized both morphologically and immunologically. Myelogram count was made by two morphologists (250 cells each) on Giemsa stained BM smears. The following M-types were identified basing on standard morphological criteria for the number of blasts: M1—specimens with ≤5% of blasts, M2—specimens with 5.0–25.0% of blasts, and M3—specimens with ≥25% of blasts. After that, a more detailed analysis was made, and the morphological criteria were compared with immunological findings. Detailed analysis of MRD levels was made in M1, M2, and M3 specimens. Subgroups of specimens within each group were identified with respect to the number of MRD cells. The subgroups were identified according to the BFM protocol for MRD assessment on induction chemotherapy as follows: standard risk <0.1% (including negative cases), medium risk 0.1–10.0%, and high risk ≥10%/the level of 0.01% of tumor cells in a specimen was taken as a standard threshold for MRD negativity.

Statistical analysis was made using IBM-SPSS Statistics v.17 software. Parametric data were analyzed by comparison of means using Student's t-test. Comparison of nonparametric data was made by Pearson's $\chi 2$-test.

3. Results

3.1 General patient characteristics

A total of 186 patients with ALL (five patients were treated in debut and in relapse of the disease) managed at the N.N. Blokhin Cancer Research Center during 2006 through 2017 were entered in the study.

The B-ALL group included 155 patients 1–18 years of age: 78 boys (48.7%) and 82 girls (51.3%) (**Table 5**). In 142 cases (88.8%), the diagnosis was made at the disease onset. In 18 cases (11.2%), the diagnosis was made in recurrence stage. Five patients were followed up both in the debut and recurrences stages.

The T-ALL group consisted of 31 patients aged 2–17 years including 26 boys (83.9%) and 5 girls (16.1%) (**Table 6**).

Treatment was given according to the ALL IC BFM 2002 and 2009 protocols. Data on risk stratification are shown for 79 patients with B-ALL receiving treatment by the ALL IC BFM 2009 protocol (data for 63 patients with B-ALL onset are not available). The majority of patients (67.1%) were classified into an intermediate risk group. Only 5.1% of patients were a high risk group. And 27.8% of patients were assigned to a standard risk group.

71.0% of T-ALL patients received treatment by ALL IC BFM 2009 protocol. The majority of patients (72.7%) were classified into an intermediate risk group. Other patients (27.3%) were assigned to a high risk group.

Characteristic		Number	Percentage
Gender	M	78	48.4
	F	83	51.6
Age	1–18 years (mean 5.9 ± 0.3 years)		
Time of diagnosis	Debut	142	88.8
	Recurrence	18	11.2
Immunosubtype	Pre-pre-B	150	93.8
	Pro-B	10	6.2

Table 5.
Patient characteristics in the B-ALL group.

Characteristic		Number	Percentage
Gender	M	29	83.9
	F	7	16.1
Age	1–17 years (mean 7.3 ± 4.7 years)		
Time of diagnosis	Debut	31	100
	Pre-T-cell	12	38.7
	Cortico-thymocytic	19	61.3

Table 6.
Patient characteristics in the T-ALL group.

MRD monitoring was made on days 15, 33, and 78 of chemotherapy according to the treatment protocols used at the Research Institute of Pediatric Oncology and Hematology of the FBI N.N. Blokhin NMRCO and international recommendations.

3.2 ALL from B-cell precursors

3.2.1 Immunological characterization of blasts at primary diagnosis

Table 7 shows B-lymphoblast immunophenotype characteristics at primary diagnosis. These immunological characteristics were used further in MRD monitoring.

CD58 and CD38 are currently most commonly used antigens as main criteria of tumor B-lymphoblast aberrance. Blasts are considered aberrant if they are characterized by overexpression of CD58 in combination with low or no expression of CD38 (immunophenotype $CD58^{++}CD38^{low/-}$, **Figure 1**).

Analysis for this antigen combination demonstrated that blasts do not always have the $CD58^{++}CD38^{low/-}$ immunophenotype, that is, aberrance by both antigens is found in 54.3% of cases only (**Table 8**). **Figure 2** shows an example of no aberrance by CD58 and CD38 antigens.

The following markers were studied as additional criteria of aberrance: CD123 (**Figure 3**), CD66c, CD81 (**Figure 4**), CD9, CD21, CD24 with their expression presented in **Table 9**. **Figures 3** and **4** show an example of aberrant expression of CD123 and CD81 in B-lymphoblasts at primary diagnosis.

3.2.2 Immunophenotypic characterization of blasts at relapse

A total of 18 patients were examined at relapse of the disease. Five patients were examined at both disease onset and relapse. Blast characterization was made using a

Antigen	No. of cases analyzed	Frequency of Ag-positive cases (%)
CD10	142	93.0
CD34	139	74.1
nuTdT	24	100.0
smCD22	137	50.3
CD20	129	14.7
Pan-myeloid (CD13, CD33)	137	46.0
$CD58^{++}CD38^{low/-}$	50	54.0
$CD38^{+/++}$	93	54.8
Additional criteria of aberrance		
CD123	8	100.0
CD66c	8	75.0
CD81	7	14.3
CD9	8	87.5
CD21	7	0.0
CD24	7	100.0

Table 7.
Tumor blast immunophenotype characteristics at primary diagnosis at disease onset.

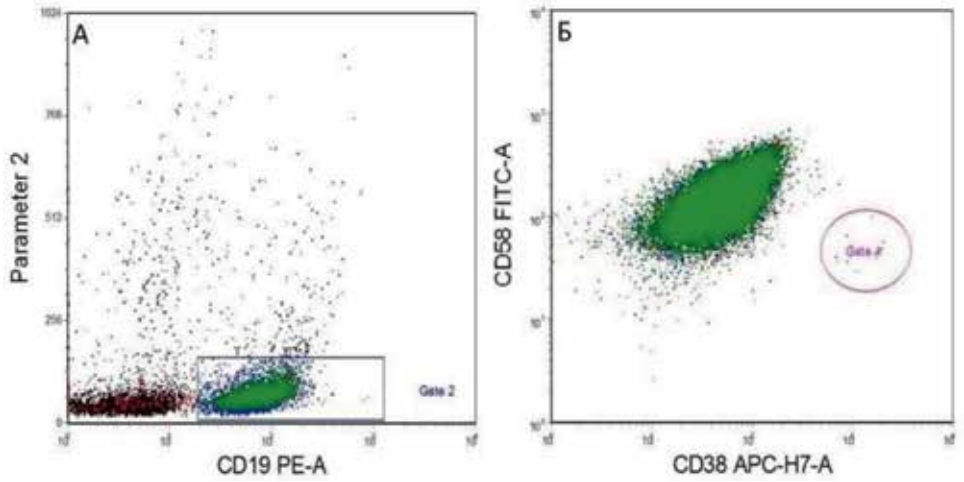

Figure 1.
Blasts with aberrant immunophenotype CD58^{++}/CD38^{low}. Cytogram A: blasts (gate 2, green) demonstrate bright CD19 expression (X-axis) against side scattering parameters (SSC, Y-axis). Cytogram B: B-lymphoblasts demonstrate bright CD58 expression (Y-axis) in combination with low CD38 expression (X-axis) as compared to normal BCP (CD58^{+}CD38^{+}, gate 4).

CD58/CD38 expression	Number	Percentage
CD58^{++}/CD38$^{low/-}$	25	54.3
CD58^{+}/CD38$^{low/-}$	2	4.3
CD58^{++}/CD38^{+}	8	17.4
CD58^{+}/CD38^{+}	11	24.0
Total	46	100.0

Table 8.
Blast immunophenotypes with respect to CD58 and CD38 expression at primary diagnosis.

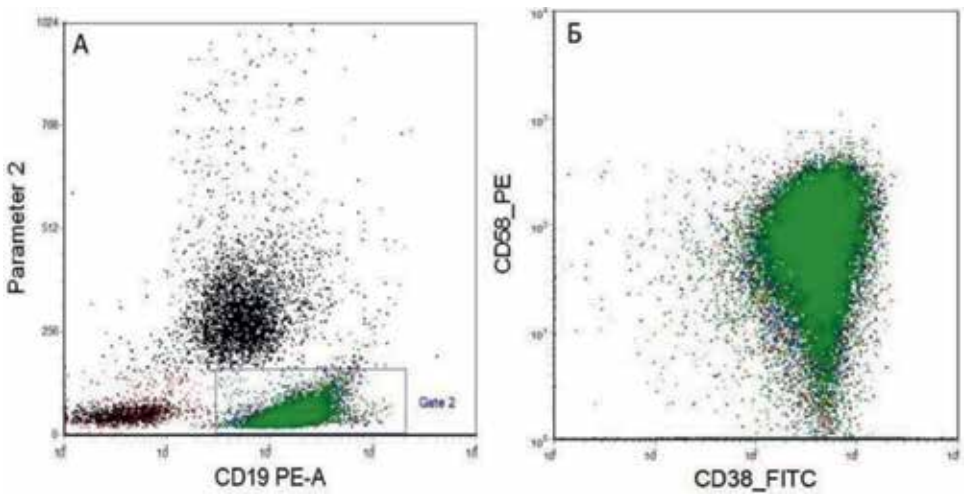

Figure 2.
Tumor B-lymphoblasts with immunophenotype CD58^{+}/CD38^{+}. Cytogram A: tumor B-lymphoblasts are identified basing on CD19 expression (X-axis) and low SSC characteristics (Y-axis). Blasts (CD45^{low}CD19^{+}) are characterized by bright CD38 expression (X-axis) in combination with normal CD58 expression (Y-axis) (cytogram B).

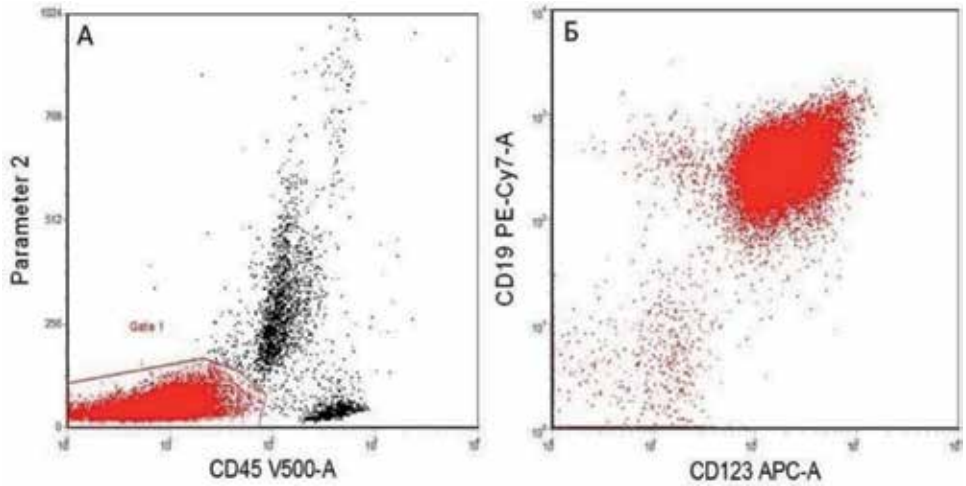

Figure 3.
Tumor B-lymphoblast aberrance by CD123 at primary diagnosis. Cytogram A: blasts (gate 1, red) are detected by low CD45 expression (X-axis) and low SSC characteristics (Y-axis). Cytogram B: tumor lymphoblasts demonstrate bright expression of pan-B-cell antigen CD19 (Y-axis) in combination with bright (aberrant) CD123 expression (X-axis).

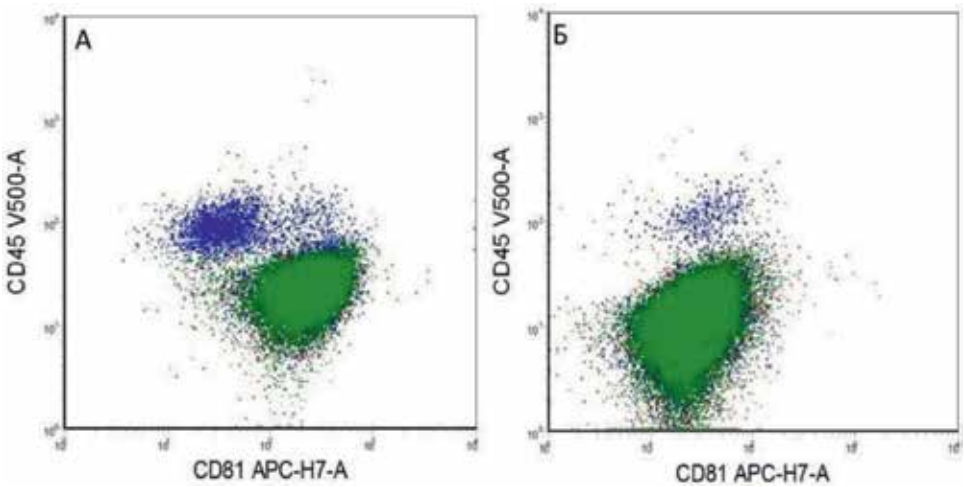

Figure 4.
Normal (A) and aberrant (B) expression of CD81 on B-LP. Cytogram A: normal bright CD81 expression on tumor B-lymphoblasts (green) in X-axis (CD81) against Y-axis (CD45) coordinates. Cytogram B: tumor B-lymphoblasts (green) with aberrant (weak) CD81 expression in X-axis (CD81) against Y-axis (CD45).

3-color FC protocol in 66.7% (12/18) patients. In six patients (33.3%), immunophenotyping used the EuroFlow consortium 8-color protocol.

Expression of main CD58/CD38 aberrance markers at disease recurrence was assessed in 13 patients.

Blast immunophenotype with respect to CD58 and CD38 expression intensity as compared with the primary diagnosis changed in 2 of 5 cases. In the first case, blasts at primary diagnosis were immunophenotypically close to normal BCP as concerns CD58 and CD38 expression ($CD58^{+}CD38^{++}$), while acquiring aberrant CD58 and CD38 expression ($CD58^{++}CD38^{+}$) at relapse. In the second case, on the opposite, blasts lost aberrant CD38 sign and became close to normal non-tumor B-lymphoblasts by this antigen expression.

Antigen	No. of cases studied	Frequency of Ag-positive cases (%)
nuTdT	31	80.6
CD34	31	19.4
CD10	31	38.7
$CD4^+CD8^+$	31	35.5
CD1a	31	54.8
CD5	31	90.3
CD7	31	96.8

Table 9.
Detection frequency of antigens associated with various TCP differentiation stages in T-ALL.

3.2.3 MRD assessment on day 15 of therapy

We compared morphological and immunological findings from BM analysis on day 15 of therapy. In group M1, morphological and FC data were fully similar (no blasts by morphology and complete MRD negativity [MRD cells < 0.01%] by FC) in 11.5% of cases.

Specimens from the M1 group were characterized by a marked percentage of lymphocytes (mean > 60.0%). The presence of MRD-positive specimens (FC) in the M1 group (good morphological response) may be explained by blast mimicry, that is, similarity with lymphocytes. This makes difficult accurate morphological verification of residual leukemic blasts.

In group M1, there was no contradiction between morphological and FC data. Most M3 specimens (85.7%) contained ≥10.0% of MRD-positive cells by FC. The fraction of MRD-positive cells was 5.5% in one case only; that is, in terms of FC, this patient might be referred to the intermediate risk group and did not require therapy intensification.

Comparison of two FC protocols for MRD monitoring on day 15 (3-color St. Jude [28] based on identification of $CD10^+/CD34^+$ BCP among BM mononuclear fraction, and BFM protocol based on identification of BCP with aberrant immunophenotype among nucleated cells [NC]) demonstrated that the mononuclear approach was a more strict criterion for risk stratification of patients.

MRD on day 15 of induction therapy in B-ALL is always represented by BCP. Their identification is based on detection of CD10 and CD34 expression or no expression of pan-leukocyte antigen CD45 and combination of the above mentioned markers in B-cells. Quantification of MRD cells on the basis of each of the progenitor markers demonstrated that CD10 was the most reliable criterion for evaluation of BCP cells on day 15 of chemotherapy in children with pre-pre-B ALL. While in pro-B B-ALL, the count of BCLP should be made basing on nuTdT-positive cyCD22-expressing B-cells.

CD58/CD38 expression was assessed on day 15 of induction chemotherapy in 28 patients. All BCP, that is, MRD cells demonstrated aberrant immunophenotype $CD58^{++}/CD38^{low}$ only in 15 of 28 patients (54%) (**Figure 5**).

$CD58^{++}/CD38^{low/-}$ BCP content was analyzed in patients with no aberrance by this antigen combination at primary diagnosis (n = 11). The proportion of $CD10^+$ B-cells was similar to that of $CD58^{++}/CD38^{low/-}$ BCP in four specimens only. This example is illustrated in **Figure 5**. In the remaining cases, the number of $CD58^{++}/CD38^{low}$ BCP was less than the number of $CD10^+$ B-cells.

The analysis demonstrated a significant predominance of MRD on day 15 of therapy in pro-B ALL as compared to pre-B ($p = 0.016$).

Day 15 of induction chemotherapy is characterized by BM hypocellularity and a marked fraction of debris that can interfere with results (**Figure 6**). To avoid this,

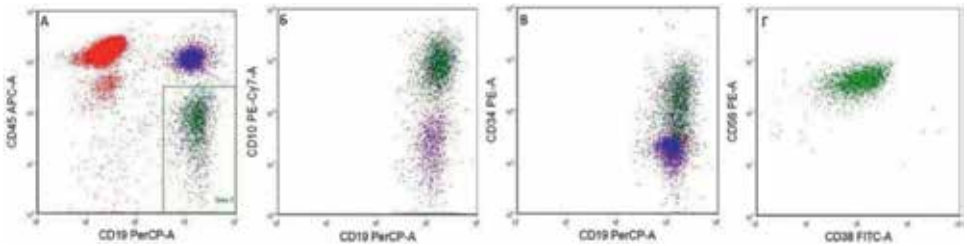

Figure 5.
BCP immunophenotype features on day 15 of induction therapy. Cytogram A: 16.6% CD19^{+}CD45^{low} BCP (gate 3, green) in CD19 (X-axis) against CD45 (Y-axis) coordinates. The cells demonstrate bright CD10 (cytogram B), CD19 (X-axis) expression against CD10 (Y-axis) and marked CD34 expression (cytogram C), CD19 (X-axis) against CD34 (Y-axis). MRD cells show aberrant immunophenotype CD58^{++}CD38^{low} (cytogram D, CD38 [X-axis] against CD58 [Y-axis]).

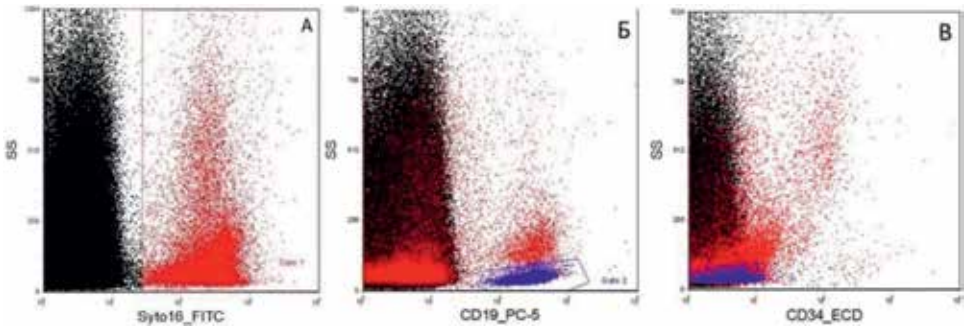

Figure 6.
Day 15 of induction therapy. The specimen has much debris. Cytogram A: NC detection basing on expression of nucleotropic die Syto16 against SSC. The specimen has 3.65% of NC (gate 1, red). Cytogram B: evaluation of B-cell fraction (gate 2, blue): CD19 (X-axis) against SSC. The B-cell fraction without NC is 0.57%, with recount for NC CD19^{+} cells are 15.6% in this specimen. CD34^{+} BCP are 0.0021% without NC, that is, MRD-negativity. Recount with respect to Syto16 results in CD34^{+} BCP being 0.06%, that is, MRD-positivity.

a nucleotropic Syto die (Sito16 and Sito16 in our study) was included in the MRD detection panel.

In the group, as a whole MRD-negative status was found on day 15 of induction chemotherapy in 11.5% of patients, which allowed reduction in anthracycline dose to be considered with respect to clinical prognostic factors without the risk of recurrence in this subgroup.

3.2.4 MRD assessment on day 33 of therapy

MRD quantification was based on count of CD58^{++}CD38$^{low/-}$ BCP. If specimens demonstrated no aberrance by this antigen combination at primary diagnosis, MRD was assessed by CD58^{++}CD10^{++} or CD38^{low}CD10^{++} combinations.

According to the morphological risk stratification criteria, all specimens contained less than 5.0% of blasts (1.2% ± 0.1, n = 80) and were included in the M1 group. There were no blasts in 6.25% of specimens (5/80). MRD-positivity by immunology was identified in 40.0% of cases (32/80). In two patients (2.5%), MRD cells were >1.0%. There were no high risk specimens (MRD ≥ 10.0%) in our study.

CD58 and CD38 primary tumor aberrance on day 33 of induction chemotherapy was assessed in 50.0% of patients. CD58 aberrance was found in 28 of 40 cases (70%), and CD38 aberrance was detected in 60.0% of cases (24/40). 55.0% of cases (22/40) were CD58/CD38 aberrant and 45.0% (18/40) demonstrated no aberrance.

Cases without CD58/CD38 aberrance at the primary diagnosis were examined for markers identifying cells of residual tumor by the literature.

We analyzed CD81, CD123, CD9, CD21, CD24 expression in 21 BM specimens. As a result, most informative marker combinations for detection of aberrant B-lymphoblasts were identified: combinations of CD58 or CD38 with CD10 (aberrant immunophenotypes $CD58^{++}CD10^{+}$ or $CD38^{+}CD10^{++}$) were the most informative in terms of FC. In case of CD20-positive ALL MRD could be detected by asynchronous CD20 and CD34 expression (aberrance characterized by $CD34^{+}CD20^{+}$ phenotype, **Figure 7**). In most cases, residual tumor may be detected by marked expression of CD123 in combination with weak or no expression of CD81 (aberrant immunophenotype $CD123^{++}CD81^{low}$, **Figure 8**). In some cases, CD9 monomorphic tumor expression especially in combination with one of clear-cut

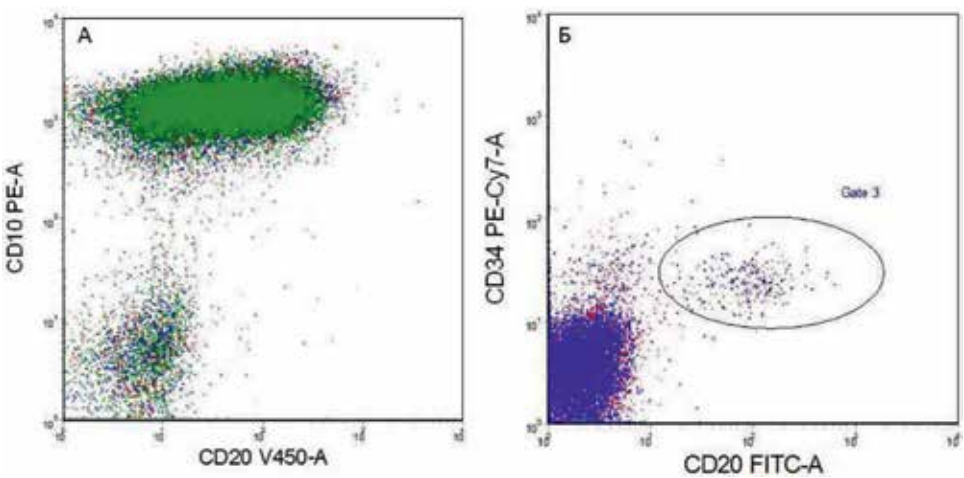

Figure 7.
MRD quantification basing on asynchronous expression of stage-specific Ag CD34/CD20 in a patient with $CD20^{+}$ pre-pre-B B-ALL immunosubtype. Cytogram A: tumor B-lymphoblasts ($CD45^{low}CD19^{+}$) at primary diagnosis (X-axis CD20 against Y-axis CD10), of which 60.0% express CD20. Cytogram B: MRD detection basing on simultaneous CD20 (X-axis) and CD34 (Y-axis) expression. MRD cells are 0.027% of NC specimen, that is, MRD-positivity.

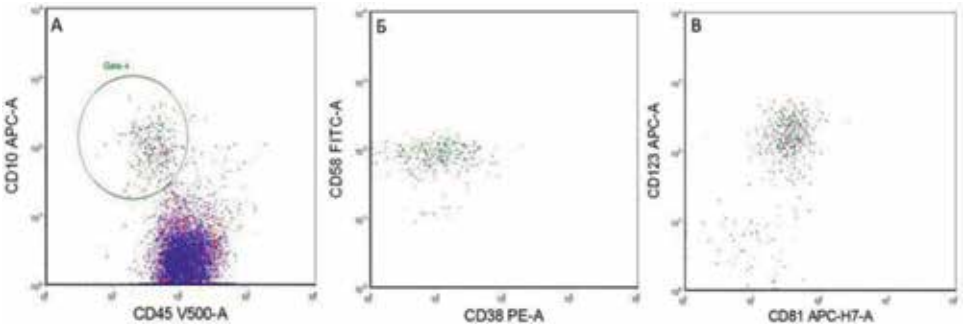

Figure 8.
MRD quantification basing on aberrance by CD58/CD38 and CD123/CD81. Cytogram A: BCP detection basing on bright CD10 (Y-axis) expression in combination with low CD45 expression (X-axis), gate 4 (0.03% of NC) within $CD19^{+}$ B-cells. Cytogram B: BCP are characterized CD58 overexpression (Y-axis) in combination with no CD38 expression (X-axis) and demonstrate $CD123^{++}CD81^{low}$ immunophenotype (cytogram C: CD123 [Y-axis] against CD81 [X-axis]). Therefore, B-lymphoblasts are aberrant by all four Ag analyzed, and there are no normal BCP in this specimen. Aberrant BCP are 0.027% of NC specimen, that is, MRD-positivity.

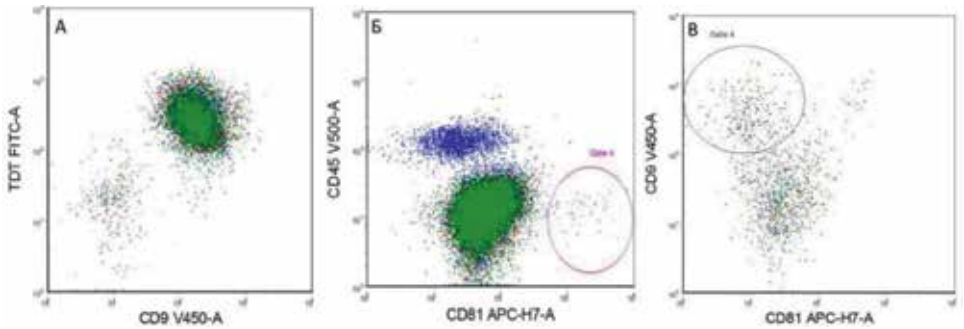

Figure 9.
MRD quantification basing on aberrance by CD9 and CD81. Cytogram A: tumor B-lymphoblasts ($CD45^{low}CD19^{+}$) at primary diagnosis with bright nuTdT expression (Y-axis) in combination with monomorphous CD9 overexpression (X-axis). Cytogram B: tumor cells are aberrant by CD81 (X-axis CD81 against Y-axis CD45). As compared to residual normal BCP (gate 4, pink) with bright CD81 expression, tumor B-lymphoblasts demonstrate low (aberrant) Ag expression. Cytogram C: MRD detection basing on bright monomorphous CD9 overexpression (Y-axis) in combination with low CD81 expression (X-axis) (gate 4, dark blue). They are 0.015% of NC specimen: MRD-positivity.

aberrance criteria ($CD58^{++}$, $CD123^{++}$, $CD38^{low}$, $CD81^{low}$, **Figure 9**) are a sign of tumor aberrance and criterion for MRD assessment.

In cases with monomorphic co-expression of myeloid antigens (CD66c, CD13 or CD33), MRD assessment may be based on these markers.

Of the whole patients, cohort (80 patients) MRD-negative status was determined by FC in 60.0% (48 patients), and 40.0% (32 patients) were found MRD-positive. Comparison of basic morphological and immunological characteristics in these groups identified significant differences in the number of blasts ($p = 0.012$) and $CD19^{+}$ B-cells ($p = 0.044$).

Basing on FC quantification of MRD, most patients (65.0%) receiving treatment by ALL IC BFM 2009 protocol were included in the intermediate risk group.

3.2.5 MRD assessment on day 78 of therapy

MRD assessment on day 78 of therapy was made in 42 patients and was based on detection of BCP with aberrant immunophenotype ($CD58^{++}CD38^{low/-}$).

As recommended by BFM protocols, BM specimens were divided into MRD-positive and MRD-negative subgroups. Most BM specimens were MRD-negative ($n = 27$), and 15 of 42 specimens (35.7%) were MRD-positive.

In 14 patients, MRD status was assessed at three points of treatment protocol (days 15, 33 and 78). Seven of them achieved MRD-negativity by day 78 and met the criterion of slow response to therapy.

3.2.6 MRD assessment after 1 block of anti-relapse therapy (day 35)

After 1 block of anti-recurrence therapy, MRD was detectable in 15 BM specimens. By FC, 40.0% of patients (0.004 ± 0.0008%, $n = 6$) were MRD-negative and 60.0% of patients (7.3 ± 4.8%, $n = 9$) were MRD-positive with 77.0% of the MRD-positive specimens (0.9 ± 0.6%, $n = 7$) containing <5.0% of blasts by morphological study.

3.2.7 MRD monitoring on targeted therapy

In our study, one patient with early recurrence of ALL (pre-pre-B immunosubtype) received anti-recurrence targeted agent blinatumomab (biospecific

anti-CD19 monoclonal antibody). Blinatumomab mechanism of action involves specific binding with $CD19^+$ cells and recruitment of effector T-cells to enhance response. According to the literature data, CD19 is not expressed on cells during blinatumomab therapy which makes difficult BCP identification. We chose an alternative BCP identification procedure to monitor MRD in this patient category: BCP identification and characterization on blinatumomab (anti-CD19) therapy should be based on expression of cyCD22 and nuTdT due to lost of CD19 expression.

3.3 ALL from T-lineage precursors

A total of 31 patients with T-ALL were included in our study. About 61.3% of patients (n = 19) presented with cortico-thymocytic immunosubtype (CD1a expression and/or the presence of $CD4^+CD8^+$ population) as determined by primary immunophenotyping. **Table 9** shows characteristics of blast immunophenotype at the stage of primary diagnosis.

Since T-cell ontogenesis is characterized by the absence of T-cell progenitors in normal BM, MRD monitoring by FC at all therapy stages involves detection and count of TCP.

According to BFM protocol, MRD detection in T-ALL is based on identification of T-cells with aberrant immunophenotype within CD7-positive fraction. However, CD7 demonstrates a broad range of expression and is present not only on TCP (NK-cells and myeloid progenitor cells); therefore, a cytometric protocol for TCP detection in BM (MRD cells) was developed that was based on expression of pan-T-cell antigen CD3 in cytoplasm (cyCD3) as the most stable lineage-specific pan-T-cell antigen. Choice of appropriate antigen clone is an important requirement for detection of cytoplasmic CD3 expression. For instance, SK7 clone is used to detect membrane determinant, while UCHT-1 binds to cytoplasmatic determinant. So, only UCHT-1 monoclonal antibody should be used in analysis of cyCD3.

In our study, quantification of TCP in BM was based on detection of subpopulations $smCD3^-CD7^+$, $smCD3^-TdT^+$, $smCD3^-CD1a^+$, and $CD4^+CD8^+$ within cyCD3-positive BM T-cells at all time points of MRD monitoring.

To simplify recount and exclusion of debris, the quantification was made within NC of specimens as identified by Syto dies. The highest values were used as recommended by MRD detection protocols.

3.3.1 MRD assessment on day 15 of therapy

Quantification of residual blasts on day 15 of induction chemotherapy was made in 30 BM specimens.

The morphological and immunological findings were as a whole similar. The group M2 was heterogeneous demonstrating variability in blast numbers and MRD levels; however, these specimens belonged to intermediate risk group both by M-subtypes and MRD levels and therefore did not interfere in patient stratification.

BM characteristics on day 15 of treatment were analyzed with respect to T-ALL immunosubtype. The pre-T immunosubtype was characterized by significantly higher MRD level on day 15 as compared to cortico-thymocytic one ($p = 0.044$).

About 76.2% of patients composed the intermediate risk group (according to ALL-IC-BFM 2009 protocol), and 23.8% were stratified into the high risk group. There were no patients meeting criteria for standard risk in our study

Basing on MRD level on day 15 of induction chemotherapy, 25.0% were transferred from the intermediate to the high risk group.

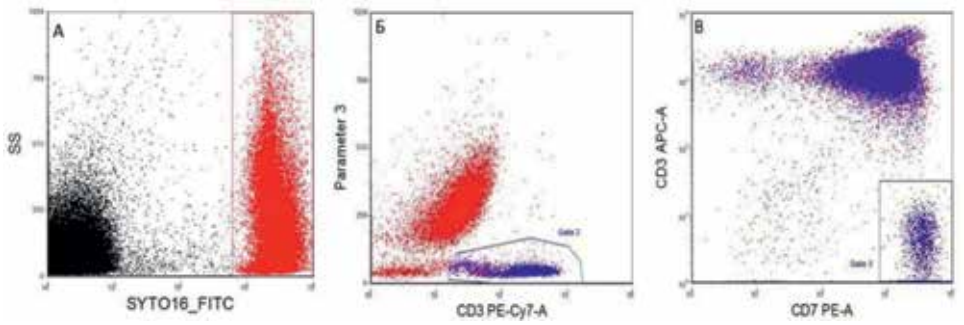

Figure 10.
An example of MRD-positivity with no blasts in the BM specimen by morphology on day 33 of therapy. Cytogram A: shows NC of the specimen (gate 1, red) basing on reactivity with Syto16 (X-axis) against SSC. Cytogram B: cyCD3⁺ T-cells within NC (gate 2, blue) with low SSC parameters (cyCD3, X-axis against SSC Y-axis). Cytogram C: CD7, X-axis against smCD3, Y-axis (gate 2) shows TCP (MRD cells) with CD3⁺CD7⁺ smCD3⁻ immunophenotype that are 2.69% of myelokaryocytes: MRD-positivity.

It is important to note that MRD negativity was not reported on day 15 of induction chemotherapy in any of the patients, that is, none of the patients achieved complete leukemic cytoreduction in the middle of remission induction therapy.

3.3.2 MRD assessment on day 33 of therapy

Quantification of residual blasts on day 33 of induction chemotherapy was made in 26 patients.

Most BM specimens (25/26) were included into group M1, while there was only one specimen meeting the M2 criteria. None of the specimens was included in group M3 basing on morphological characteristics.

We compared morphological and immunological findings. Specimens from the standard and intermediate risk groups were analyzed in detail with respect to MRD content.

All BM specimens from group M1 were MRD-positive. Basing on the immunological criteria, 16.0% of BM specimens (n = 4) were included in the standard risk group (MRD < 0.1%). The intermediate risk group consisted of 20 of 25 BM specimens (80.0%). One BM specimen from this group contained no blast cells by morphology, though FC discovered 2.69% of MRD cells (**Figure 10**).

One specimen was included in the high risk group by MRD assessment (MRD ≥ 10.0%).

Note that specimens from this sample demonstrated rather high lymphocytosis (more than 28.0%).

By morphological criteria, only one BM specimen was included in the M2 group (blasts 10.8%), while by immunological characteristics, this specimen might be referred to the standard risk group (MRD cells 0.04%).

CD7 in combination with CD3 are used to assess MRD in T-ALL in the BFM protocol. However, CD7 is commonly found on myelokaryocytes including both lymphoid and myeloid progenitors. Basing on ontogenetic characteristics of expression of T-lineage antigens, we propose to detect TCP (that is MRD cells) by expression of cytoplasmatic CD3 as the earliest lineage-specific T-cell marker.

Comparison of these two approaches to MRD monitoring (the standard BFM protocol basing on MRD cell count within CD7-positive cells and MRD count within cyCD3⁺ cells), both on days 15 and 33 of induction chemotherapy allowed us to make the conclusion that MRD quantification in T-ALL by detection of cyCD3⁺ smCD3⁻ populations is more accurate.

4. Conclusions and discussion

Successful treatment of patients with acute leukemia is associated with escalation of chemotherapy doses which requires effective monitoring of response. Assessment of response in key points of chemotherapy protocols helps both to stratify patients by risk groups more accurately and to avoid serious long-term side effects or patient overtreatment.

Search for adequate approaches to assessment of response was started since the 1970s. For a long time, response to therapy was assessed basing on the number of blasts in BM specimens as determined by morphological study, and as a result so called M-types were defined as follows: M1: <5.0% of blasts, M2: 5–25% of blasts, and M3: ≥25% of blasts [22].

However, the progress in molecular methods such as polymerase chain reaction (PCR) and flow cytometry (FC) allowed quantification of minimal number of residual blasts at submicroscopic level (<0.01%).

In recent years, quantification of residual tumor cells in BM, that is, minimal residual disease (MRD) was a main criterion of response to chemotherapy. Opposite to myeloid leukemias when molecular genetic methods play the leading role in MRD detection, it is immunological approaches involving FC alone that can detect MRD cells in more than 95.0% of cases [23].

Both PCR and FC have their advantages and disadvantages. Implementation of multiparameter FC able to assess up to 12 parameters of a single cell and development of computer software that allows tens of millions of cells to be examined makes FC close to PCR by sensitivity [24]. FC is currently the most rapid and less costly approach to MRD monitoring.

In adults, problems of the most important detection points are a matter of discussion [2]. The MRD significance is most vivid in pediatrics. MRD is found to play a role both in ALL prognosis and recurrence prediction [1]. Key points of MRD monitoring and their clinical significance have been determined as well as MRD levels that allow detailed risk stratification of patients.

It is in childhood oncology that the MRD effects of the patient faith have been studied [1, 25]. The current protocols defined three mandatory time points such as the middle and end of remission induction chemotherapy (days 15 and 33) and the end of consolidation induction (day 78).

MRD monitoring plays different roles at different chemotherapy stages. The most important point in terms of the patient faith is the middle of induction chemotherapy when primary tumor response and prognosis are determined.

For a long time, there were multiple attempts to make the earliest possible assessment of response. One of the approaches is count of blasts in peripheral blood on day 8 of treatment. Patients with blast reduction below 1000 cells/mcl are a group of good response and good prognosis. However, the blast clearance on day 8 is found to reflect response to steroids only [5], while MRD level on day 15 of induction chemotherapy is the most accurate reflection of leukemic cytoreduction, that is, primary response to treatment [5, 14].

The key moment is the selection of good response patients (MRD < 0.01%) in whom reduction of anthracycline dose may be considered [11, 26]. In our study, FC discovered MRD-negativity in 11.5% of patients in whom reduction of anthracycline dose could be considered. These patients did not develop recurrence.

The situation in BM by day 15 of therapy is unique in terms of immunology. Since BCP are highly sensitive to glucocorticoids [17] that are the basis for treatment at the given stage, these cells are fully eliminated from BM [16]. This observation was a basis for a FC protocol developed by the St Jude Children's Research Hospital (Memphis, USA) study group for detection of MRD cells on day 15. This protocol

involves MRD monitoring within BM mononuclear fraction, while the BFM protocol uses recount for NC in the specimen.

We compared the two approaches to conclude that recount for NC (the St. Jude Hospital protocol) is the most robust criterion for risk stratification of patients. Moreover, analysis of MRD cell immunophenotype with respect to CD58 and CD38 expression demonstrated that the number of $CD10^+$ B-cells was similar to that of $CD58^{++}/CD38^{low/-}$ BCP in 36.4% of cases only. While the number of $CD58^{++}CD38^{low}$ BCP was less as compared to $CD10^+$ B-cells in the remaining specimens of aberrant BCP. We conclude therefore that quantification of MRD should be based on detection of BCP rather than of aberrant blasts.

MRD cells on day 15 are BCP. We used a variety of monoclonal antibodies for their monitoring. In pre-pre-B and pre-B ALL immunosubtypes, it is just enough to count $CD34^+$ and/or $CD10^+$ BCP. In case of pro-B immunosubtype ($CD10^-$ and/or $CD34^-$), MRD detection should involve a combination of nuclear TdT with cytoplasmatic CD22 or CD79α that show stable expression as demonstrated already in early studies of B-cell ontogenesis [27, 28].

Important properties of BM on day 15 are hypocellularity and a marked proportion of debris, that is, destroyed cells under the effect of systemic chemotherapy [29, 30]. This requires modification of FC protocols for MRD monitoring, namely use of special dies able to identify viable, not destroyed cells in specimens. Our study showed reasonable to use Syto nucleotropic dies that can clearly select NC and as a consequence help to avoid debris dilution that may result in a considerable underestimation of MRD count and interfere with risk stratification.

By the end of induction chemotherapy, appearance (regeneration) of normal B-lineage progenitors in BM makes MRD detection more difficult. Clear-cut distinguishing between MRD cells and regenerating BCP is possible basing on LAIP or aberrant immunophenotype.

Aberrance is currently defined as follows:

1. Different levels of Ag expressions (overexpression of some molecules [CD58, CD10, CD9], low or no expression of Ag such as CD38, CD81)

2. Expression of Ag of uncommon differentiation lineages (e.g., co-expression of myeloid Ag such as CD13, CD33, CD66c, CD123 on tumor blasts)

3. Asynchronous expression of lineage-specific Ag (expression of Ag of mature B-cells, e.g., CD20 on $CD34^+$ BCP)

A combination of CD58 and CD38 is the most frequently used Ag combination for assessment of B-lymphoblast immunophenotype aberrance in the AIEOP-BFM and COG FC protocols.

Normal BCP are characterized by $CD58^{+/low}/CD38^{+/++}$ immunophenotype, while aberrant immunophenotypes demonstrate CD58 overexpression and no or low CD38 expression.

As shown by the I-BFM-ALL-MRD Study group, CD58 overexpression is present on overwhelming majority of tumor B-lymphoblasts and is found in 93.5% of cases [31, 32, 19].

CD38 molecule with receptor and enzymatic activities that is involved in B- and T-cell ontogenesis is brightly expressed on normal BM progenitor cells. Low CD38 expression in BCP is considered aberrant [19, 41] and is found in 61.2% of B-ALL according to Min Xia et al.

In our study, CD58 was overexpressed in 71.7% of B-ALL cases, that is, less frequently that by literature data. An explanation may be that the boundary between

Ag normal and overexpression may be unclear. To make a more accurate evaluation of aberrant immunophenotype in primary diagnosis, Ag tumor expression should be compared with the number of residual normal BCP that is not always large. To make visualization more determinant, one has to select a considerable amount of cells (≥1 million), which was not possible in early studies.

There were only 58.6% of ALL with aberrant (low) CD38 expression which was in accord with foreign data.

If one of two Ag is not aberrant, MRD may be assessed basing on alternative combinations such as CD58/CD10, CD58/CD34, CD38/CD10, and CD38/CD34.

Of note, in our study, about one fourth of all cases (24.0%) had tumor B-lymphoblasts with no aberrance by this Ag combination. Therefore, additional criteria to identify aberrant immunophenotype are needed.

This requires MRD criteria to be used at time of diagnosis and most informative MRD markers to be selected in every specific case at the stage of ALL primary diagnosis.

Multicolor FC that simultaneously evaluates up to 12 various cell characteristics makes the most complete characterization of LAIP. This approach may be implemented by use of the EuroFlow consortium 8-color panels with optimal combination of stable markers and fluorochromes [33–35].

The panel for more accurate B-ALL diagnosis includes 3 basic (repeated in all specimens) Ag and 20 specific Ag. This helps to make multiple aberrance evaluation by expression of nonlineage-specific CD45, CD58, CD38, CD9, CD123, and CD81; by expression of noncommon differentiation lineages such as pan-myeloid CD13, CD33, CD66c, CD15, CD65; and by asynchronous expression of Ag of different stages of B-cell differentiation CD10, CD34, CD, CD22, CD20, CD21, CD24. It is of much importance that expression of all above-mentioned markers may be assessed within a single cell population, that is, $CD19^{+}CD34^{+}CD45^{low}$ BCP.

The protocol makes possible indirect prediction of clinically significant abnormalities basing on a proper immunophenotype [36]. For instance, immunophenotypes of cells with mutations BCR-ABL, MLL, Tel-AML-1, and E2APBX1 are described.

When analyzing capacities of EuroFlow 8-color protocols with respect to MRD monitoring, we paid attention to CD81 [35]. The CD81 molecule belongs to the tetraspanin family and is directly associated with CD19, thus forming a signal complex CD19-CD21-CD81 that realizes its functional activity in normal B-cell ontogenesis. By the literature [37], aberrant (weak) CD81 expression is found in 82% of B-ALL cases. Our evaluation of CD81 expression in primary diagnosis coincided with international study data. For instance, aberrant expression was found in 85.7% of primarily diagnosed ALL.

Another molecule from the tetraspanin family is of interest as a criterion for MRD detection, that is, CD9 [38–40]. Its expression is evaluated both in 4- and 6-color FC protocols of the COG study group (M. Borowitz). CD9 is brightly expressed on early BCP, disappears at the pre-B stage, and appears again on mature B-cells. Its monomorphic overexpression on BCP is considered aberrant. In our study, 87.5% of B-ALL cases demonstrated CD9 aberrant expression on tumor B-lymphoblasts.

In case of monomorphous co-expression of myeloid Ag such as CD13, CD33 and CD66c [31, 41] and also CD123 [42], they may be used as LAIP of tumor B-lymphoblasts. Prognostic significance of myeloid Ag co-expression on tumor cells is disputable, though CD66c (KORSA) is shown to be associated with BCR/ABL1 reconstructions [43, 44]. N. Guillaume et al. analyzed immunophenotype of ALL from BCP to discover CD66c co-expression to be the most frequent (40.0%), while pan-myeloid Ag CD13 and CD33 were detected in 15.0% of cases only.

In our evaluation of primary immunophenotype of tumor B-lymphoblasts, CD66c co-expression was found in 75.0% of cases, while CD13 and CD33 were present in 46.0%, which is similar to international study data.

As concerns interleukin-3 receptor α-chain (CD123), all B-ALL were aberrant (i.e., the Ag was expressed on tumor cells), which is in accord with L. Munoz et al. [45].

Asynchronous expression of Ag of noncharacteristic differentiation lineages is an additional criterion for tumor B-lymphocyte LAIP. CD20 is such a marker in CD20-positive B-ALL whose expression is characteristic of mature B-cells only. However, CD20 co-expression on tumor B-lymphoblasts is found in 6.4–15.3% of B-ALL [29, 30]. CD20 is included in most FC panels for MRD diagnosis though it is appropriate rather for more clear-cut identification of $CD20^{-}$ BCP than for evaluation of its aberrant expression. This finding may be explained by a small cohort of B-ALL patients (up to 16.0% of all B-ALL patients) in whom residual blasts may be identified on the basis of $CD34^{+}CD20^{-}$ immunophenotype. In our study, only 14.7% of ALL patients demonstrated CD20 co-expression on tumor B-lymphoblasts.

So in terms of FC approaches to evaluation of small populations which may also include MRD determination of LAIP is most effective using a combination of two Ag with aberrance manifesting itself as overexpression or a combination of molecules with over- and low/no expression. We propose the following Ag combinations: $CD123^{+}CD81^{low}$, and $CD9^{++}CD81^{low}$.

Remission completeness is clinically assessed on day 33 of treatment. In our study, MRD-negative status was detected in 600% of patients.

Most patients (65.0%) receiving treatment by ALL IC BFM 2009 schedule were stratified into the intermediate risk group by MRD content.

Day 78 of treatment (end of consolidation induction) is the third time point with undoubted prognostic value for MRD detection. Basing on this evaluation, a group of so called slow response may be identified basing on MRD status. These patients are MRD-positive on days 15 and 33 and reach MRD negativity by day 78 only. This cohort is characterized by good prognosis.

In our study, 64.3% of patients were MRD negative, with 7 of 14 patients followed-up in all the three points belonging to the slow response group.

Tumor immunophenotyping in disease recurrence is of special interest. Changes in Ag expression during treatment are studied and described rather well. According to international study data, changes as compared to disease onset may occur also in tumor cell immunophenotype in disease recurrence.

We assessed blast immunophenotype both in disease onset and relapse in five patients. Tumor blast immunophenotype was the same by the main diagnostic Ag in disease onset and recurrence in four of the five patients. Of interest is that change in blast phenotype as concerns intensity of CD58/CD38 expression versus the primary diagnosis was found in two of the five patients.

Clinical value of immunophenotype change as concern aberrance markers needs further clarification and may become an additional criterion in the protocol.

Novel targeted therapies that influence disease at molecular level are implemented in clinical practice every year and many are at various stages of clinical trial.

A new stage of progress in targeted therapy is associated with development of biospecific antibodies that are a new class of monoclonal antibodies (MAb) binding with a surface antigen target, on the one hand, and with T-cell receptor, on the other hand, thus recruiting effector T-cells and enhancing tumor response. Blinatumomab (Blinzito) is the first therapy in this class that is approved by FDA for the treatment of Ph-negative refractory/relapse ALL from B-LP [38]. This approval was based on results of phase II clinical trials with 43% of 189 adults achieving complete response and 82% achieving MRD-negativity [46].

Notwithstanding initial treatment results, some patients fail to respond to blinatumomab or develop progressive disease after initial response. Recurrence rate is 30%.

Characterization of blast immunophenotype in recurrence on blinatumomab therapy discovered no CD19 expression on tumor cells. This interferes with BCP identification and requires new methodological approaches to MRD assessment.

Inclusion of blinatumomab into B-ALL therapy requires an alternative MRD evaluation strategy.

There was one patient receiving blinatumomab in our study [47]. The MRD monitoring and immunophenotyping at second recurrence was difficult due to the absence of $CD19^+$ B-cells. An algorithm for BCP detection was proposed and tested that was based on alternative B-lineage differentiation markers. Nuclear nuTdT in combination with cytoplasmatic CD22 as most stably expressed Ag were chosen.

So, given the appearance of new targeted therapies, FC algorithms for both MRD diagnosis and monitoring require certain flexibility and timely rational changes.

There is an equivocal situation with T-ALL. On the one hand, search for aberrant immunophenotype for TCP ended in failure. Expression of main Ag studied with respect to LAIP, that is, CD99 and nuTdT is variable [21]. On the other hand, taking into account normal T-cell ontogenesis, MRD assessment may be based on the absence of TCP of the $cyCD3^{++}smCD3^{-}nuTdT^{+}CD7^{+}$ level in BM. One has therefore just to assess TCP number in order to quantify MRD at any therapy stage.

Correct choice of antibody CD3 clone is of much importance for TCP identification. For instance, clone UCHT1 should be used to detect CD3 cytoplasmatic expression, while CD7 is needed to detect the membrane determinant.

The AIEOP-BFM protocol suggests that MRD detection in T-ALL should be based on CD7.

We compared the two approaches to TCP identification to find optimal TCP detection with $CD7^{+}/^{++}smCD3^{+}$ immunophenotype within $cyCD3^{+}$ population basing on T-cell ontogenesis.

All T-ALL patients in our study were stratified into the intermediate and high risk groups. According to MRD levels on day 15, most patients remained in the initial stratification group though 25.0% were transferred from the intermediate into the high risk group.

Of note that all patients demonstrated MRD-positivity on day 15, that is, none of the T-ALL patients achieved complete leukemic cytoreduction on ALL-IC-BFM 2009 therapy.

So, clinical significance of MRD in ALL arises no doubt. It is reasonable to make MRD quantification immunologically using FC assay.

Since there are no normal BCP on day 15 of induction chemotherapy, MRD quantification in case of pre-pre-B and pre-B ALL immunosubtypes should be based on detection of $CD10^{+}/CD34^{+}$ population within $CD19^{+}$ B-cells. In case of pro-B ($CD10^{-}$ and/or $CD34^{-}$), ALL immunosubtype MRD detection should be based on expression of nuclear nuTdT in combination with cyCD22.

During BM regeneration (end of remission induction therapy and long-term treatment stages), MRD quantification is based on identification of BCP with aberrant immunosubtype. Alongside with the most common CD58/CD38, the following Ag should be used in LAIP assessment: CD81, CD9, CD13, CD33, CD66c, CD123, and CD20. At the stage of diagnosis, the most informative personalized Ag combinations should be selected for further MRD monitoring to make a more accurate risk stratification of patients at different therapy stages and are an effective tool for its modification.

Tumor immunophenotype in disease recurrence should be compared with that in disease onset taking into account its possible changes.

In case of targeted therapy, for example, anti-recurrence treatment, FC protocol should be changed with respect to treatment features. For instance, BCP evaluation

on blinatumomab therapy should be based on expression of nuTdt in combination with cyCD22.

In T-ALL, MRD assessment at any therapy stage may be limited to quantification of $CD7^{+/++}smCD3^{-}$ cells within $cyCD3^{+}$ population.

Conflict of interest

The authors declare no conflict of interests.

Appendixes and nomenclature

Ab	antibody
Ag	antigene
ALOT	acute leukemia orientation tube
ALL	acute lymphoblastic leukemia
ALL-IC-BFM	intercontinental acute lymphoblastic leukemia protocol of Berlin-Frankfurt-Munster study group
B-ALL	ALL from B-cell precursors
BCP	B-cell precursors
BFM	Berlin-Frankfurt-Munster study group
BM	bone marrow
CD	cluster of differentiation
cyCD	cytoplasmic cluster of differentiation
COG	children oncology group
FC	flow cytometry
LAIP	leukemia-associated immunophenotype
MAb	monoclonal antibodies
MRD	minimal residual disease
MNC	mononuclear cells
NC	nucleated cells
smCD	membrane cluster of differentiation
T-ALL	ALL from T-cell precursors
TCP	T-cell precursors

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Section 4

Splenectomy Associated Changes in B-Cell Subsets

Chapter 7

Splenectomy in Gastric Cancer: Influence of B Lymphocytes

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Abstract

The spleen is the largest peripheral organ of the immune system. The standard volume of lymphodissection in stomach cancer during gastrectomy or proximal resection is D2, which implies splenectomy. Immunity disorders in patients after splenectomy primarily affect the B cell immune response. Peripheral blood B-lymphocytes subpopulations have been studied in patients with gastric cancer. Group 1 - patients with gastrectomy, D2 lymphodissection, group 2 - patients with gastrectomy, D2-lymphodissection, splenectomy. Evaluation of the expression of antigens (CD20, CD21, CD23, CD38, HLA-DR, CD71, CD10, CD95, CD25, CD5, CD56, κ- and λ-light was performed in the gate of CD19+ cells. Among peripheral blood lymphocytes the presence of CD19+CD5+ B cells (B1a cells), some of which express the activation antigens CD38 and CD23 is found; a small part of CD5+ B cells is CD25+CD38−. The number of CD23+ cells ranged from 25 to 40% in different patients. A significant number of B cells with a low level of CD21+ expression were detected. In group 2 after surgery, the percentage of cells with CD5+ expression significantly increased, the relative amount of CD19+ lymphocytes, CD19+CD21+ B cells decreased. Given data on B1 and BMZ populations, this can lead to a weakening of both general and antitumor immunity.

Keywords: B lymphocytes, B1 cell, cells of the marginal zone of the spleen, thymus-independent antigens, humoral immunity, stomach cancer, splenectomy, splenomegaly, D2 lymphodissection, IgM

1. Introduction

The spleen is one of the most important peripheral organs of the immune system. The content of lymphocytes in the white pulp of the spleen reaches 85% of the total number of cells. This amount is almost 25% of all body's lymphocytes, almost half of them being B cells. Thus, it is the spleen, along with the lymph nodes, which is the organ providing humoral immunity. In the spleen, a red pulp of 70–80% of the body weight is distinguished and white pulp, which accounts for 6–20% of the mass. The red pulp of the spleen is represented by venous sinuses and pulpal strands. In the red pulp, destruction of red blood cells

and their absorption by macrophages occur. Lymphocytes predominate in the white pulp of the spleen. They accumulate around the arterioles in the form of so-called periarteriolar clutches. B cell follicles are located closer to the edge of the clutch [1–3].

Complex processes of activation of B cells occur in the spleen. Immature B cells come from the bone marrow to the spleen and lymph nodes, where their further maturation, antigen presentation, proliferation, and differentiation occur. Molecules of IgD, CD21, and CD22 appear on their surface.

The process of activation of B cells can be carried out in response to thymus-dependent antigens or without the involvement of a T lymphocyte [4]. T-independent antigens are generally polyvalent lipopolysaccharides, polysaccharides, or proteins. T-independent antigens are divided into type I and type II antigens, which differently ensure the full development of B cells in cells that synthesize antibodies [5–8].

Immature B cells react to T cell-independent antigens of type 1, which elicit a rapid antibody response. Most of mature B cells are within the lymphoid follicles of the spleen and lymph nodes, where they collide and react to T-dependent antigens associated with follicular dendritic cells and proliferate and either differentiate into plasma cells [1].

B cells, specific for autologous antigens, do not enter the follicles; they linger in the outer zone of the periarteriolar lymphoid clutches and die [9]. During the immune response to various antigens, the B lymphocyte-specific immunoglobulin receptor is bound after which the movement of all B cells in the outer zone of the periarteriolar lymphoid clutches has significantly slowed down. Activated B lymphocytes die in the event that there is no interaction with the T cells necessary for the realization of an immune response to thymus-dependent antigens. In the presence of cooperation with T cells, naive B cells enter mainly into follicles, where they undergo differentiation in the germinal centers during primary immune responses.

In secondary immune responses of memory B cells to thymus-dependent antigens, pronounced B cell proliferation and differentiation into plasma cells within the outer zone of periarteriolar lymphoid clutches are observed; follicular B cell proliferation is somewhat weaker than with primary responses [10, 11].

Also in the spleen, there is a special population of cells that delimits white pulp from the red pulp. This area is called the marginal or marginal zone, where both T and B cells are located with a relative predominance of the latter [12]. The population of the B cells of the marginal zone is not homogeneous: it includes naive B cells, as well as B cells of immunological memory, generated both during T-dependent and T-independent antibody responses of the first type [13].

The spleen is the main place for the synthesis of IgM [14–16]. IgM is a polymer in which multiple immunoglobulins are linked together by covalent bonds known as disulfide bonds. IgM-class antibodies are the earliest in immunogenesis and constitute about 6% of all immunoglobulins. The time of their half-life is 5–6.5 days. They are produced by activated B cells at a primary immune response in peripheral lymphoid organs, which also includes lymph nodes and lymphoid formations of mucous membranes [17].

At the same time, the spleen cells are capable of producing various cytokines. In the experiment, it was shown that splenocytes produce interleukin-2, interferon-gamma, and interleukin-7 during antigen stimulation, which in turn stimulate the proliferation of B cells and the production of immunoglobulins [18].

Surgical treatment of patients with gastric cancer involves the implementation of lymphodissection. The standard volume of lymphodissection for stomach cancer

during gastrectomy or proximal resection is D2, which implies a splenectomy that is performed to completely remove the lymph nodes of the spleen gates. However, the splenectomy leads to an increase in the frequency of postoperative complications and mortality, as confirmed by European randomized trials [19–23].

The spleen is the largest peripheral organ of the immune system. Immunity disorders can be more pronounced and prolonged after splenectomy. Clinical observations indicate that the improvement of health and clinical and laboratory parameters after splenectomy is in some cases replaced by the development state of immunodeficiency. Currently, this is confirmed experimentally and is referred to in the foreign literature as overwhelming postsplenectomy infections (OPSI) [24–26]. An analogue of the name "overwhelming postsplenectomy infections" is the term "postsplenectomy hypersplenism"; the signs of which are decrease in general tone and performance and susceptibility to viral, bacterial, parasitic, and fungal infections [26, 27].

Splenectomy for the purpose of adequate lymphodissection in stomach cancer causes pronounced and long-term dysfunction of various immunity units: the presentation of macrophages by foreign antigens to T and B lymphocytes is disrupted, the subpopulation of B lymphocytes changes, and the levels of all classes of immunoglobulins (IgG, IgA, IgM) decrease [28–30]. However, immunity disorders in patients after splenectomy primarily affect the B cell immune response, including thymus-independent type 2 antigens, which is provided by the population of B1 lymphocytes [31, 32].

B1 lymphocytes are relatively small group of B cells, found in humans and mice, and are considered to be the most phylogenetically oldest branch of antibody-producing cells. The population of B1 cells was first described in 1983 by Lee Herzenberg (Hayakawa et al.) as a CD5+ population that differs from normal B (B2) cells by phenotype, anatomical localization, self-healing ability, and the production of natural antibodies. It includes two subpopulations: B1a and B1b [32, 33]. B1 lymphocytes develop in the fetal liver from progenitor cells.

The predecessors of B1a lymphocytes in ontogenesis appear before other subpopulations and migrate from the embryonic hematopoietic tissues (fetal liver and omentum) to the abdominal and pleural cavities as early as the embryonic period. B1b lymphocytes also originate from fetal precursors, but their pool in adults can be partially replenished by the bone marrow and migrate to the serous cavities during the embryonic period where they exist throughout the life of the organism [34, 35].

Thus, during life, the B1 lymphocyte pool is maintained by the activity of progenitor cells through their very slow proliferation. B1 cells are localized mainly in serous cavities—the abdominal and pleural. Some B1 cells migrate (through the omentum) to the intestinal mucosa and mesenteric lymph nodes (up to 50% of IgA producers in the lymphoid tissue of the intestinal B1cells). In the lymph nodes of the mouse, they are absent.

B1 cells are characterized by an "activated phenotype," which is manifested in the expression on their surface of costimulatory molecules CD80 and CD86. This property provides the ability of B1 lymphocytes to function as antigen-presenting cells. Subpopulations of B1 lymphocytes are similar, but subpopulation of B1b is characterized by the absence of CD5 expression [36].

The antibodies produced by B1 lymphocytes are almost exclusively IgM. The response of B1 cells is predominantly thymus-independent [37]. IgM plays an important role in the induction of apoptosis of tumor cells [38–40]. Approximately half of the serum IgM is secreted by B1 cells. A small number of B1 lymphocytes, mainly cells secreting antibodies, are detected in the spleen, where they account for up to 5% of the number of B cells.

To study the peculiarities of the B cell link in patients with gastric cancer after splenectomy, the subpopulation composition of B lymphocytes was analyzed. The study included two groups of patients who underwent surgery. The first group of patients received treatment in the volume of gastrectomy with spleen-protective D2 lymphodissection. In the second group, patients underwent gastrectomy with D2 lymphodissection and splenectomy. The study of subpopulations of B lymphocytes was carried out at the preoperative stage and 3 months after the surgical treatment.

2. Materials and methods

We studied the subpopulation composition of B lymphocytes of peripheral blood in patients with gastric cancer. The possible influence of splenectomy on the subpopulation composition of B lymphocytes was investigated. The B cell link of peripheral blood lymphocytes in patients with gastric cancer was studied in dynamics (3 months after the operation).

To assess the effect of the splenectomy on the B cell link, the subpopulation composition of B cells of peripheral blood after surgery was assessed both in patients after gastrectomy with standard D2 lymphodissection (splenectomy) (14 people) and in patients after gastrectomy with spleen-protective D2 lymphodissection (12 people).

The reaction was taken into account on flow cytometers (FACScan, Lysys II and FACSCanto II, FACSDiva program). Data processing: WinMDI 2.8 and FCS 3.0 applications. Evaluation of the expression of membrane antigens was carried out in the gate of CD19+ B cells. Cells were stained at the same time with three monoclonal antibodies labeled with different fluorochromes. In 42 peripheral blood samples before the operation and 23 samples after the operation on the hematological analyzer Micros 60, the hemogram was calculated, which allowed to estimate the absolute number of B cells.

The expression of the following antigens is analyzed: CD20, CD21, CD23, CD38, HLA-DR, CD71, CD10, CD95, CD25, CD5, CD56, IgG-λ, and IgG-κ light chain immunoglobulins (**Table 1**). Direct conjugates of monoclonal antibodies with fluorochromes were used: FITC, fluorescein; PE, phycoerythrin; PerCP, peridinin

Antigen	Antibody clone	Antibody isotype	Manufacturer	Fluorochrome
CD19	4G7, HIB19	IgG1-κ	BD Biosciences	PerCP
CD20	2H7	IgG1-κ	BD Biosciences	PE
CD23	LT23	IgG1-κ	Sorbent, Russia	FITC
CD5	L17F12	IgG2a-κ	BD Biosciences	PE
CD21	BL13	IgG1-κ	BD Biosciences	FITC
CD10	HL10a	IgG2a-κ	BD Biosciences	PE
CD71	L01.1	IgG2a-κ	BD Biosciences	FITC
CD95	DX2	IgG1-κ	BD Biosciences	PE
CD25	2A3	IgG1-κ	BD Biosciences	FITC
CD56	NCAM 16.2	IgG2a-κ	BD Biosciences	PE
CD3	SK7	IgG1-κ	BD Biosciences	FITC
IgG-λ/IgG-κ	—	—	BD Biosciences	PE/FITC

Table 1.
Monoclonal antibodies and antigens.

chlorophyll protein; and PE-Cy5, double (tandem) dye-combining phycoerythrin with cyanine 5. The expression of the above antigens was detected by monoclonal antibodies directly labeled with various fluorochromes.

3. The effect of the splenectomy on humoral immunity in patients with gastric cancer

An analysis of the B cell subpopulation was performed in the gate of CD19+ cells. At the first stage of the analysis, the number of CD19+ B cells within the lymphocytes was cytometrically estimated (**Figure 1a**). Further, within the limits of only B cells [gate CD19+, **Figure 1b**], the expression of two antigens associated with B cells was analyzed. In these samples, there is an estimate of CD56 antigens concurrently with CD21 (**Figure 1c**).

The main indices of peripheral blood samples before and after surgery are given in **Table 2**. In general, in the group before the surgical intervention in comparison with the physiological norm, the average indices of both the relative quantity and the absolute content of B cells fit within the limits of the norm.

In 33% of patients with gastric cancer at the preoperative period, a decrease in the relative number of B cells (less than 5%) were detected. Moreover in 38% of patients decrease in the absolute number were revealed. Three months after the operation, in 52% of cases, the relative number of B cells was reduced, in 31% of the absolute content of B cells.

The average relative number of B cells, as well as their mean absolute content in 1 μl of peripheral blood, was slightly elevated 3 months after surgery in comparison with these parameters in preoperative assessment. The average percentage of B lymphocytes after the operation was slightly lower than before surgery. However, these differences were statistically unreliable (T-test when comparing two independent variables). This can be explained by a significant spread of the analyzed indicators both in patients before and after surgery.

3.1 B2 cells

Investigation of subpopulations of peripheral blood B lymphocytes in patients with gastric cancer at the preoperative stage established that all B cells had immunophenotype of naïve mature B2 cells: CD19+ CD20+ HLA-DR+ CD10-CD21low/+. The data of subpopulation composition of B lymphocytes of peripheral blood of gastric cancer patients are given in **Table 3**. B2 cells have a long life, localized mainly

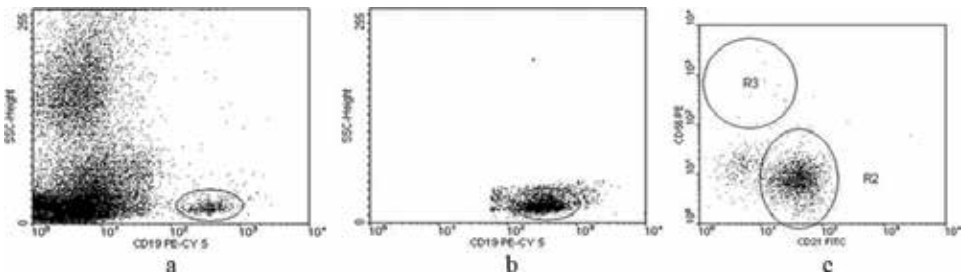

Figure 1.
(a) Estimating the total number of CD19+ B cells within the lymphocytes. The abscissa is the expression of pan-B cell antigen CD19+; along the ordinate axis—the parameter of lateral light scattering of the laser beam, reflecting the working cytometric concept—the granularity of the cells. (b) Cells that fall only in the region of CD19+, i.e., B cells accumulated during sample collection. (c) Evaluation of the expression of two antigens associated with B cells in the CD19+CD56 and CD21.

	Before operation		After the operation	
	Average	**Median**	**Average**	**Median**
Leucocytes ($\times 10^3/\mu L$)	6.3 ± 0.34 n = 43	5.9	6.76 ± 0.49 n = 24	6.4
Lymphocytes (%)	30.1 ± 1.8 n = 43	30.0	36.9 ± 3.06 n = 24	35.5
B lymphocytes (CD19+) %	6.44 ± 0.51 n = 48	5.5	6.0 ± 0.57 n = 28	4.8
B leucocytes (CD19+) %	2.12 ± 0.2 n = 42	2.04	2.5 ± 0.4 n = 22	1.86
B cells (CD19+)/μL	122.5 ± 10.8 n = 42	110.0	156.4 ± 21.5 n = 22	131.0

Table 2.
Parameters of peripheral blood of the general group of patients.

in the spleen, bone marrow, lymph nodes, Peyer's patches, and individual follicles of the lymphoid tissue of the intestine. The histological unit, which is the site of the concentration of B2 cells, is the lymphoid follicle. These cells constitute the vast majority of circulating B lymphocytes. B2 cells undergo selection in the bone marrow and participate in the formation of an adaptive humoral immune response to thymus-dependent antigens.

Some patients noted the presence of transient T2B and T3B cells. They are characterized by pronounced expression of CD23 and CD21. The number of CD23+ cells ranged from 25 to 40% in different patients. In this case, as a rule, CD23+ B cells had a weaker expression of the antigen of mature B cells of CD20. **Figure 2a** shows samples of peripheral blood with a pronounced proportion of CD23+ B cells. In 40% of cases, CD23 coexpression was more than 25%, and in 22.4% of patients, the number of CD23+ B cells exceeded 40% of B cells. Some patients have an insignificant number of CD23+ B cells (6%) (**Figure 2b**).

Transient (immature) B cells were first characterized in mice [41]. Immature B cells migrate from the bone marrow to the spleen. Throughout their migration to the spleen and after spleen entry, they are considered T1 B cells; within the spleen, T1 B cells transition to T2 B cells. Under normal conditions, CD19+CD21lowCD23 B cells (T1-transient) pass positive selection in the course of B cell ontogeny: cells receive a signal through the B cell receptor without the participation of any

	M ± m	Median
CD19+CD5+	14.2 ± 1.91	9.8 n = 46
CD19+CD23+	24.5 ± 2.4	20.5 n = 49
CD19+CD20+	97.2 ± 0.4	97.5 n = 48
CD19+CD71+	9.6 ± 1.1	7.7 n = 48
CD19+CD10+	0.48 ± 0.09	0.2 n = 45
CD19+CD38+	19.8 ± 2.9	11.1 n = 45
CD19+HLA-DR+	98.6 ± 0.3	99.1 n = 48
CD19+CD25+	2.1 ± 0.82	0.5 n = 40
CD19+CD95+	9.6 ± 1.96	7.8 n = 31
CD19+CD21+	80.6 ± 1.43	82.1 n = 43
CD19+CD56+ (IgG-κ+/IgG-λ+)	2.7 ± 2.0	0.15 n = 40, 1.5–2.0 n = 29, 1.0–1.3 n = 12

Table 3.
Subpopulations of B lymphocytes of peripheral blood in patients' stomach cancer before surgery.

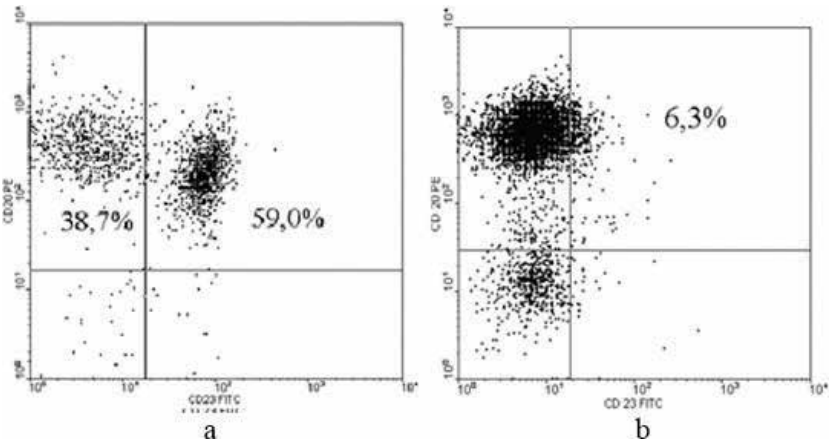

Figure 2.
Expression of CD23 antigen on peripheral B cells in cancer patients'stomach. (a) The population of CD20+CD23+ is the majority (59%) of B cells and represents as a separate discrete population. (b) Insignificant number of CD23+ B cells.

antigens, which ensures their survival. This process can occur both in the bone marrow and in the spleen [42, 43]. In the evaluation of subpopulations of peripheral blood B cells in patients with gastric cancer before surgery treatment, a significant number of B cells with a low level of CD21+ expression were detected [area R2, **Figures 3** and **4**]. This phenotype corresponds to the T1 transient stage of B cell development. Expression of CD21 antigen was characteristic of a larger number of B cells and averaged 82%.

The selection of transient T2B and T3B cells, characterized by the expressed expression of CD23 and CD21 antigens, passes in the peripheral lymphoid organs (the spleen, lymph nodes) high levels of IgM, IgD, and CD23 and lower levels of CD21. The expressed expression of CD23 antigen is intrinsic to the B cells of the embryonic center of the follicle. The follicular B cells that make up the majority and B cells of the marginal zone are lining outside the marginal sinus and bordering the red pulp. Follicular B cells also express high levels of IgM, IgD, and CD23 and lower levels of CD21. These cells are absent in CD1 or CD5, which distinguishes them from B1 cells and B cells of the marginal zone.

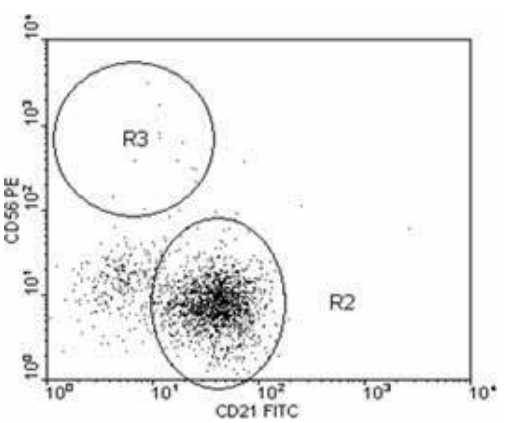

Figure 3.
The population of CD19+CD21low B cells (region R2).

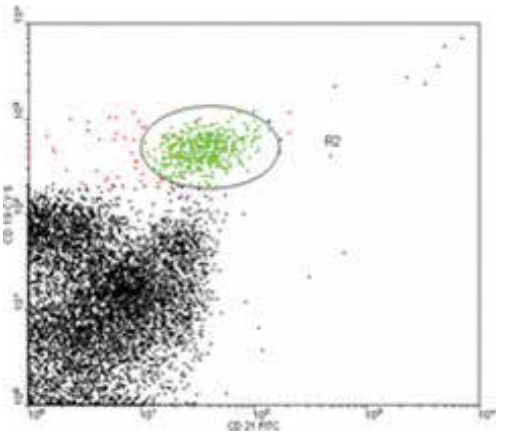

Figure 4.
Expression of CD21 antigen on B cells of blood of patient with stomach cancer. The abscissa axis is the expression of CD21; on the ordinate axis—CD19 expression, in the figure all cells of the sample, including granulocytes. Area R2, CD19+CD21+ cells, this region is located above the granulocytes, which indicates a weak expression of CD21 B cells.

Thus, in patients with gastric cancer, a violation of the immunological repertoire of B2 cells was established: the presence of a pronounced proportion of CD21+ B cells with weak expression, a significant number of CD23+ cells, and cases of clonal B cells. In most of the studied samples, B cells were polyclonal with a predominance of Ig-κ (**Figure 5**). When the thymus-dependent pathway of the immune response is realized, these patients will have a disruption in the synthesis of antibodies.

It is known that a B cell can be activated without the involvement of a T lymphocyte, which leads to the full development of B lymphocytes to antibody-producing forms. It is a characteristic that in this case the immunoglobulin M is synthesized predominantly [5–7]. The process of activation of B cells without T lymphocyte is provided by thymus-independent antigens of type I and II [4]. They are lipopolysaccharides, polysaccharides, and proteins.

Antibodies of IgM class are the earliest in immunogenesis and make up about 6% of all immunoglobulins; their half-life is 5–6.5 days. Approximately half of the

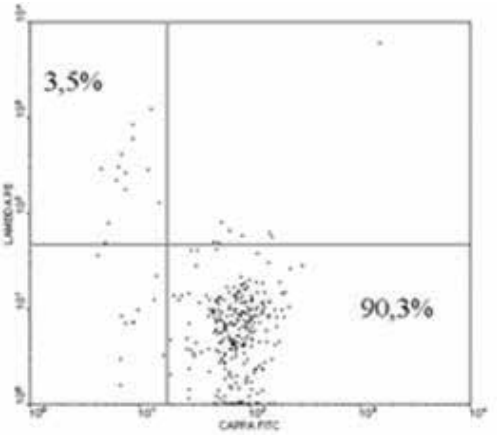

Figure 5.
Polyclonality of B cells with predominance of Ig-κ.

serum IgM is secreted by a pool of B1 lymphocytes. Many of the antibodies are polyspecific, i.e., are able to interact with several antigens, including autologous ones. These antibodies have a low affinity for antigens, including autoantigens, and are not capable of causing damage to tissues. B1 cells are constantly circulating between the spleen and the abdominal cavity but do not enter the follicles, since they do not express the chemokine receptor. Therefore, the processes of "improving" the humoral immune response in the form of switching isotypes and increasing the affinity for antigens do not affect or minimize B1 cells.

3.2 B1 cells

The population of B1 cells is the most phylogenetically ancient branch of antibody producing cells found in humans and mice. B1 lymphocytes develop in the liver of the fetus from progenitor cells. The B1 precursor, which differs from the B-line progenitor that develops primarily in the B2 population, is identified in the murine bone marrow to a lesser degree in the adult bone marrow [34]. B1 cells are considered as key players of the early humoral response against pathogens and considered primary antibody producers in response to T cell-independent type 2 antigens [44]. A specific thymus-independent response is realized by two subpopulations of B1 cells: B1a and B1b.

Both subpopulations of B1 cells are characterized by an "activated phenotype," which is manifested in the expression on their surface of costimulatory molecules CD80 and CD86. This property provides the ability of B1 lymphocytes to function as antigen-presenting cells. B1b cells are phenotypically similar to B1a cells, but they are characterized by the absence of CD5 expression. B1b lymphocytes realize an adaptive immune response, and B1a lymphocytes produce natural antibodies that are specific to microbial agents and opsonize pathogen-mediated innate immunity [45–47].

Such a B1a population of lymphocytes has the phenotype CD19+CD21lowCD23-CD5+IgM++. A number of experimental studies have revealed that immunity disorders after splenectomy primarily affect the B cell immune response, including thymus-independent (TN) type 2 antigens, which is provided by the population of B1a lymphocytes [31, 32].

Among B lymphocytes of peripheral blood of patients with gastric cancer, expressed subpopulation of CD5+ cells was noted. The number of CD19+CD5+ B cells averaged 17.7%. In 23% of patients, CD19+CD5+ lymphocytes were more than 20%, and in three patients, more than 40% of CD19+CD5+ lymphocytes were detected. Normally, the population of CD19+CD5+ B cells is no more than 10% of the total number of peripheral blood B lymphocytes. Among the B cells of the group with a higher CD19+CD5+ B lymphocyte count (more than 15%), the percentage of cells expressing CD25+ and CD21+ antigens was significantly higher; differences in the number of CD38+ B cells were found to be significant, but the range of values for one group of samples with respect to this antigen was higher. The data are presented in **Table 4**.

The presence of CD38 and CD25 antigens on circulating peripheral B cells indicates their activation. It is possible in coexpression of CD23 antigen, which was observed in a group of samples containing more than 25% of B1a lymphocytes.

A high probability of the presence of the CD19+CD23+CD38+CD5+ population (the presence of a reliable correlation relationship for B cells with the expression of CD38 and CD23 antigens, $R = 0.885$, $p = 0.008$) was established. There was also a high probability of the presence of the CD19+CD5+CD25+ population (significant

Population	Group 1	Group 2	Group 1/group 2	*t*-Test	Relevance
CD21	85.3 ± 1.6	79.1 ± 1.9	13/27	2.436	0.02
CD25	3.9 ± 2.0	0.7 ± 0.1	11/26	2.435	0.02
CD38	27.7 ± 6.5	16.1 ± 2.9	16/38	1.858	0.07

Table 4.
Significant populations of B cells in groups of patients with stomach cancer with (CD5+) lymphocytes (group 1 ≥ 15%) and their normal content (group 2 < 15%).

correlation for CD1+CD5+ and CD19+CD25+ B cells, R = 0.879, p = 0.05). However, in most samples (65%), the number of CD19+CD38+ B cells was insignificant—less than 15.0% (**Figure 3b**).

It is interesting to note that the expression levels of the CD5 antigen on B cells proved to be significantly weaker [region R3, (**Figure 5d**)] as compared to the expression of CD5 antigen on peripheral CD3+ T cells.

Thus, in 17.7% of patients with gastric cancer in the peripheral blood, there is a pronounced proportion of a special subpopulation that provides a specific response to thymus-independent type II antigens, which is accompanied primarily by the synthesis of immunoglobulin M: B1a lymphocytes, some of which express the activation antigens CD38 and CD23. It is known that the precursors of these cells migrate early from the hematopoietic tissue to their anatomical niche, into the abdominal and pleural cavities, where autonomy from the central organs of the immune system is maintained by the number of its population. Some B1 cells migrate (through the omentum) to the intestinal mucosa and mesenteric lymph nodes (up to 50% of IgA producers in the lymphoid tissue of the intestinal B1 cells).

3.3 Cells of the marginal zone

Coexpression of CD38 and CD25 can be observed within the B cell population of the marginal zone of the spleen. B cells of the marginal zone (BMZ) originate from a pool of recycled B lymphocytes that have returned to the marginal zone of the spleen. Phenotypically, these cells are more similar to B2 cells than to B1 cells. They come from the same bone marrow precursor cells. Separation of the line of BMZ cells from the general line of B2 cells occurs in the transitional stage of transient cells (T3), when future BMZ cells weaken the expression of non-IgM (like B2 cells) and IgD and lose the CD23 molecule.

These cells have specific morphological features: IgM molecules are expressed on their membrane, but there are no IgD molecules [48]. IgM is expressed more strongly than on B2 cells. For BMZ, high expression of CD21 is characteristic, which allows them to successfully bind T cell-independent type 2 antigens, presented in particular on encapsulated bacteria [49]. The molecules of CD69, CD25, and CD38 in a small amount of CD23 are expressed on BMZ. B lymphocytes with this phenotype are also found in other lymphoid tissues, but only the marginal zone of the spleen accumulates the largest number of these cells in the body. At the BMZ lymphocytes, the chemokine receptor is not expressed, which allows the cells to migrate to the follicles. The cells of the marginal zone are located in the primary blood sine network of the spleen, which allows them to interact with antigens carried by the blood [38, 51]. Information on antigens of BMZ is obtained by "shuttle" migration to lymphoid follicles and back. When answering antigens, the BMZ cells differentiate into short-lived antibody-forming cells. Due to the strong expression of MHC-11 molecules and costimulatory

molecules, BMZ cells have pronounced an ability to interact with T-helper cells. The period of their life is comparable with the life of an organism.

In the spleen, there are complex processes of differentiation, selection of B lymphocytes, and replenishment of the pool of recirculating B lymphocytes and the pool of BMZ and B1 cells. To evaluate the features of the B cell link of immunity in patients with gastric cancer after gastrectomy with splenectomy, an investigation of subpopulations of B lymphocytes in the dynamics before and after the operation was carried out. Immunophenotypic profile of B cells was studied in 14 patients with gastric cancer with spleen-protective D2 lymphodissection and gastrectomy and in 16 patients with gastric cancer after gastrectomy and D2 lymphodissection with splenectomy.

In the group of patients with spleen-protective D2 lymphodissection, a significant correlation between the relative values (the proportion of lymphocytes and leukocytes) and the absolute (cells in 1 μl of blood) in the number of CD19+ B cells was established in a pairwise comparison (T-test for paired variables) ($p = 0.015$, $p = 0.04$, and $p = 0.05$, respectively). The number of cells in the CD19+CD21+ population ($p = 0.034$) also significantly correlated before and after the operation.

In the group of patients who underwent gastrectomy with splenectomy, a significant correlation between the relative number of B lymphocytes ($p = 0.018$) and CD5+ B lymphocytes ($p = 0.012$) and the number of CD19+CD38+ cells was found in a pairwise comparison of mean values before and after surgery ($p = 0.035$).

Three months after surgery in comparison with preoperative parameters in patients with gastric cancer after splenectomy, the percentage of cells with CD5+ expression significantly increased ($t = -6.015$ sig <0.0001, $p = 0.013$), and the relative amount of CD19+ lymphocytes and CD19+CD21+ B cells was decreased (before surgery, their number was 83.6%, and after, 73.9%, $p = 0.08$). The largest number of CD19+CD21+ B cells in the body is accumulated in the marginal zone of the spleen. When comparing the two groups after surgery, a high percentage of CD19+CD5+ B cells was detected (21.7 vs. 14.5%) in the group of patients with splenectomy ($p = 0.049$), which are precursors of functionally more advanced and clonally more diverse true B cells.

4. Discussion

In the spleen, there are complex processes of differentiation, selection of B lymphocytes, and replenishment of the pool of recirculating B lymphocytes and the pool of BMZ and B1 cells.

Coexpression of CD38 and CD25 can be observed within the B cell population of the marginal zone of the spleen. B cells of the marginal zone (BMZ) originate from a pool of recycled B lymphocytes that have returned to the marginal zone of the spleen. Phenotypically, these cells are more similar to B2 cells than to B1 cells. They come from the same bone marrow precursor cells.

These cells have specific morphological features: IgM molecules are expressed on their membrane, but there are no IgD molecules [49]. IgM is expressed more strongly than on B2 cells. For BMZ, high expression of CD21 is characteristic, which allows them to successfully bind TH2 antigens (T cell-independent type 2 antigens), presented in particular on encapsulated bacteria [50, 51]. The molecules of CD69, CD25, and CD38 in a small amount of CD23 are expressed on BMZ. B lymphocytes with this phenotype are also found in other lymphoid tissues, but only the marginal zone of the spleen accumulates the largest number of these cells in the body.

Information on antigens of BMZ is obtained by "shuttle" migration to lymphoid follicles and back. When responding to antigens, the BMZ cells differentiate into short-lived antibody-forming cells. Due to the strong expression of MHC-11 molecules and costimulatory molecules, BMZ cells have a pronounced ability to interact with T-helper cells.

B1 lymphocytes are small group of B cells, found in humans and mice. CD5+ population differs from B2 cells by their phenotype, anatomical localization, self-healing ability, and the production of natural antibodies, includes two subpopulations: B1a and B1b [32, 33]. B1 lymphocytes develop in the fetal liver from progenitor cells.

The predecessors of B1a lymphocytes in ontogenesis appear before other subpopulations and migrate from the embryonic hematopoietic tissues (fetal liver and omentum) to the abdominal and pleural cavities as early as the embryonic period. B1b lymphocytes also originate from fetal precursors, but their pool in adults can be partially replenished by the bone marrow and migrate to the serous cavities during the embryonic period where they exist throughout the life of the organism [34, 35]. Thus, during life, the B1 lymphocyte pool is maintained by the activity of progenitor cells through their very slow proliferation. B1 cells are characterized by an "activated phenotype" which is manifested in the expression on their surface of costimulatory molecules CD80 and CD86. This property provides the ability of B1 lymphocytes to function as antigen-presenting cells. Subpopulations of B1 lymphocytes are similar, but subpopulation of B1b is characterized by the absence of CD5 expression [36].

The data obtained show a disruption in the composition of B cell subpopulations. Most peripheral blood B cells showed weak levels of CD21 (low) antigen expression, marked presence of a pronounced amount of CD2+ B cells, and cases of clonal B cells. This probably reduces the function of antibody formation in the case of the realization of the main path of development of antibody producers in response to thymus-dependent antigens.

The antibodies produced by B1 lymphocytes are almost exclusively IgM. The response of B1 cells is predominantly thymus-independent [37]. IgM plays an important role in the induction of apoptosis of tumor cells [38–40]. Approximately half of the serum IgM is secreted by B1 cells. A small number of B1 lymphocytes, mainly cells secreting antibodies, are detected in the spleen, where they account for up to 5% of the number of B cells.

In the works which were devoted to the study of the function of the spleen and performed on animals, it has been shown that after splenectomy in the serum, the IgM level and the phagocytic activity of neutrophilic granulocytes decreased. However, if the spleen is reimplanted, the concentration of IgM is increased [52].

In a clinical study conducted at our research center, the levels of the main IgG, IgM, and IgA immunoglobulin classes were studied in patients with gastric cancer who underwent a standard operation in the volume of gastrectomy and lymphadenectomy with splenectomy [53].

The level of immunoglobulins was determined by the method of radial immunodiffusion in Mancini with the use of test systems produced by the SPC Medical Immunology (Russia). The level of immunoglobulins A, G, and M in serum in patients without splenectomy, from 14 days after the operation, was slightly elevated and decreased to the initial value by 3 months (**Figure 6a**).

The level of all investigated immunoglobulins in the blood serum in patients with splenectomy before the operation was within the physiological norm. The content of IgA initially and for the entire monitoring period was within physiological values. The authors found that the levels of immunoglobulins G and M in

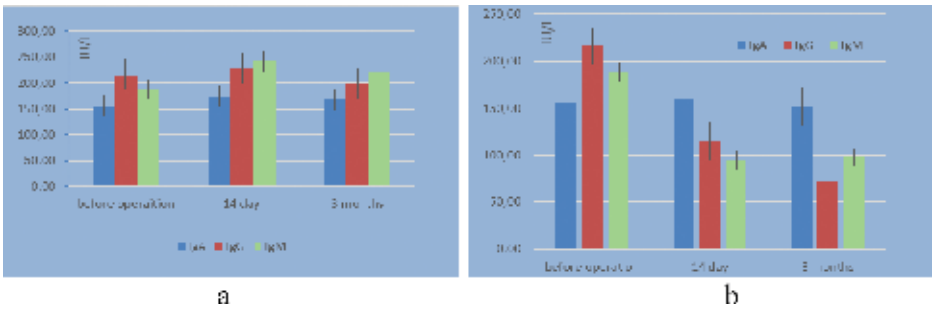

Figure 6.
(a) The level of immunoglobulin IgA, IgG, and IgM in the blood serum in patients in the dynamics in the postoperative period without splenectomy and (b) the level of immunoglobulin IgA, IgG, and IgM in the blood serum in patients in the dynamics in the postoperative period with a splenectomy.

patients with gastric cancer who underwent surgical treatment with splenectomy were reduced almost twofold from the baseline, from the 14th day to the 3rd month of **Figure 6b**.

This fact agrees with the data obtained by our study. Change in the immunological repertoire of B2 cell antigens, weak expression of the CD21 membrane antigen, and a significant amount of CD23+ cells in the case of realization of the main pathway of development of antibody producers in response to thymus-dependent antigens may lead to decrease in antibody production.

Among peripheral blood lymphocytes, the presence of CD19+CD5+ B cells (B1a cells), some of which express the activation antigens CD38 and CD23, is found; a small part of CD5+ B cells is CD25+CD38−. Given the membrane immunophenotype circulating in the peripheral blood of B cells, patients with gastric cancer probably have an alternative TH2 response to pathogens.

In the group of patients after surgical intervention in the volume of gastrectomy with standard D2 lymphodissection and splenectomy, the relative total number of CD19+ B lymphocytes and the number of CD19+CD21+ B cells decreased (the differences are close to reliable) compared to preoperative values. In the group of patients with standard D2 lymphodissection and splenectomy, the percentage of CD5+ B lymphocytes significantly increased from 12.9 to 21.8%, after the operation. Given data on B1 and BMZ populations, this can lead to a weakening of both general and antitumor immunity. Since maintaining the population B1 population is very slow, and the renewal of the BMZ pool is possible only in the spleen.

5. Conclusion

Immunosuppression in patients who underwent surgery (including splenectomy) develops as a result of a disruption in the composition of B cell link. Disorders of the immune response primarily affect the population of B1a lymphocytes, which provides a response to thymus-independent antigens of the second type. Patients in the experimental group may experience decreased production of antibodies, including IgM, which plays an important role in inducing apoptosis of tumor cells.

Conflict of interest

We hereby inform you that there is no conflict of interest.

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Normal and Malignant B-Cell is a collection of harmonious chapters contributed by different authors. This book sets out to describe the B-cell during different stages of ontogeny and the molecular mechanisms of its antigen receptor diversity. It also discusses the main clinical and etiopathogenic aspects when it is transformed into a malignant cell. The book will be interesting and useful for clinicians, biologists, researchers, teachers, and graduate students of both doctoral and master's degrees in the field of immunology.

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