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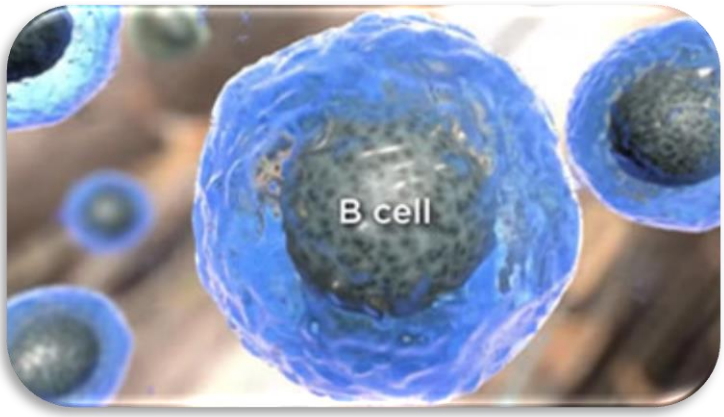
Μελέτη της αλληλεπίδρασης των κλωνοτυπικών ανοσοσφαιρινών με αντιγόνα σε νεοπλάσματα από ώριμα Β λεμφοκύτταρα

Ιωάννης Τυριτίδης



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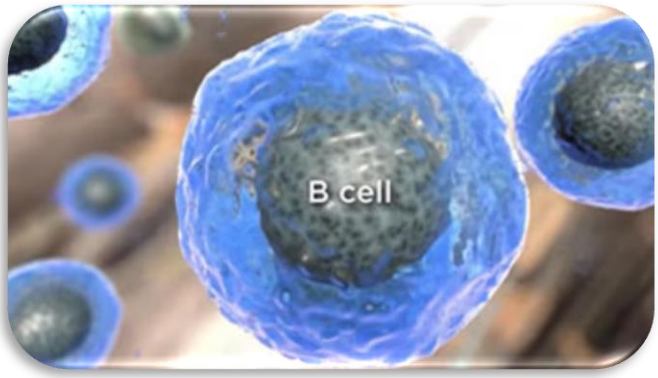
Postgraduate Diploma Thesis

Study of the interactions between the clonotypic immunoglobulin and antigens in mature B cell neoplasms

Ioannis Tyritidis



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Contents

Prologue

The role of B cell Receptor immunoglobulins (BcR IGs) is crucial in the ontogeny and evolution of malignancies of mature B cells. The BcR IG mediates the interaction with the microenvironment, including the recognition of specific antigens that may lead to intracellular signaling activation. However, the nature of the implicated antigens and the consequences of the selection of leukemic B cells by antigens are still elusive. Therefore, the definition of the implicated antigens is vital in order to elucidate the *in vivo* stimulation of the malignant clone.

In the present thesis we used recombinant DNA technology in order to produce as monoclonal antibodies (mAbs) the clonotypic BcR IGs from cases with Chronic Lymphocytic Leukemia (CLL) and Splenic Marginal Zone Lymphoma (SMZL) and subsequently characterize their antigen binding profile.

The present study was conducted at the Institute of Applied Biosciences (INAB) of the Centre for Research and Technology Hellas (CERTH), Thessaloniki, Greece. I would like to thank Dr Kostas Stamatopoulos, Director of the Institute, for giving me the opportunity to conduct my master thesis at INAB, Dr Eleni Papadaki, my thesis supervisor, for her support and mentorship and prof. Charalambos Spilianakis for his evaluation of this project and his advices. Moreover, I am really grateful to the postdoctoral researcher Maria Gounari, my daily supervisor. This thesis would not have been possible without her assistance and guidance.

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Last but not least, I would like to point that I owe everything to my family. With their love and endless support, I managed to complete my studies.

Περίληψη

Η ανάλυση των κλωνοτυπικών ανοσοσφαιρινών του Β κυτταρικού Υποδοχέα (ΒκΥ) έχει αποδειχθεί κρίσιμη για την κατανόηση της μοριακής βάσης των κακοηθειών από ώριμα Β λεμφοκύτταρα. Τα ευρήματα λειτουργικών και ανοσογενετικών μελετών υποστηρίζουν ισχυρά το ρόλο της αντιγονικής διέγερσης στην ανάπτυξη της νόσου. Ωστόσο, παρά τη σχετική πρόοδο, η ακριβής φύση των εμπλεκόμενων αντιγόνων αλλά και οι λειτουργικές συνέπειες της αντιγονικής επιλογής παραμένουν σε μεγάλο βαθμό άγνωστα. Σε αυτό το πλαίσιο, η ταυτοποίηση των αντιγόνων που αναγνωρίζονται από τον λευχαιμικό ΒκΥ είναι ζωτικής σημασίας καθώς μπορεί να παρέχει σημαντικές πληροφορίες σχετικά με την *in vivo* διέγερση των λευχαιμικών κυττάρων.

Στην παρούσα μελέτη εξετάστηκαν 2 τύποι κακοηθειών από ώριμα Β λεμφοκύτταρα: Χρόνια Λεμφοκυτταρική Λευχαιμία (ΧΛΛ) και Σπληνικό Λέμφωμα Οριακής Ζώνης (ΣΛΟΖ). Οι κλωνοτυπικές ανοσοσφαιρίνες του ΒκΥ από 41 ΧΛΛ και 12 ΣΛΟΖ ασθενείς παράχθηκαν ως ανασυνδυασμένα μονοκλωνικά αντισώματα (mAbs) και το πρότυπο της αντιγονικής ειδικότητάς τους εξετάστηκε με δοκιμές ELISA και κυτταρομετρία ροής. Ειδικότερα, με ανοσοανίχνευση τύπου ELISA ελέγχθηκε η αντιδραστικότητά τους ενάντια σε (αυτο)αντιγόνα τα οποία έχουν καθιερωθεί ως κοινοί στόχοι φυσικών και παθολογικών αντισωμάτων: δίκλωνο DNA, ακτίνη, μυοσίνη, θυρεοσφαιρίνη, πρωτεΐνη του β-αμυλοειδούς, καρβονική ανυδράση, F(ab')2 θραύσμα της ανθρώπινης ανοσοσφαιρίνης IgG, απτένιο TNP. Επιπλέον, με κυτταρομετρία ροής ελέγχθηκε η αναγνώριση επιτόπων της επιφάνειας ζωντανών HEK293 κυττάρων.

Ένα ή περισσότερα από τα παραπάνω αντιγόνα αναγνωρίστηκαν από πολλά ΧΛΛ mAbs, ιδίως από mAbs που προέρχονται από ασθενείς με επιθετική κλινική πορεία, αλλά και από ΣΛΟΖ mAbs. Αυτό το πρότυπο αντιγονικής αναγνώρισης προσομοιάζει με το αντίστοιχο των φυσικών και παθολογικών αυτοαντισωμάτων. Ωστόσο, παρατηρήθηκαν διαφορές μεταξύ των διαφόρων υποσυνόλων ασθενών με ΧΛΛ αλλά και μεταξύ ΧΛΛ και ΣΛΟΖ, υποδηλώνοντας ότι τα διακριτά υποσύνολα της ΧΛΛ καθώς και οι δυο διαφορετικές κακοήθειες συνδέονται με ιδιαίτερους τύπους αντιγονικού ερεθίσματος.

Τέλος, μέχρι πρότινος η μεταβλητή περιοχή των ανοσοσφαιρινών θεωρούταν υπεύθυνη για την αντιγονική ειδικότητα. Πρόσφατες μελέτες όμως έχουν αρχίσει να

αναδεικνύουν το ρόλο της σταθερής περιοχής των ανοσοσφαιρινών στην αντιγονική αναγνώριση. Σε αυτό το πλαίσιο, τα ευρήματα της παρούσας μελέτης αναδεικνύουν το ρόλο της εναλλαγής ισοτύπου και κατά συνέπεια της σταθερής περιοχής στην ρύθμιση ειδικότητας/συγγένειας των ανοσοσφαιρινών προς το αντιγόνο και στην ΧΛΛ.

Abstract

Analysis of the clonotypic B cell Receptor (BcR) immunoglobulins (IG) has been critical for understanding the molecular basis of mature B cell malignancies, supporting the notion of antigen selection in lymphomagenesis. However, despite relevant progress, the exact nature of the implicated antigens remains elusive. On these grounds, definition of the antigens recognized by the BcR IGs expressed by the malignant cells is crucial for elucidating the *in vivo* stimulation of the leukemic cells and could shed light on the ontogeny of B cell malignancies.

In the present study we examined 2 types of B cell malignancies: Chronic Lymphocytic Leukemia (CLL) and Splenic Marginal Zone Lymphoma (SMZL). The clonotypic BcR IGs from 41 CLL and 12 SMZL patients were produced as recombinant monoclonal antibodies (mAbs) and their antigenic specificity profiles were assessed by ELISA and flow cytometry. In particular, the reactivity against a variety of autoantigens already shown to be common targets of natural and disease occurring antibodies (dsDNA, actin, myosin, thyroglobulin, β-amyloid, carbonic anhydrase, F(ab')2 fragment of human IgGs, the non-self hapten TNP) was assessed by ELISA. In addition, recognition of epitopes on the surface of viable HEK293T cells was evaluated by flow cytometric analysis.

One or more of the above antigens were recognized by the CLL mAbs derived from patients with aggressive disease, as well as many SMZL mAbs. This pattern of antigenic recognition is reminiscent of that of natural and disease occurring antibodies. However, differences in the reactivity pattern were observed between mAbs derived from CLL patients assigned to distinct disease subsets as well as between the two distinct entities, implying differences in the antigen exposure history and/or in the subsequent immune responses to the cognate (auto)antigen(s).

Finally, until recently the constant region of the immunoglobulins was considered responsible for the antigen fine specificity. However, recent studies highlight the role of the immunoglobulin constant region in the regulation of the antigen recognition. In the current thesis we showed that isotype switching and subsequently the constant region affect the antigenic specificity/affinity of CLL BcR IGs too.

Introduction

1. Immune system

1.1. Structure and function

The immune system is very complex, able to defend the human organism against a massive amount of exogenous and endogenous threats. The immune system can be divided into innate and adaptive. Innate immune response represents the first line of defense, is short-lasting and does not provide immunological memory [1]. On the other hand, the adaptive immune response follows the innate response, presents high specificity for particular pathogens and provides memory. In addition, the two branches of the immune system use different mechanisms and receptors for the recognition and elimination of pathogens. More specifically, leukocytes (such as dendritic cells and macrophages) as well as endothelial and epithelial cells play significant roles in the innate immune responses, whereas T and B lymphocytes are the main cellular components of the adaptive immune responses [2].

Leukocytes derive from heamatopoietic pluripotent stem cells in the bone marrow. These stem cells give rise to myeloid lineage cells such as neutrophils, monocytes, dendritic cells and to lymphoid lineage cells that include T lymphocytes (T cells), B lymphocytes (B cells) and natural killer cells (NK) [3]. Both NK cells, which kill virally infected cells and cancer cells, and lymphocytes are located in the organs of the lymphoid system [4]. The bone marrow and the thymus constitute the primary or central lymphoid organs, while the lymph nodes, blood, mucosa associated lymphoid tissue, tonsils and Peyer's patches are classified as secondary or peripheral lymphoid organs [5].

1.2. Innate immunity

The innate immunity is the first line of defense against a wide range of pathogens and includes many different mechanisms of protection. In particular, anatomic barriers, such as the skin, cilia and the respiratory tract, block the invasion of pathogens, while biochemical barriers such as sweat, tears and gastric acid may trap pathogens. In addition, general innate responses include inflammation, phagocytosis, complement activation and the action of NK cells [1].

Innate immunity depends on a limited repertoire of receptors, whose expression and specificity are genetically regulated. These receptors are known as pattern recognition receptors (PRRs). PRRs bind (i) conserved features of pathogens known as pathogen-associated molecular patterns (PAMPs) or/and (ii) endogenous molecules derived from damaged cells known as damaged-associated molecular patterns (DAMPs) [6][7]. PAMPS include different types of molecules such as unmethylated CpG motifs, ssRNA, dsRNA, bacterial lipopolysaccharides, endotoxins, bacterial flagellin, lipoteichoic acid and peptidoglycan [6]. DAMPs are secreted or exposed by damaged cells under stress or tissue injury. Some examples of DAMPs are heat shock proteins and oxidized low density lipoprotein (OxLDL) [8].

Toll like receptors (TLRs) are a major type of PRRs [7]. TLRs are type I transmembrane proteins and can be divided in two subgroups, according to their localization. More specifically, TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the surface of innate immune cells such as macrophages and dendritic cells and recognize microbial components such as lipids and proteins, while TLR3, TLR7, TLR8, TLR9 and TLR10 are localized into intracellular vesicles and recognize microbial nucleic acids [9].

1.3. Adaptive Immunity

Adaptive immunity is based on T and B cells. This branch of the immune system is characterized by specificity and memory. Specificity refers to the ability to bind a particular chemical structure (the antigen, chapter 4) rather than, or only weakly, similar alternatives. Antigen recognition is achieved by specific receptors on B cells and T cells termed B cell receptor (BcR) and T cell receptor (TcR), respectively. Immunological memory refers to the production of memory lymphocytes, leading to faster and stronger future response against antigens to which the organism was previously exposed [10].

Adaptive immunity is distinguished into humoral and cell-mediated immunity. In humoral immunity, B cells produce antibodies against foreign and extracellular antigens. More specifically, the antibodies contribute to the immune response in three ways: 1) neutralization of pathogens and their toxins by binding to them and, thus, preventing them from entering host cells [11]; 2) opsonization, which enhances the phagocytosis of pathogens, by coating their surface [1]; and, 3) complement activation [11].

Cell-mediated immunity is responsible for the elimination of pathogens which replicate inside the cells, such as viruses. T cells are the main effector cells in this branch of the adaptive immunity. There are two types of T cells: cytotoxic (Tc-$CD8^+$) cells and helper (Th-$CD4^+$) [12].

Adaptive immune responses can be divided into three stages: 1) presentation of antigens by Antigen Presenting Cells (APCs) and their recognition by Th-$CD4^+$ cells, 2) proliferation and differentiation of lymphocytes mainly to effectors cells and to a lesser extent, to memory cells and 3) antigen elimination either from Tc-$CD8^+$ cells or from antibodies produced by B cells.

2. Antigens

2.1. General

Infectious agents contain numerous substances that can elicit immune responses, termed immunogens or antigens. Nevertheless, there is a distinction between these terms: the former refers to substances capable of eliciting humoral and/or cellular immune responses, whereas the latter refers to substances capable of binding to the key players of the immune responses, more particularly antibodies and T cell receptors (TcRs). Therefore, an immunogen can be always considered an antigen, while an antigen may not necessarily be an immunogen [12]. For simplicity, in this thesis, the term antigen will be used interchangeably for both antigens and immunogens.

Antigen receptors on the surface of lymphocytes and secreted antibodies recognize and bind to specific regions of the antigens, termed epitopes or antigenic determinants. Every antigen possesses numerous epitopes, since almost every region of an antigen has configurations that could act as epitopes [11].

Several factors influence the immunogenicity of a given substance. In particular, immunogenicity is determined by properties such as (i) “non-self” property, (ii) molecular weight (the most active immunogens tend to have a high molecular mass), (iii) chemical complexity (molecules with more complex chemical structure are more immunogenic), (iv) antigen dose (above or below a certain dose of antigen, the immune response is not optimal) and (v) route of administration (the subcutaneous route of administration increases immunogenicity, whereas the intravenous or intragastric route causes weaker immune responses).

The vast majority of immunogens are proteins (pure proteins, glycoproteins or lipoproteins). Polysaccharides and lipopolysaccharides are good immunogens too. Nucleic acids are usually poorly immunogenic but their immunogenicity increases in single stranded form or in complexes with proteins. Lipids are also non-immunogenic, unless they are associated with a carrier protein [12].

2.2. Types of antigens based on their recognition by lymphocytes

The humoral immune response, mediated by the recognition of the antigen by the BcR, may require or not the presence of Th-CD4$^+$ cells to stimulate antibody production by B cells. Based on this distinction, the antigens are classified as Thymus Dependent (TD) or Thymus Independent (TI), respectively [11].

TI antigens are further divided into two sub-categories: i) TI-1 antigens such as bacterial lipopolysaccharides (LPS) are completely independent of T cells and in high concentrations may induce polyclonal activation of B cells, and ii) TI-2 antigens (e.g. ficoll), which are not polyclonal B cell activators, necessitate the presence of a small number of T cells [11].

The typical TD antigens are soluble proteins. This is due to the fact that the T cells recognize only linear hydrophobic regions of proteins presented on the surface of APC cells in combination with major histocompatibility complex (MHC) molecules. Thus, in TD immune responses, protein antigens are recognized by both B and T cells and antigen specific B cells receive signals and cytokine stimulation from a Th-CD4$^+$ cell, activated by the same antigen [11].

Finally, superantigens are a special category of antigens capable of stimulating a great number of T cells, followed by massive release of cytokines. They first bind to MHC Class II expressed on APCs and then to the variable region of the TcR β-chain, leading to great release of cytokines and chemokines such as ILs, TNF and IFNs. Pathogenic viruses, mycoplasma, and bacteria can produce superantigens that may activate up to 20 % of the T cell compartment [13].

3. Antibodies

3.1. Structure and function

Antibodies or immunoglobulins (IGs) are Y-shaped molecules and consist of 2 identical heavy chains (50-75 kDa) and 2 identical light chains (25kDa), connected by noncovalent and covalent (disulfide) bonds (Figure 1) [14]. There are two types of light chains: kappa (κ) and lambda (λ). On the other side, there are 5 types of heavy chains: μ, δ, γ, α and ε. Based on the type of heavy chains, the IGs are classified into 5 classes, IgM, IgD, IgG, IgA and IgE, each of which has different properties and function. IgM, IgG and IgA are the most abundant classes of IGs [14]. IgM is the first and major IG class produced during the B cell development. Additionally, IgM is the first IG isotype secreted during an immune response on the first exposure to an antigen. The secreted form of IgM is pentameric, consisting of five 4-chain molecules [11]. IgG is the major class of IG during secondary immune responses and is secreted as a monomer. IgA is found in high amounts in secretions such as milk, tears and gastrointestinal secretion in the form of dimers, but it is also found in the blood as a monomer [11].

Every chain and, consequently, every IG has a variable region (V) at the N-terminus, which is responsible for the specificity of the IG (i.e. the recognition of the antigen) and a constant region (C) at the C-terminus, which defines the biological function of the IG (Figure 1). The C region of γ, α and δ heavy chains consists of three IG domains (CH1, CH2, CH3), whereas μ and ε heavy chains have 4 IG domains in the C region. Each domain consist of two β-pleated sheet structures held together by interactions between conserved cysteines and other charged amino acids. On the contrary, the V region of heavy chains and the V and C regions of the light chains are composed of one such domain [15].

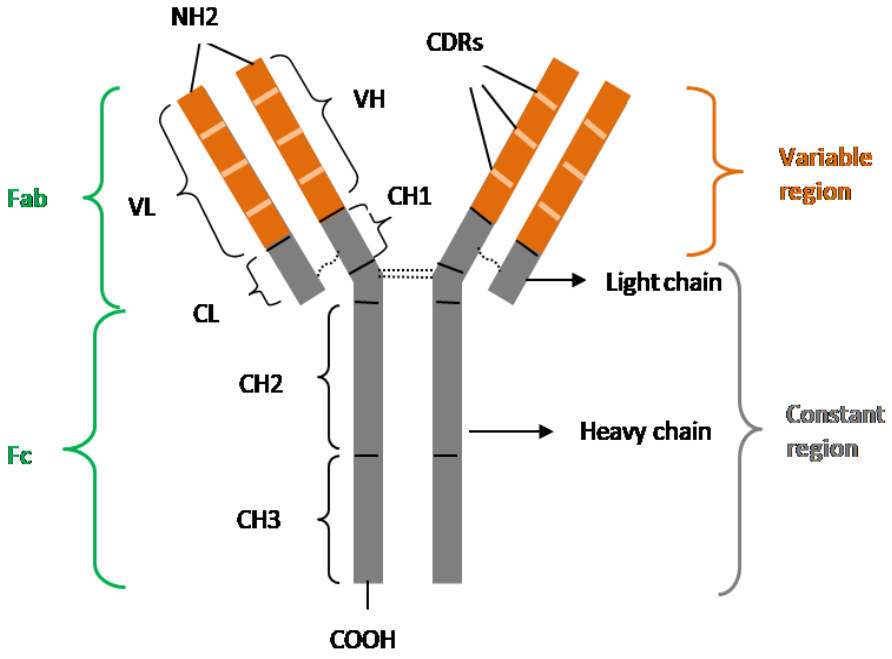

Figure 1. The immunoglobulin structure [14][15]

The V domain of the IG heavy and light chains presents great variability between different antibodies, albeit the variability is not uniformly distributed. In particular, there are parts in the V domain with enhanced diversity, called hypervariable regions (HR) or complementary determining regions (CDRs), due to the fact that these regions are responsible for the formation of the antigen-binding site on the antibodies [16]. There are three CDRs regions in each chain (CDR1, CDR2, CDR3) that are alternated by the 4 more conserved regions termed framework regions (FR1, FR2, FR3 and FR4) [1][17].

3.2. IG genes

The heavy and light chains of IGs are produced by a group of specific IG genes: V (Variable), J (Juctional) and C (Constant) for both heavy and light IG chains, while the IG heavy chain genetic locus also has D (Diversity) genes that are located between the V and the J genes [18]. There is a large number of germline V, D and J genes: 44 functional V, 23 D and 6 J genes for the heavy chain [18], 31-35 functional V, and 5 J genes for the κ light chain [19] and 29-33 functional V and 4 J genes for the λ light chain [20]. One gene from each group is randomly selected for the IG synthesis. Regulatory genes such as the leader sequences upstream of the V genes are also included in the IG loci.

The IG heavy chain genes are located on chromosome 14 [18], the IG κ light chain genes on chromosome 2 [19] and the IG λ light chain genes on chromosome 22 [20].

3.3. Immunoglobulin diversity

The IGs present great variability which underlies their ability to bind to and subsequently protect the human from a great number of threats. This diversity is achieved by specific mechanisms operating during B cell development, namely combinatorial diversification, juctional diversification, somatic hypermutation and class switch recombination.

3.3.1. V(D)J recombination and Combinatorial Diversity

The V, (D) and J genes are assembled randomly to form the variable domain of the IG chains. This processes, termed V(D)J recombination, takes place in the bone marrow, during the B cell differentiation and is directed by DNA sequences, known as Recombination Signal Sequences (RSS). RSS are located at the 3' end of V genes, at the 5' end of J genes, whereas the D genes have RSS at both 5' and 3' ends. RSS consist of conserved heptamer and nonamer elements, separated by a less conserved "spacer" sequence of either 12 or 23 nucleotides; genes of the same group have identical RSS and recombination is possible only between genes bearing RSS with different spacer lengths (the so-called '12/23 rule") [21]. Combinatorial diversity also includes the pairing of one heavy chain with one κ or λ light chain.

At the biochemical level, the V(D)J recombination is distinguished in 2 phases: (i) DNA cleavage, accomplished by the RAG (RAG1, RAG2) proteins, encoded by Recombination Activation Genes (RAGs), associated with the non-lymphoid specific DNA binding factors HMG1A or HMG1B and (ii) joining of the cleaved DNA ends through the Non Homologous End Joining (NHEJ) pathway, carried out by several enzymes such as DNA-PK, proteins Ku, XRCC4 and DNA ligase IV [22][21].

The V(D)J recombination is regulated in three levels:

i) at the transcriptional level, the expression of RAG proteins is limited to the precursors of B and T cells.

ii) a hierarchical order is followed: the genes of the heavy chains are recombined before the genes of the light chains, and κ light chain gene rearrangement precedes that of λ light chain genes.

iii) successful rearrangement of one chromosome prevents the rearrangement on the other allele, a phenomenon known as allelic exclusion [23].

3.3.2. Juctional Diversity

In both heavy and light chains, the CDR1 and CDR2 regions of the V domain are encoded by the V genes, while the CDR3 region is formed by the V(D)J recombination. Juctional diversity is the diversity introduced at the junctions between the V, (D) and J genes as some nucleotides may be removed from the end of recombined genes while other nucleotides may be added randomly [22]. More specifically, during V(D)J recombination, a DNA hairpin is generated at each V, D, or J coding end, that is subsequently cleaved by the protein Artemis. The hairpin opening position may not be at the hairpin tip, resulting in extra bases remaining on one strand, known as palindromic nucleotides, (P nucleotides). In addition, the Terminal deoxynucleotidyl transferase (TdT) adds short GC-rich inserts, called N-nucleotides in the absence of a template, increasing even more the diversity of IGs (Figure 2). Finally, nucleotides at gene junctions can also be deleted by exonucleases before their ligation, contributing further to the juctional diversity [22].

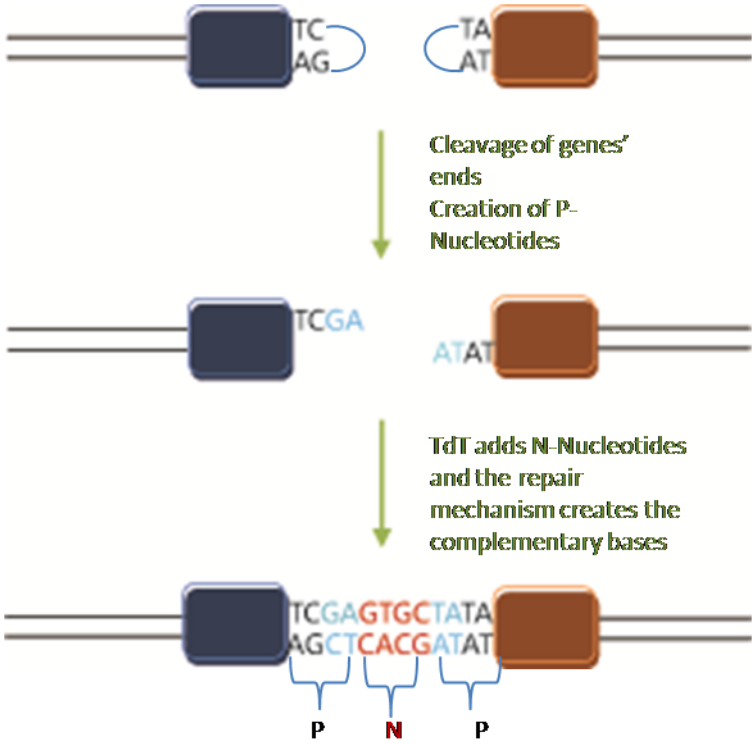

Figure 2. The juctional diversity mechanisms [22]

3.3.3. Somatic Hypermutation (SHM)

SHM occurs after the encounter of antigens by the B cells in specific anatomic sites of the secondary lymphoid organs (germinal center-GC), and aims at producing IGs with higher affinity towards these antigens. SHM introduces point mutations in the rearranged V domains of the heavy and light IG chains [24]. The activation-induced cytidine deaminase (AID) plays a critical role in the SHM mechanism as it creates DNA lesions. Next, error-prone repair mechanisms process these lesions, resulting in the creation of point mutations [25][26] clustering in specific regions of V genes [27]. These hotspots are motifs of three or four nucleotides such as RGYW, WRCY and GNW (R:purine, Y: pyrimidine, W: A or T) [28].

The location of point mutations is very critical. Point mutations in the conserved FR regions may alter the basic IG structure and are often negatively selected. In contrast, point mutations in the CDR domains may lead to the generation of IGs with increased affinity to the antigen and will be positively selected [1].

3.3.4. Class switch recombination (CSR)

Another mechanism that takes place in the GC is CSR whereby the C region of the μ heavy chains that are expressed by naïve mature B cells is replaced by the C region of the γ, ε or α heavy chain. CSR occurs after encountering antigen and leads to the generation of IG molecules with different isotypes (IgG, IgA and IgE) that present the same antigenic specificity but with new effector functions [29]. As in SHM, AID is critical for CSR too, replacing cytidines in S regions (conserved sequences inside the intron downstream of the J_H and upstream of C_H genes except for the Cδ gene) with uracils. These changes trigger the DNA repair mechanisms, which in turn lead to the CSR [30][31].

The CSR is the result of the interaction between CD40 receptor on B cells with CD40 ligand expressed by T cells. This process is also boosted by several cytokines, such as IFNγ, IL-4 and TGFβ [30].

4. B cell development

B cell development is a highly regulated process that allows the generation of fully immunocompetent B cells from hematopoietic stem cells (HSC). The maturation process includes several stages of differentiation, each of which is defined by the IG gene rearrangements and the expression of significant cell surface molecules [32].

The rearrangement of the IG genes starts in the stage of early pro-B cell between the D and J genes of the heavy chain IG locus (IGHD and IGHJ genes, respectively), on both alleles [33]. Then, the rearrangement of IGHV with the IGHD-IGHJ gene complex follows at the stage of late pro-B cell [34], on one allele only, by silencing the other allele, a process known as allelic exclusion [35]. This differentiation stage requires the expression of IL-7, the transcription factors Pax5 and YY1 and the RAG1/RAG2 proteins [34].

Pro-B cells which fail to express successfully the recombined IGHV-IGHD-IGHJ genes are programmed for apoptosis [33]. On the other hand, pro-B cells with functional recombination proceed to the stage of large pre B-cell [36]. In this stage, the heavy chain is detected in the cytoplasm, while the genes for the light chain are not yet rearranged. Large pre-B cells express the pre-BcR, consisting of μ heavy chain paired with the surrogate light chain (SLC) and the co-receptors Igα and Igβ [37][38]. The pre-BcR is crucial for further B cell development. Signaling from the pre-BcR results in protection from apoptosis and the proliferation of the pre-B cell, as well as, the downregulation of RAG protein expression [39]. The maturation process continues with the downregulation of pre-BCR expression and the reactivation of the RAG genes, leading to the recombination of IG light chain genes. The key step for the transition to immature B cell is a productive IG light chain gene rearrangement that leads to the production of a light chain that associates successfully with the μ heavy chain, thus forming a functional IG molecule [40]. The BcR, consisting of the membrane-bound IG (mIG) associated non-covalently with a heterodimer of Iga and Igβ co-receptors, is then expressed on the surface of the immature B cell and mediates both negative and positive selection of B cells. Negative selection eliminates the potentially auto-reactive B cells, whereas the positively selected B cells further mature in peripheral lymphoid organs [33].

In negative selection, the binding of the BcR of immature B cells to autoantigens activates many processes, collectively termed as central tolerance that are essential to avoid auto-reactivity [41][42]. These processes include:

1) clonal deletion: immature B cells, which recognize auto-antigens with high affinity are eliminated through apoptosis

2) anergy: immature B cells that bind to autoantigen(s) with low affinity enter into a state of lethargy and become unresponsive to their cognate antigen; anergic B cells lose their surface IgM and express high levels of surface IgD

3) receptor editing: binding of the BcR to an autoantigen can result in the reactivation of RAG proteins, leading to new recombinations of the light chain genes. The new light chains results into a BcR with different antigen-specificity [43].

B cells with no or low reactivity to autoantigens leave the bone marrow and, after an intermediate stage in their development during which they are called transitional B cells, finally differentiate to mature B cells in secondary lymphoid organs such as the spleen. Transitional B cells are selected to become either Follicular (FO) B cells or Marginal Zone (MZ) B cells.

4.1. Follicular B lymphocytes (FO B cells)

FO B cells are the majority of the B cells in both the lymph nodes and the spleen and are involved in T cell-dependent responses [33]. They are located in the primary follicles, next to the T cell region, so as to have interaction with T cells. After antigen exposure, these cells start to proliferate and then migrate to the center of the follicles, creating the GC [44].

The GC provides the ideal microenvironment for B cell differentiation. GC consists of the light and the dark zone. B cells are first located in the dark zone and are called centroblasts. Centroblasts express low levels of surface IG, proliferate very fast and undergo extensive SHM and CSR. Next, B cells migrate to the light zone, hosting Th-$CD4^+$ cells and APCs, which present the antigens to the B cells [45]. At this stage, the B cells that are now referred to as centrocytes, start to express the mutated IG on their surface and are subjected to selection dependent on the affinity of their surface IG to the cognate antigen. B cells with low affinity for antigens undergo apoptosis, whereas those with high affinity become plasma cells or memory B cells [45].

Plasma cells live only 4 weeks in the bone marrow or the mucosal sites and secrete antibodies independent of the presence of antigen, as they do not express surface IG and MHCII molecules [45]. Memory B cells live longer and do not produce antibodies; they express BcR with high affinity against specific antigens and after antigen exposure, they are quickly transformed to plasma cells, in order to effect fast immune responses [46].

4.2. Marginal Zone B cells (MZ B cells)

MZ B cells are located in the spleen, lymph nodes, tonsils and Payers' patches and are characterized by high levels of IgM, CD21, CD9 and CD1 on their surface. Although MZ B cells represent only the 5% of the B cells in the spleen [47], they have a significant role in primary immune responses against circulated antigens in the blood. The fast response of MZ B cells is due to their lower threshold for activation and differentiation compared to other types of B cells [48].

MZ B cells are mainly involved in T cell-independent immune responses against highly repetitive antigens, such as bacterial polysaccharides [49]. After antigen exposure, MZ B cells differentiate to short-lived plasma cells and secrete mainly IgM antibodies [33]. MZ B cells can also participate in T cell dependent immune responses, by presenting antigens to $CD4^+$ T cells [50]. In addition, MZ B cells may be subjected to CSR and SHM [51].

4.3. B1 cells

B1 cells are $CD5^+$ B cells, which express high levels of surface IgM and low levels of surface IgD. They are found in the peritoneum and mucosal sites of rodents and are considered to be the first line of defense against pathogens, as they secrete antibodies in the absence of antigen stimulation [49], known as natural antibodies. Natural antibodies have the ability to recognize self and foreign antigens such as phospholipids, single stranded DNA, surface glycoproteins [52] and byproducts of the cell metabolism [53]. B1 cells very rarely undergo SHM or CSR.

There is great debate about the existence of B1 cells in humans. Recently, a $CD20^+$ $CD27^+$ $CD43^+$ $CD70^-$ B cell population was identified in humans, which was considered to be the human counterpart of murine B1 cells [54], although this view is still contested.

5. BcR signaling

BcR is a transmembrane multiprotein complex, expressed on the surface of B cells. In particular, the BcR is composed by an antigen binding subunit (surface IG molecule) and a signaling subunit containing an Igα/Igβ (CD79a/CD79b) heterodimer. CD79a and CD79b are subdivided into 3 domains: the extracellular IG-like domain, the transmembrane domain and the cytoplasmic ITAM (Immunoreceptor Tyrosine-based Activation Motif) sequence that is responsible for signal transduction [55].

The BcR IG stimulation by antigen leads to its aggregation and, subsequently, to the phosphorylation of the ITAMS of Igα and Igβ by Src-family kinases such as Fyn, Blk, Hck, Fgr and Lyn. Then, the Syk kinase, which is activated by phosphorylation of Src-family kinases or by autophosphorylation, is recruited to the phosphorylated ITAMs. The activation of Syk is a key event in BcR signaling, initiating the formation of the BcR signalosome which assembles signaling molecules, such as Syk itself, phospholipase-Cγ2 (PLCγ2), PI3K, Bruton's tyrosine kinase (BTK), VAV1 and adaptor molecules, such as B-cell linker (BLNK) [56].

The activation of PLCγ2 and PI3K are crucial signaling events. PLCγ2 generates key second messengers such as increased Ca^{2+}, which in turn, activate several kinases such as IKK and ERK and several transcription factors including Nuclear Factor-κB (NF-κB) and Nuclear Factor of Activated T cells (NFAT) [57]. Activation of the PI3K pathway induces the expression of anti-apoptotic factors and inhibits the expression of pro-apoptotic factors [58].

6. Chronic Lymphocytic Leukemia (CLL)

6.1. General

Chronic Lymphocytic Leukemia is a chronic B cell malignancy, characterized by extensive accumulation of $CD5^+$ B cells in the bone marrow, secondary lymphoid tissues and blood. CLL is the most common adult leukemia in Western countries with an incidence of ~5 new cases per 100.000 individuals annually. CLL is more frequent in elderly people, as the median age of diagnosis is 72 years and is also more frequent in men than women (male to female ratio of ~2:1) [59].

6.2. Diagnosis

Following the revised guidelines from the International Workshop on CLL (iwCLL) [60][61] CLL diagnosis requires the presence of $\geq 5x10^9$ B lymphocytes/L in the blood, sustained for at least 3 months. B cell clonality should be confirmed by flow cytometric demonstration of IG light chain restriction. CLL cells should also co-express CD5, CD19, CD23 along with low levels of surface IG, in the absence or low expression of CD20 and CD79b.

6.3. Staging and prognosis

There are two staging systems that correlate the clinical features with the median survival time. The Rai staging system is used more frequent in United States [62], whereas the Binet staging system is widely used in Europe [63].

CLL is a heterogeneous disease, as some patients follow an indolent clinical course and may even not require treatment, whereas others present an aggressive disease and need treatment early in the disease course [64]. Besides the clinical findings, many molecular/biological factors including the mutational status of IGHV genes, the expression levels of ZAP70 [65], CD38 [66] and CD49d [67] and mutations in specific genes such as *TP53* [68] allow a biologically-grounded risk stratification of CLL patients.

6.4. Mutations

6.4.1. Cytogenetic mutations

Almost 80% of CLL patients carry chromosomal alterations. More than half of CLL patients have a deletion at 13q14.3, which is associated with good prognosis when found in isolation. In this region there are genes, which negatively regulate the apoptosis [69]. del(17p) is present in 7% of CLL cases, resulting in the loss of the *TP53* tumor suppressor gene [70]. del(11q) is another frequent chromosomal alteration, present in 18% of patients, leading to disruption of the cell cycle regulator ATM. Both del(17p) and del(11q), particularly the former, are associated with adverse prognosis [71]. Trisomy 12, which is found in 16% of CLL patients is linked to intermediate prognosis [59].

6.4.2. Somatic mutations

Mutations have been found in many genes in CLL, mainly by next generation sequencing analyses. Some of these genes participate in DNA-damage repair (*TP53, ATM*), mRNA processing (*SF3B1*), NOTCH signaling (*NOTCH1)* and inflammatory pathways (*MYD88*) [72].

6.5. CLL stratification based on the somatic hypermutation (SHM) status of the clonotypic rearranged IGHV genes

The load of the somatic hypermutation (SHM) in the IGHV genes is one of the most accurate molecular markers for CLL patient risk stratification [73][74]. CLL

patients can be divided into two groups, based on the presence or absence of SHMs in the IGHV genes of their clonotypic BcR IG (Figure 3). Patients with clonally rearranged IGHV genes with equal or greater than 98% identity to the germline gene are assigned to the unmutated CLL category (U-CLL) and are generally associated with aggressive disease [75]. On the other side, CLL patients with rearranged IGHV genes, that differ more than 2% from the germline IGHV sequence belong to the mutated CLL category (M-CLL) and generally experience a considerably more indolent disease [76][66].

However, the 2% cut-off is not always biologically relevant in CLL, since even a single mutation could be functionally significant [77]. Besides the SHM status, other molecular features of the clonotypic BcR IG may be important for conal behavior [78].

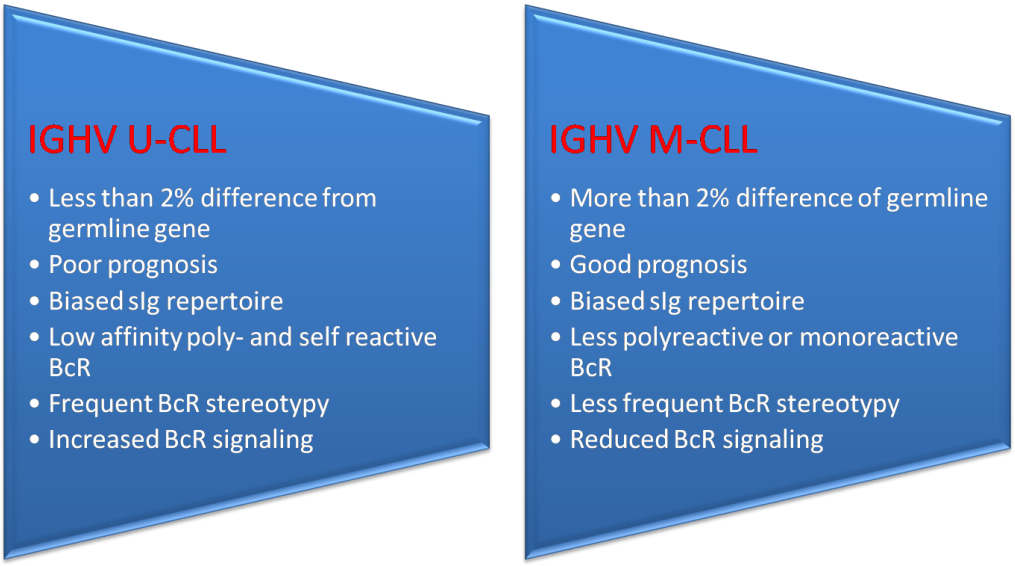

Figure 3. The differences between U- CLL and M-CLL [78].

6.6. IGHV restrictions and BcR stereotypy in CLL

In CLL the IGHV gene repertoire is restricted compared to normal B cells [79]. Some genes such as IGHV1-69, IGHV3-7, IGHV4-34 and IGHV3-23 are very frequent in CLL. In addition, there is also a correlation between the utilization of specific IGHV genes and the SHM status. For instance, IGHV1-69 is more frequent in U-CLL cases [80], whereas IGHV4-34 is more frequent in M-CLL cases [82].

A remarkable feature of the IG gene repertoire in CLL concerns the fact that unrelated CLL patients may carry similar or identical BcR IGs, a phenomenon termed BcR stereotypy [81]. Agathangelidis et al. studied 7428 CLL patients and found that approximately 30% of CLL cases can be assigned to different subsets with stereotyped

BcR [82]. Considering that the chance to find the same or highly similar BcR IG among different B-cell clones is extremely low (10^{-12}), this observation indicates that CLL ontogeny is not stochastic, pointing instead to selection of the CLL clones by common antigens or structurally similar epitopes [64]. Even more intriguing is the fact that patients assigned to the same stereotyped subset present similar clinicobiological features [83][84][85][86].

The stratification of CLL patients into subsets is based on the following criteria: i) utilization of IGHV genes belonging to same phylogenetic clan [87], ii) identical VH CDR3 (antigen binding domain) length and conserved patterns, iii) at least 50% amino acid similarity and 70% similar amino-acid physico-chemical properties [82].

Stereotyped CLL cases are classified in hundreds of stereotyped subsets. However, there are 19 major subsets, which represent the 41% of all stereotyped patients and 12% of all CLL cases [82]. Amongst these, subset #1 is the largest within U-CLL, subset #2 is the largest overall, including both M-CLL and U-CLL cases, and finally, subset #4 is the largest within M-CLL (Figure 4) [64][82].

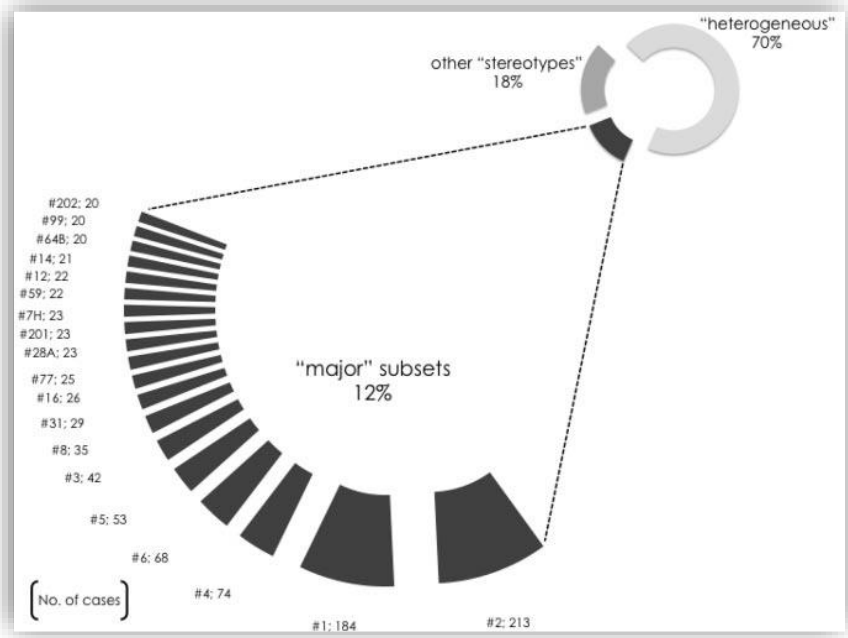

Figure 4. The distribution of stereotyped CLL cases into 19 major subsets (Agathangelidis et al. Blood 2012) [82].

6.7. Antigens in CLL

Biases in the IGHV gene repertoire in CLL, culminating in BcR stereotypy, strongly imply that the antigen selection is critical for CLL pathogenesis. This is also supported

by the fact that CLL B cells have a phenotype of antigen-experienced B cells. More specifically, CLL B cells of both U-CLL and M-CLL cases express the memory marker CD27 [88] as well as other markers of activation (e.g. CD69, CD71 etc). In addition, the low levels of IgM, IgD and CD79b indicate a phenotype of mature, activated B cells [75].

The exact nature of the antigens implicated in CLL pathogenesis is still elusive. Many studies have shown that CLL BcR IGs recognize a variety of autoantigens. Early studies reported that CLL BcR IGs bind the Fc (fragment crystallizable region) of IgG, ssDNA, dsDNA, histones and cytoplasmic structures such as actin, tubulin and myosin [89][90]. Interestingly, it was shown that U-CLL BcR IGs were highly autoreactive/polyreactive, whereas most M-CLL BcR IGs were not [91].

More recent studies showed that CLL BcR IGs react with molecules present on apoptotic cells, including cytoskeletal proteins, as well as bacterial antigens [92][93][94][95]. Interestingly, CLL monoclonal antibodies (mAbs), derived from cases belonging to the same stereotyped subset, possess very similar antigen binding profile. For example, vimentin is recognized by subset #1 and subset #8 antibodies [93][95], cofilin by subset #5 and #2 antibodies [93], whereas nonmuscle myosin heavy chain IIA (MYHIIA) is a target of subset #6 antibodies [96][94]. Other apoptotic targets of CLL antibodies are modified proteins and lipoproteins. Lipids, proteins and lipoproteins are oxidized during apoptosis or during sustained inflammation and CLL antibodies have the ability to recognize these modified molecules (Catera et al., 2008) (Lanemo Myhrinded et al., 2008). For instance, oxidized low density lipoprotein (oxLDL) is recognized by CLL antibodies from subsets #1, #6 and #32 [93].

Besides autoantigens, CLL mAbs are able to bind microbial epitopes e.g. from *Streptococcus pneumoniae, Streptococcus pyogenes, Enterococcus faecium, Enterococcus faecalis* and *Enterobacter cloacae* [97]. Viral infections could also have a relevance for CLL pathogenesis. There is an association between Epstein-Bar (EBV) virus and cytomegalovirus (CMV) with stereotyped subset #4, expressing the IGHV4-34 gene that is known to be overexpressed after EBV and CMV infection [98][99]. Finally, a recent study showed that the somatically hypermutated CLL mAbs from a minor stereotyped subset that are encoded by the IGHV3-7 gene and carry a very small CDR3 region have the ability to bind the β-(1,6)-glucan, which is a major antigenic determinant of yeasts and filamentous fungi [100].

Overall, the available evidence supports the view that CLL derives from B cells which are able to produce poly- and auto-reactive antibodies. The fact that CLL mAbs recognize antigens on the surface of apoptotic cells and/or antigens been modified by typical chemical reactions during apoptosis has led to the hypothesis that CLL may originate from the (still elusive) human counterpart of B1 cells in mice, which produce natural antibodies that have the ability to clear debris and molecular modifications during normal cellular catabolism and constitute the first line of defense against microbial infections.

However, the majority of the aforementioned studies have a major drawback, since the clonotypic CLL IGs were produced as recombinant IgGs, whereas the majority of CLL cases express surface IGs of the mu isotype. Although, the constant region is thought to be responsible only for the effector functions of the IGs, recent reports demonstrated that it can also affect the fine specificity of the interactions between the antibody binding site and the antigen [101][102]. In particular for CLL, a structural study of the CLL BcR IG, has shown that the IG constant region is directly involved in the formation of antibody-antigen-like, homotypic BcR IG interactions [103].

7. Splenic Marginal Zone Lymphoma (SMZL)

7.1. General

SMZL is a rare, indolent B cell lymphoma, with an incidence of 0.13 new cases per 100.000 persons per year [104]. SMZL affects mainly elderly people (median age at diagnosis is 69 years). Massive splenomegaly is the most common clinical feature, observed in 75% of patients, whereas 25% of patients present anemia and thrombocytopenia. The bone marrow and the peripheral blood are also frequently involved [105][106].

SMZL clonal B cells do not have a specific immunophenotype [107]. However, in most cases the clonal SMZL cells express surface IgM and IgD or IgM alone along with CD20, CD22, CD24, CD27, FMC7 and CD79b [107]. Malignant cells are typically negative for CD5, CD10, BCL6, cyclin D1/BCL1, CD43, annexin A1, LEF1, CD103, and CD123 [105].

7.2. Mutations in SMZL

Cytogenetic abnormalities are very frequent among SMZL patients, although there is not a SMZL-specific genetic hallmark. Salido et al showed that the majority of

SMZL cases (72%) present an aberrant karyotype, of which 53% had more than 3 cytogenetic abnormalities. The predominant abnormalities are gains of chromosome 3/3q and 12q, deletions of 7q, 17p and 6q and translocations involving 8q/1q/14q [108]. Among these abnormalities, 7q deletion is the most common, presenting in almost 45% of SMZL cases [109].

In addition, several genes are recurrently altered in SMZL. For example, *NOTCH2* mutations, that are associated with adverse clinical outcome, are very frequent (25%) [110][111][112]. Mutations in *TP53* are also associated with poor prognosis and are present in 16% of SMZL patients [110]. The transcription factor Krüppel-like Factor 2 (KLF2) is defective in 20-40% of SMZL cases and is associated with poor outcome too [113][110]. Somatic mutations have also been found in NF-κB regulators. In positive regulators like CARD11 and MYD88 there are activating mutations, while inactivating mutations occur in negative regulators such as TNFAIP3 and BIRC3 [114][115]. Finally, genes involved in chromatin remodeling such as *MLL2*, *ARID1A* and *SIN3A* are also frequently mutated in SMZL [112][114].

7.3. BcR IG gene restrictions in SMZL

Similarly to CLL, SMZL is also characterized by restrictions in the IG gene repertoire. In 2012 Bikos et al assessed the IG heavy chain gene rearrangements of 337 SMZL patients demonstrating that three IGHV genes (IGHV1-2, IGHV4-34 and IGHV3-23) are used by almost 50% of SMZL patients. Furthermore, the IGHV1-2*04 is the dominant allele, utilized by almost 30% of SMZL patients [116]. Except for the IGHV genes, there is also restricted repertoire in IG light chain genes: the IGKV3-20, IGKV4-1, IGKV1-5, IGKV1-8, IGKV1-39 and IGLV2-14 are collectively expressed in 64% of SMZL cases [117].

In addition, the IGHV1-2*04 allele showed biased recombination with certain IGHD genes, namely IGHD3-3, IGHD3-9 and IGHD3-10 [116] (Figure 5). Furthermore, biases are found in the light chain variable genes paired with IGHV1-2*04 allele. In particular, IGKV3-20, IGKV1-8 and IGLV2-14 are very frequently expressed in association with IGHV1-2*04 [117] (Figure 6). Other distinctive features of the IGs in SMZL concern the recurrent mutations generated by SHM and the pronounced intraclonal diversification within the IG genes of IGHV1-2*04 expressing cases, indicating ongoing interactions with antigen(s) [116].

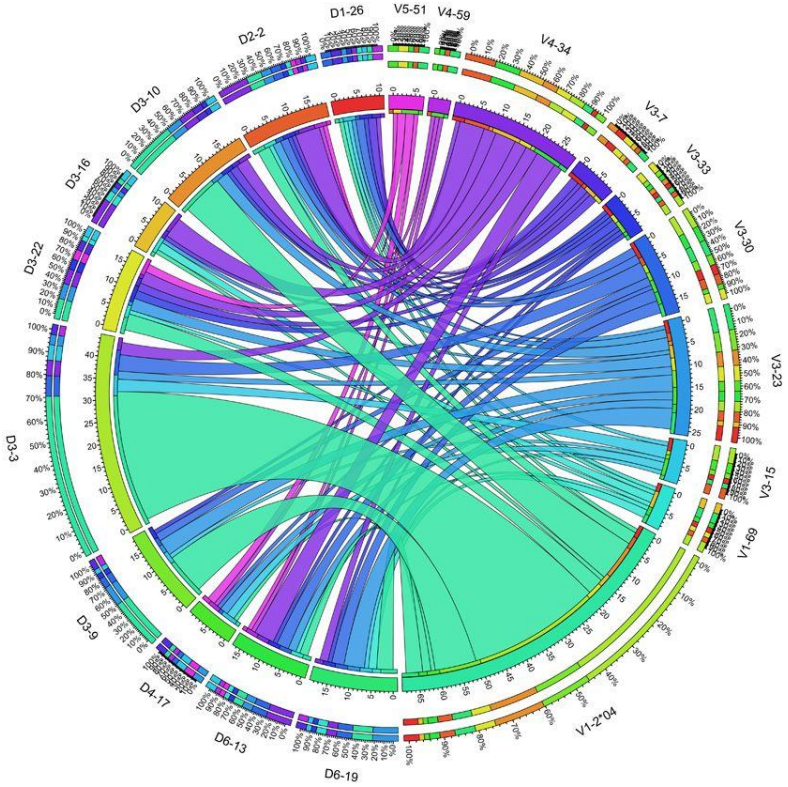

Figure 5. The biased associations of IGHV genes with IGHD genes in SMZL[116]

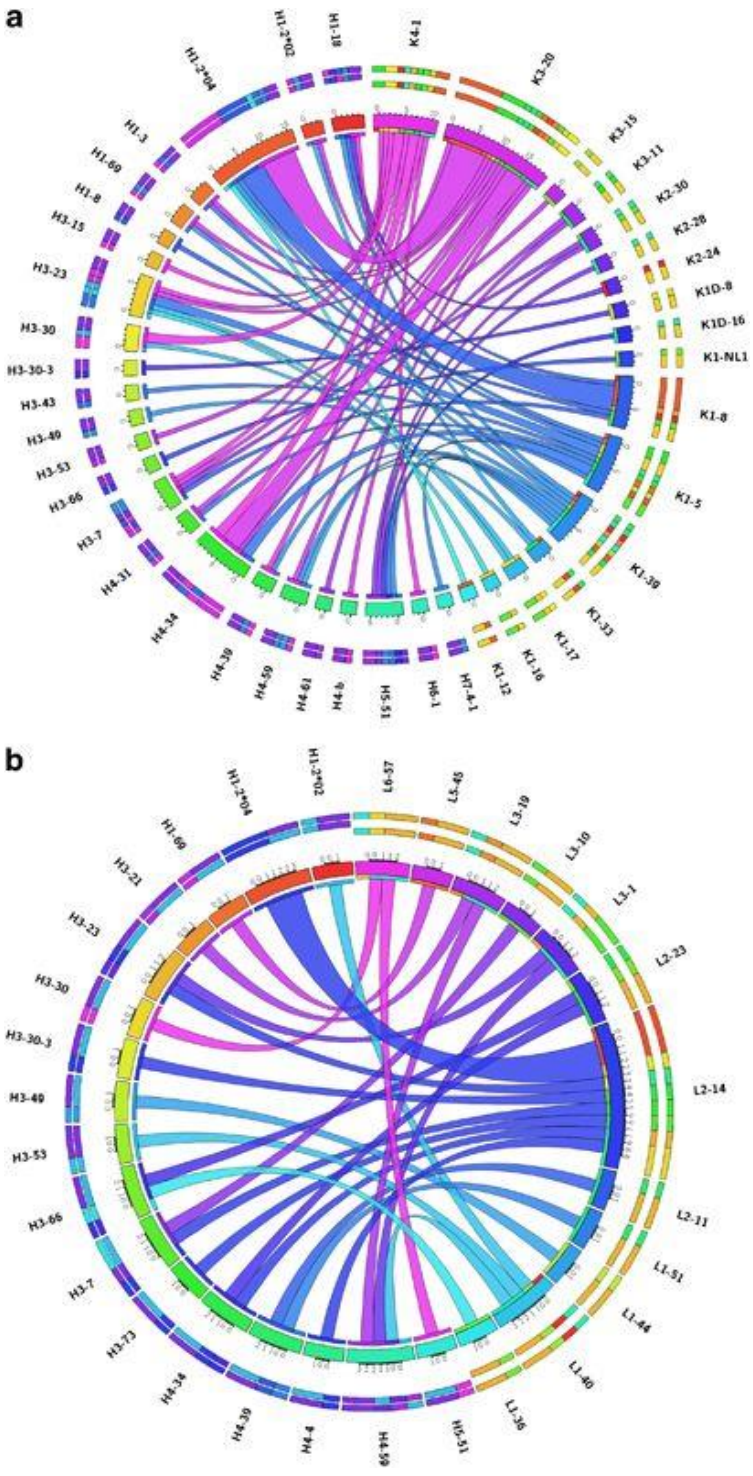

Figure 6. The biased associations of IGHV genes with (a) IGKV and (b) IGLV genes [117]

7.4. Antigens in SMZL

The impressive IG restrictions described above indicate that specific antigens could be implicated in SMZL ontogeny and progression. Indeed, several epidemiological studies and meta-analyses showed that HCV infection is correlated with several non-Hodgkin's lymphomas, including SMZL [118]. Studies in Italy

demonstrated that almost 25% of SMZL cases were positive for HCV infection [119][120]. Interestingly, antiviral treatment with interferon or HCV inhibitors may lead to partial lymphoma regression, proving that HCV is associated with SMZL [121][122][123].

Thus far, there is only one study concerning the actual SMZL BcR IG antigen reactivity profile, in which the BcR IG of 5 IGHV1-2*04 expressing cases were produced as IgG mAbs and were shown to bind to nuclear, cytoplasmic and membranous antigens on human cells, as well as antigens present in human serum [124]. The SMZL mAbs reacted also with autoantigens on the surface of living cells but not apoptotic cells [124], unlike what is known for CLL mAbs [92][93].

Aim of the Study

Numerous findings highlight the significance of BcR IG in the pathogenesis and evolution of B cell non-Hodgkin lymphomas (B-NHLs) [125]. The importance of antigen triggering in driving lymphomagenesis is underscored by the fact that certain B-NHLs have been linked with infections and chronic antigenic stimulation (e.g *Helicobacter pylori* infection in gastric mucosa-associated lymphoid tissue lymphoma) [126]. From a clinical perspective, the importance of BcR - microenvironment interactions is also highlighted by the remarkable effectiveness of BcR signaling-inhibitors [127][128].

Immunogenetic evidence also alludes to antigen selection in the ontogeny and evolution of CLL and SMZL. These evidence pertains to (i) biases in the IGHV and IGK/LV gene repertoire, [79][82][116][117], (ii) the presence of SHM (often in distinctive pattern) within the IG genes of many CLL [66] and SMZL [116] cases, (iii) intraclonal diversification within the IG genes observed in certain CLL and SMZL subgroups that suggests an ongoing interaction between the BcR IG and the antigen(s) [129][116][129] and (iv) the expression of (quasi)identical BcR IG by unrelated patients, a phenomenon termed “BcR stereotypy” [81].

Recent studies revealed that the CLL BcR IGs, mainly those deriving from the U-CLL, are polyreactive, recognizing both foreign and self-antigens. The recognized antigens include auto-antigens exposed in the context of apoptotic bodies produced during physiological cell turnover or neo-epitopes produced during apoptosis [89][90][91][92][93][94][96][95], as well as molecular structures of microbes [97][100]. This antigen binding profile is reminiscent of natural antibodies [53] and suggests that CLL clones, mostly those of U-CLL, may arise from a B cell population which provides a first line of defense and is also implicated in the removal of apoptotic debris [130]. However, despite the progress achieved so far, the precise nature of antigens recognized by CLL BcR IGs remains elusive.

In SMZL, the single study published thus far included only five IGHV1-2*04 expressing clones and showed binding to nuclear, cytoplasmic and membranous antigens on the surface of viable human cells but not on the surface of apoptotic cells that have been shown to react with U-CLL BcR IG [124]. The BcR IG antigen reactivity of SMZL cases expressing other IGHV genes is still unknown.

In this context, the aim of the present thesis was to provide novel knowledge about the antigen reactivity profiles of CLL and SMZL BcR IGs, ultimately also contributing to elucidating the molecular mechanisms and the microenvironmental interactions involved in the pathogenesis of these two lymphomas.

Material and Methods

1. Recombinant plasmid DNA production

1.1. Plasmid vectors

Four different expression vectors were used for the cloning of the IGs: pIgGamma1 (Figure 7), pIgMu (Figure 8), pIgLamda (Figure 9) and pIgKappa vectors (Figure 10). These vectors contain the C region of the human IgG heavy chain, and the human IgM heavy chain, the human λ light chain and the human κ light chain, respectively, providing the skeleton to insert the V region of each chain of the clonotypic IGs. For each different case, cloning was performed to the appropriate vector based on the isotype expressed by the malignant clone of each patient. The majority of CLL and SMZL cases express clonotypic IGs of the IgM isotype; CLL stereotyped subsets #4 and #8 are exceptional, since they express IgG-switched clonotypic IGs. The V regions of 4 subset #8 cases were cloned in both pIgGamma1 and pIgMu, in order to assess the role of isotype switching into the antigen recognition.

The expression is under the control of the promoter of the Human Cytomegalovirus (HCMV), while replication is achieved with the help of pUC, SV40 and F1ori replication start sites. Upstream of the constant region there are a murine leader peptide sequence and a multiple cloning site (MCS). The latter comprises multiple restriction sites, suitable for the cloning: (1) for the cloning of the V region of the μ heavy chain the AgeI and AfeI sites were used, (2) for the cloning of the V region of the γ heavy chain the AgeI and SalI sites were used, (3) for the cloning of the V region of the κ light chain the AgeI and BsiWI sites were used and (4) for the cloning of the V region of the λ light chain the AgeI and XhoI sites were used. The plasmids contain also a gene which provides resistance to ampicillin, allowing thus, only the bacteria which uptake the plasmid to grow under ampicillin selection.

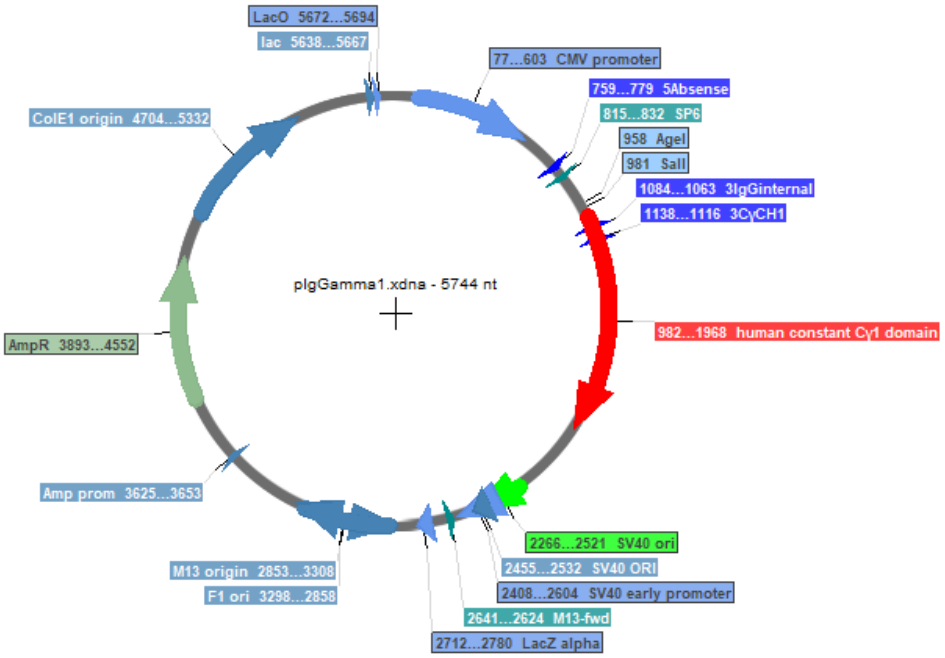

Figure 7. Map of pIgGamma1 plasmid vector

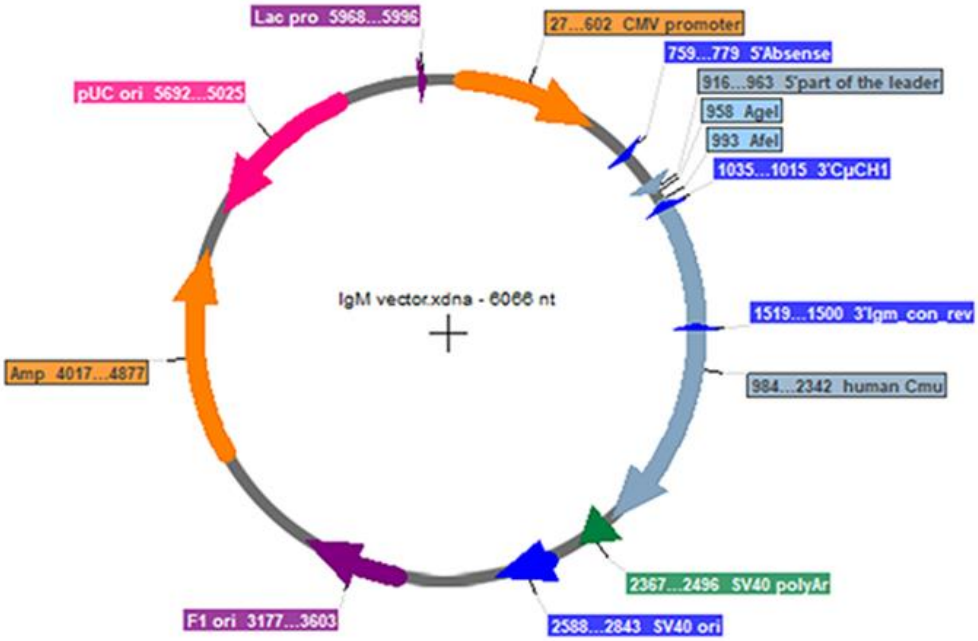

Figure 8. Map of the pIgMu plasmid vector

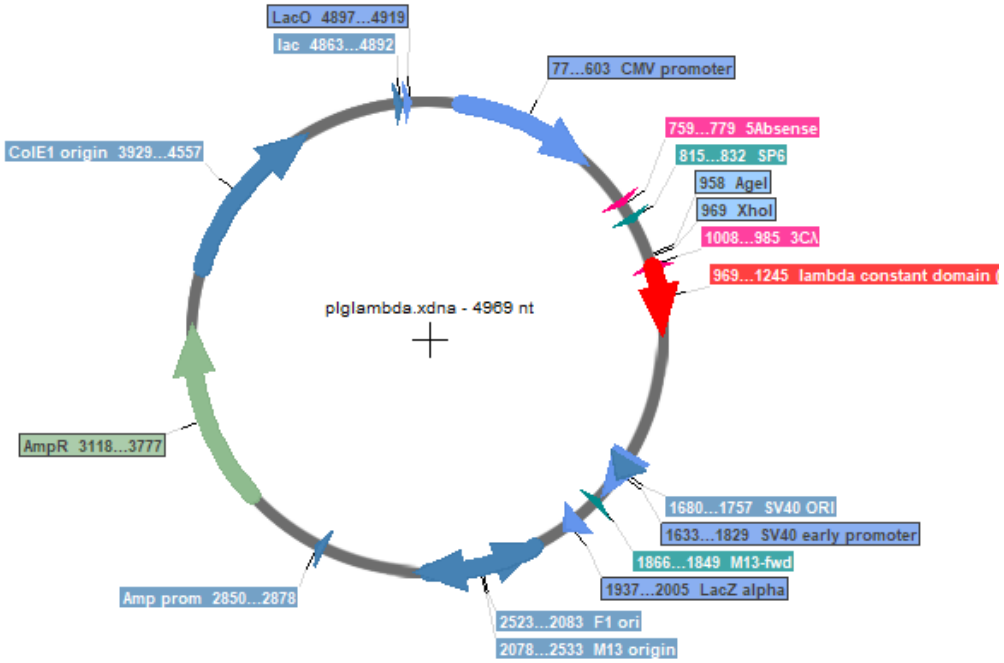

Figure 9. Map of thepIgLambda plasmid vector

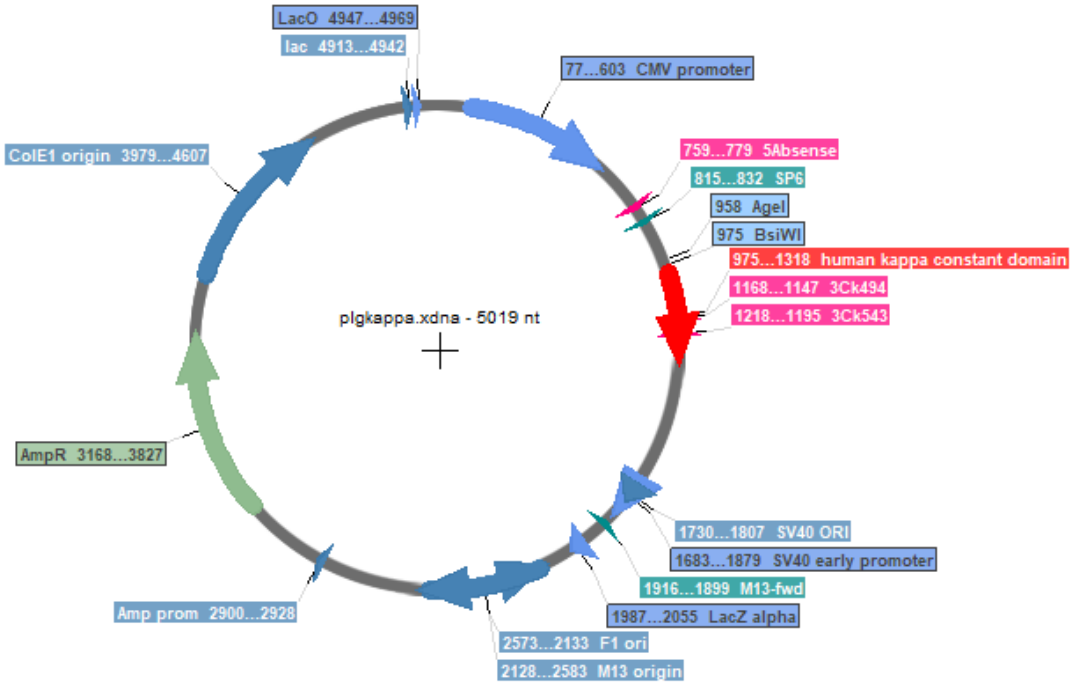

Figure 10. Map of the pIgKappa plasmid vector

1.2. Identification of Ig genes.

Before cloning, it was necessary to identify the molecular characteristics of the IGHV-IGHD-IGHJ, IGKV-IGKJ and IGLV-IGLJ clonotypic rearrangements. For this purpose, PCR was performed as described [131]. Briefly, primers annealing upstream of the V region (forward primers) and at the constant region of the IGs (reverse primers) were used. The reagents and the conditions of PCR are summarized in Tables 1 and 2. The PCR products were gel extracted, using the Monarch® DNA Gel Extraction Kit (New England Biolabs-NEB) according to the manufacturer's instructions. Then, the purified PCR products were sequenced with the appropriate primers. IG sequence analysis was performed using the IMGT/V-QUEST tool (http://www.imgt.org/IMGT_vquest/vquest).

Table 1. Reagents and their volumes for the amplification of IGHV-IGHD-IGHJ, IGKV-IGKJ and IGLV-IGLJ rearrangements

Reagents	Volumes (μl)
10X Reaction Buffer	5
dNTPs mix (10mM)	2.5
Forward primer	1.5
Reverse primer	1.5
H_2O	37.5
Pfu DNA polymerase	1
Template	1
Total Volume	50

Table 2. The condition of PCR

Thermocycler conditions		
Initial Denaturation	94°C 5 min	
Denaturation	94°C 30 sec	X35 cycles
Annealing	58°C for IgH/Igκ or 60°C for Igλ, 30 sec	
Extension	72°C 40 sec	
Final extension	72°C 5 min	

Storage	4°C	

1.3. PCR cloning

Subsequently, gene-specific PCR was performed using appropriate oligonucleotide primers for the amplification of the IG variable domain and the insertion of restriction sites for AgeI, AfeI, SalI, BsiWI and XhoI restriction enzymes. The conditions of PCR are identical to the previous PCR (expect for the duration of extension stage: 30sec) and the PCR products were gel excised using the Monarch® DNA Gel Extraction Kit (NEB). Next, the PCR products and the plasmid vectors were digested with the appropriate restriction enzymes (NEB); 10-20 units of enzymes per μg of DNA were used. All digestions were performed using CutSmart buffer (NEB) at 37°C and the reaction was terminated by increasing the temperature to 65°C for 20 min. After enzyme digestion the PCR products and the vectors were run in agarose gel and purified using the Monarch® DNA Gel Extraction Kit (NEB).

1.4. Ligation

For the ligation reaction, the molar ratio of digested plasmid vectors to the digested PCR products was 1:3. The ligation was performed using 10 units of T4 DNA Ligase (Invitrogen) in the appropriate buffer solution (Invitrogen) in a total volume of 10μl. The mixture was incubated at 16°C for 16 hours.

1.5. Bacteria transformation with the recombined plasmids

The insertion of the recombinant plasmid DNA into the bacteria was accomplished by heat shock and the transformed bacteria were selected based on their resistance against ampicillin (Sambrook et al., 1989).

Top10F'chemically competent bacteria (50μl) were transformed with 5μl of the ligation product. Briefly, after 20-30 minute incubation at 4°C, the mixture was heated at 42°C for 45 seconds. Following the heat shock step, the mixture was kept on ice for another 5 minutes and then the bacteria were plated on ampicillin containing (100μg/ml) LB Agar plates (Lennox LB Agar-Invitrogen), and incubated overnight at 37°C. Only the bacteria that incorporate the plasmids would grow, as they gained resistance to ampicillin. Next, PCR was performed to select the bacteria colonies carrying plasmids that contain insert (tables 3&4). The 5' Absense primer which hybridizes upstream of the insert was used as forward primer for all plasmids; reverse

primers were 3'CmCH1, 3'IgG internal, 3' Cκ494 or 3' Cλ, which hybridize downstream of the insert in the pIgMu, pIgGamma1, pIgKappa or pIgLambda, respectively (table 5).

Table 3. The reagents and the volumes for each reaction of bacterial colony screening

Reagents	Volumes (μl)
Reaction Buffer 10X	2
$MgCl_2$	1
dNTPs mix (10mM)	1
Forward Primers	1
Reverse Primers	1
H_2O	14
Taq polymerase	0.2
Total Volume	20

Table 4. Conditions of the PCR bacteria colony screening

Thermocycler conditions		
Initial Denaturation	94°C 5 min	
Denaturation	94°C 30 sec	X35 cycles
Annealing	58°C 30 sec	
Extension	72°C 1 min	
Final extension	72°C 10 min	
Storage	4°C	

Table 5. The sequences of each primer and what types of plasmids were used for each primer

Primers	Sequence (5'-3')	Vectors
5' Absense	GCTTCGTTAGAACGCGGCTAC	pIgGamma1, pIgMu, pIgKappa, pIgLambda
3' CmCH1	GGGAATTCTCACAGGAGACGA	pIgMu
3' IgGinternal	GTTCGGGGAAGTAGTCCTTGA	pIgGamma1
3' Cκ494	GTGCTGTCCTTGCTGTCCTGCT	pIgKappa

3' Clambda	CACCAGTGTGGCCTTGTTGGC	pIgLambda

Bacterial colonies giving the appropriate PCR products (650bp for the IgG1 and the IgM vectors, 700bp for the Igκ vector and 590 for the Igλ) were selected for 16-hour culture at 37°C in 20ml LB Broth Base (Lennox L Broth Bases-Invitrogen), containing 100μg/ml ampicillin (Sigma-Aldrich). Next, the plasmid DNA was isolated using Nucleospin Plasmid columns (Macherey-Nagel). The isolated plasmid DNA was sequenced using the 5'Absense primer and analyzed using the IMGT/V-QUEST tool to confirm the identity of the cloned IG sequences with the clonotypic IG rearrangements.

2. Production of recombinant monoclonal antibodies (mAbs)

2.1. Cultures of eukaryotic cells

For the production of the soluble clonotypic IGs as human recombinant mAbs, the Human Embryonic Kidney (HEK) 293T cell line was used. Cells were cultured at 37°C in 5% CO_2 in 100mm plates in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (FBS, Sigma-Aldrich) and 0.1% (v/v) penicillin and streptomycin (Gibco). When the cells covered the 80-90% of the plates' surface (approx. every 48 hours) they were split in new plates as follows:

- aspiration of the medium and wash of the cells with 5ml PBS
- removal of PBS, addition of 1 ml trypsin (Gibco) and incubation for 4-6 min at 37°C
- inactivation of trypsin with 10 ml DMEM supplemented with FBS
- gentle pippeting to remove all the cells from the bottom
- transfer of the cells to new plates, supplementing with fresh medium. The dilution of the initial culture ranged from 1:4 to 1:8.

2.2. Transfection of HEK 293T cells.

The day before transfection, the cells were split and 2-3 hours before the transfection the medium was replaced. For the production of each mAb, four plates were prepared. The transfection was performed using Polyethylenimine (PEI) at a 3:1 ratio of PEI to plasmid DNA containing the IG sequences. PEI is a cationic polymer that binds to the negatively charged plasmid DNA forming cationic complexes

(polyplexes) that bind on the anionic cell surface. The polyplexes then enter into the cells through endocytosis [132]. For the transfection 5μg of each of the plasmids containing the IG heavy and light chain genes were added in 0,5ml Optimem. In a new tube 30μg of PEI were added to 0,5ml Optimem. Next, the diluted PEI was transferred to the mix of plasmids-Optimem followed by incubation at room temperature for 15 minutes; 1 ml mixture was then added to the plate drop-wise. Twelve to 16 hours after the transfection the medium was replaced with DMED supplemented with antibiotics and 1X Nutridoma-SP (Roche). Nutridoma was used instead of FBS in order to facilitate the purification of antibodies from the culture supernatant.

2.3. Collection of mAbs

One week after the transfection, the culture supernatants were collected and centrifuged at 800xg for 10 minutes to remove the cell debris. Then, the supernatants were concentrated using Millipore® Amicon® Ultra-15 Centifugal Filter Concentrators with a Molecular Weight Cutoff 100kDa for IgM mAbs and 30 kDa for IgG mAbs. The concentrated mAb preparations were protected by adding proteases inhibitors and the anti-bacterial factor sodium azide (NaN3) at a 0.05% (w/v) concentration. The concentrated supernatants were stored at 4^{o}C.

2.4. Quantification of mAbs

The concentrated mAbs were quantified using the Human IgM or IgG ELISA development kits (Mabtech) following the manufacturers' instructions. The optical density (OD) values were measured at 405 nm in a ELx800™ Absorbance Microplate Reader (BioTek). The mAbs were quantified based on the OD values of the known-concentration standards to create a standard curve, using Graphpad Prism 4.0 software (La Jolla, CA, USA).

2.5. Western blot for recombinant mAbs

To check the purity and integrity of the concentrated mAbs SDS-PAGE electrophoresis was performed under denaturating and non-denaturating conditions in appropriate running buffer (Tris ultrapure-Glycine, 10% SDS). The mAbs and prestained ladder (Blue Star pre-stain marker and MagicMark XP, Invitrogen) were loaded on the 10% polyacrylamide gel and the electrophoresis was accomplished at 120 volts for approximately 90 minutes. Next, the proteins were transferred to PVDF membrane (Immobilon). The transfer procedure was performed at 35V for 2 hours

using appropriate transfer buffer (Tris ultrapure-Glycine and 10% Methanol). Next, PVDF membranes were washed three times with 1X PBS-T and blocked with 5% milk diluted in 1X PBS for 1 hour at room temperature. Then, Horseradish Peroxidase (HRP)-conjugated rabbit anti-Human IgM mu chain (Abcam) or goat anti-human IgG Fc (Abcam), diluted 1/20.000 in 5% milk, were added to the membrane for 40 minutes. Finally, Luminata Classico Western HRP Substrate (Millipore) was used for the protein immunodetection.

2.6. ELISA assays

The antigen reactivity profile of the produced recombinant mAbs was assessed by ELISA assays. In particular, the mAbs were used as primary Abs in ELISA assays against molecules that are common antigenic targets of natural occurring and pathological auto-antibodies, including dsDNA, globular actin, myosin, thyroglobulin, β-amyloid protein, human F(ab')2fragment, carbonic anhydrase and the hapten Tri-Nitro-Phenyl (TNP), as follows:

- 96-well polystyrene plates were coated with 100μl/well of 1-5μg/ml diluted in PBS, of the examined antigens
- incubation overnight at 4^{o}C.
- 5 washes with PBS-0,1%Tween
- blocking with 200μl/well of PBS-1.5% BSA for 3h at room temperature.
- 5 washes with PBS-0,1%Tween
- addition of 100μl/well of the examined CLL or SMZL mAbs at a 20μg/ml concentration and incubation for 3h at room temperature
- addition of 100μl/well of HRP or alkaline phosphatase (ALP) conjugated anti-human IgM or IgG or Igκ secondary antibodies and incubation for 2 hours at room temperature
- 5 washes with PBS-0,1%Tween
- addition of 50μl/well of 3,3,5,5' tetramethylbenzidine (TMB) or p-nitrophenyl phosphate (pNPP) substrates (Bethyl laboratories) depending on the secondary Ab used and incubation for 15-30 minutes in the dark.
    - when TMB substrate was used, 50 μl of 0.18M H_2SO_4 was added to terminate the reaction.

– Measurement of the OD values at 450nm (for HRP-TMB) or 405nm (for ALP-pNPP), using the ELx800™ Absorbance Microplate Reader (BioTek).

2.7. Flow Cytometry

$25x10^4$ MEC1 or HEK293T cells were diluted in 100μl PBS-FBS 1% and incubated with 25μg/ml recombinant SMZL or CLL mAbs for 1 hour at room temperature. Then, the cells were washed with PBS-FBS 1% and incubated with 10μl of IgM κ-FITCH/λ-PE or PE Mouse Anti-Human light chain secondary Abs (BD Biosciences) (depending on the isotype of the light chain utilized by each mAb), for 15 minutes at room temperature, in the dark. The cells were washed with PBS in order to remove the unbound secondary antibody and resuspended in 500μl PBS. The acquisition was performed in a BD FACS Calibur flow cytometer and the data were analyzed with Flowing software.

3. Statistical analysis

Data were compared with the use of either the paired Student t test or the nonparametric Mann-Whitney U test. Analyses were performed by GraphPad Prism 4 software (GraphPad Software, San Diego, CA). A P value, .05 was considered as statistically significant.

Results

1. Production of recombinant mAbs

The clonotypic IG of 42 CLL and 12 SMZL patients were cloned in appropriate expression vectors and expressed as recombinant mAbs in HEK293T cells. The mAbs were produced either as IgGs or as IgMs paired with κ or λ light chain, depending on the isotype of the clonotypic IG expressed on the surface of the malignant cells. The isotype of the mAbs and the molecular characteristics of the V domain are presented in Tables 6 and 7. Confirmation of the IG DNA sequences cloned into the expression vectors was performed by sequencing analysis (Figure 11).

It should be noted that in the context of this master thesis, the IG genes of 14 CLL and 5 SMZL patients were cloned, while the plasmids from the remaining 28 CLL and 7 SMZL cases were produced previously in the lab as part of previous projects. All the aforementioned plasmids were used for recombinant mAb production.

The CLL study group included 19 U-CLL patients and 23 M-CLL patients. The CLL cases under study utilized IGHV genes of the IGHV1, IGHV3 and IGHV4 gene subgroups, the most frequently represented in CLL, and were assigned to the stereotyped subsets according to the largest study on BcR stereotypy in CLL by Agathangelidis et al (2012) [82]. The assignment to the "fungi subset" was according to the study of Hoogeboom et al (2013) [100] (Tables 6 and 7).

In the analyses presented in the current thesis, the CLL subset #2 and #169 cases were analyzed together as the two subsets are immunogenetically closely related: (i) the IGHV3-21 (subset #2) and the IGHV3-48 (subset #169) genes present very high overall similarity (97% identity), (ii) the 2 subsets have identical VH CDR3 length (9 amino acids), (iii) both carry a landmark aspartic acid (D) residue at position 107 within the VH CDR3, and (v) both express V3-21 lambda light chains [82].

The SMZL study group included cases utilizing the most frequently represented IGHV genes in this lymphoma, namely IGHV1-2, IGHV3-23 and IGHV4-34 [116] (Tables 6 and 7). In the analyses presented in the current thesis, to assess the impact of SHM in the antigen specificity of the SMZL mAbs, cases were grouped according to Bikos et al (2012) into truly unmutated (100% identity with the closest germline IGHV gene), minimally mutated (97-99.9% identity with the closest germline IGHV gene) and mutated (<97% identity with the closest germline IGHV gene) [116].

Table 6. Molecular characteristics of the IGHV-IGHD-IGHJ rearrangements of the CLL and SMZL cases included in the present thesis. CLL stereotyped subset assignment is according to Agathangelidis et al (2012) [82] and Hoogeboom, R et al (2013) [100]. S.S: CLL stereotyped subsets, ND: no definite

mAb	Isotype	Entity and S.S.	IGHV	IGHV gene identity %	IGHD	IGHJ	VHCDR3 amino acid sequence
GM056	μ/κ	CLL, #1	1-3*01	100.00%	6-19*01	4*02	CWEREQWLAITHFDYW
P1173	μ/κ	CLL, #1	5-10-1*03	99.31%	6-19*01	4*02	CWEREQWLGIKNFDYW
P3870	μ/κ	CLL, #1	1-2*02	100.00%	6-19*01	4*02	CARGQWLVQLNFDYW
P14197	μ/κ	CLL, #1	1-2*02	100.00%	6-19*01	4*02	CWEREQWLVRVHFDYW
RS038	μ/κ	CLL, #1	1-3*01	100.00%	6-19*01	4*02	CWEREQWLGPYYFDYW
P23728	μ/κ	CLL, #1	1-2*02	100.00%	6-19*01	4*02	CWEREQWLVLRNFDYW
N4755	μ/λ	CLL, #2	3-21*01	97,92%	5-12*01	6*02	CATDRNGMDVW
US412	μ/λ	CLL, #2	3-21*01	98.25%	ND	6*02	CARDQNGMDVW
P11475	μ/λ	CLL, #2	3-21*01	97,99%	ND	6*02	CARDQNAMDVW
P326	μ/λ	CLL, #2	3-21*02	97,92%	3-16*01	6*02	CAIDRNGMDVW
P6090	μ/λ	CLL, #2	3-21*02	96.53%	ND	6*02	CVTDRNGMDVW
P16021	μ/λ	CLL, #2	3-21*01	97.22%	3-16*02	6*02	CASDRNGMDVW
P6540	μ/λ	CLL, #169	3-48*02	96.18%	1-26*01	3*01	CARDGVGAPLW
P907	γ/κ	CLL, #4	4-34*02	93,20%	5-12*01	6*02	CARGYGTSATTKRYYYYGMDVW
US240	γ/κ	CLL, #4	4-34*01	96,84%	5-18*01	6*02	CARGYADTPVFRRYYYYGMDVW
US183	γ/κ	CLL, #4	4-34*01	96,84%	5-18*01	6*02	CARGYGDTPTIRRYYYYGMDVW
P3916	γ/κ	CLL, #4	4-34*01	92,63%	3-10*01	6*02	CARGYADSDVIRRYYYYGMDVW
P103	γ/κ	CLL, #4	4-34*01	95,90%	3-10*01	6*02	CARGYPDTPVVRRYYYYGMDVW
PM013	γ/κ	CLL, #4	4-34*01	93,15%	2-21*02	6*02	CARSYGDSPSVRRYYYYGLDVW
P10824	μ/κ	CLL, #6	1-69*01	100.00%	3-16*02	3*02	CARGGGYDYIWGSYRPNDAFDIW
KM1PL	μ/κ	CLL, #6	1-69*06	100.00%	3-16*02	3*02	CARGGNYDYIWGSYRSNDAFDIW
P7150	μ/κ	CLL, #6	1-69*01	100.00%	3-16*02	3*02	CARGGSYDYVWGSYRPNDAFDIW
P11887	μ/κ	CLL, #6	1-69*06	100.00%	3-16*02	3*02	CARGGPYDYVWGSYRPSDAFDIW
P1430	μ/λ	CLL, #7	1-69*01	100.00%	3-3*01	6*02	CWEREGNYDFWSGYYPNYYYYGMDVW
P6275	μ/λ	CLL, #7	1-69*01	100.00%	3-3*01	6*02	CATINYDFWSGYYKNYYYGMDVW
US845	γ/κ	CLL, #8	4-39*01	100.00%	6-13*01	5*02	CASSTGYSSSWYSPTNWFDPW
KA012-10PL	γ/κ	CLL, #8	4-39*01	100.00%	6-13*01	5*02	CARHNLGYSSSWYSRNNWFDPW
Nov5889	γ/κ	CLL, #8	4-39*01	100.00%	6-13*01	5*02	CARRVGYSSSWYGQKNWFDPW
US657	γ/κ	CLL, #8	4-39*01	99,31%	6-13*01	5*02	CASKTGYSSSWYGRDWFDPW
Nov12151	γ/κ	CLL, #8	4-39*01	99,60%	6-13*01	5*02	CASRSGYSSSWYSNPNWFDPW
P5071	μ/λ	CLL, #10	4-39*01	100.00%	2-2*01	6*02	CARHRLGYCSSTSCYYYYYGMDVW
P14438	μ/λ	CLL, #77	4-59*01	89.47%	6-19*01	4*02	CARGPDESGWNGLLYW
P2959	μ/λ	CLL, #77	4-4*02	95.09%	6-19*01	4*02	CARGPDISGWNGFEYW
P5623	μ/λ	CLL, #77	4-59*03	91.93%	2-21*02	1*01	CARGPNESGWLGLLHW
P8407	μ/λ	CLL, #77	4-4*02	92.01%	6-19*01	6*02	CTRGPDESGWNGMDVW

P6360	μ/κ	CLL, #148B	2-5*02	94.85%	3-3*01	4*02	CARRGEDLTGWMNAYFTFW
L7911	μ/λ	CLL #201	4-34*01	94.39%	1-26*01	3*01	CARRRERWDQKEGDAFDVW
P775	μ/λ	CLL #201	4-34*01	94.74%	1-26*01	3*01	CARRPERWDLYKNDAFDVW
P22229	μ/λ	CLL #201	4-34*01	91.58%	3-9*01	3*01	CARREETWTEIKGDAFDVW
L3961	μ/λ	CLL #201	4-34*01	91.23%	3-10*01	3*02	CARRPSNWELFRMDAFDIW
F_3687	μ/κ	CLL, fungi	3-7*01	93.40%	5-24*01	4*02	CIESSTNW
F_725	μ/κ	CLL, fungi	3-7*01	95.49%	3-10*01	5*02	CAEGTVGW
P19994	μ/κ	SMZL	1-2*04	100.00%	3-22*01	4*02	CARDQGGGSGYYHYFDYW
P8761	μ/κ	SMZL	1-2*04	100.00%	3-3*01	5*02	CARGGRASVFGVVLIGDPIFGPW
P23776	μ/λ	SMZL	1-2*04	98.96%	3-3*01	5*02	CARGPRITIFGVVIGRGTTLDPW
P411	μ/κ	SMZL	1-2*04	97.57 %	3-3*01	5*02	CARGGRITIFGVLMGRENWFDPW
P13836	μ/λ	SMZL	1-2*04	94.10%	3-10*01	4*02	CAKDRARTYYYGSGSYYNRPPQYYFDYW
P5729	μ/κ	SMZL	3-23*01	100.00%	6-19*01	4*02	CAKSPVTGGGQWLDEYYFDYW
P11388	μ/κ	SMZL	3-23*01	98.61 %	3-3*01	4*02	CAKNSKSYDFWSGYYGPYFDYW
P2120	μ/κ	SMZL	3-23*01	97.92%	4-17*01	4*02	CAKKGDQAPYGDNLYYFDYW
P6820	γ/κ	SMZL	3-23*01	90.97%	3-10*01	4*02	CAKDYGDGNPLDYW
P8257	μ/κ	SMZL	4-34*01	100.00%	2-21*01	6*03	CARVWGGLVGSGYFYSGYYYYYMDVW
P11038	μ/λ	SMZL	4-34*01	100.00%	3-16*01	4*02	CARKCFRAKYYFDYW
P2301	μ/κ	SMZL	5-51*01	97.92%	3-22*01	6*02	CARQASITSVVGMDVW

Table 7. Molecular characteristics of the IGKV-IGLV and IGKJ-IGLJ rearrangements of the CLL and SMZL cases included in the present thesis. CLL stereotyped subset assignment is according to Agathangelidis et al (2012) [82] and Hoogeboom, R et al (2013) [100]. S.S: CLL stereotyped subsets.

mAb	Isotype	Entity and S.S.	IGKV-IGLV	IGKV-IGLV gene identity %	IGKJ-IGLJ	VK/VL CDR3 amino acid sequence
GM056	μ/κ	CLL, #1	V1-39*01	100.00%	J2*01	CQQSYSTPGYTF
P1173	μ/κ	CLL, #1	V1-39*01	100.00%	J2*01	CQQSYSTPPHTF
P3870	μ/κ	CLL, #1	V1-39*01	100.00%	J2*02	CQQSYSTPPYTF
P14197	μ/κ	CLL, #1	V1-39*01	100.00%	J4*01	CQQSYSTPPYTF
RS038	μ/κ	CLL, #1	V1-39*01	100.00%	J3*01	CQQSYSTPPLFTF
P23728	μ/κ	CLL, #1	V1-39*01	100.00%	J4*01	CQQSYSTPPLTF
N4755	μ/λ	CLL, #2	V3-21*01	97,85%	J3*02	CQVWDSGSDHPWVF
US412	μ/λ	CLL, #2	V3-21*01	99,28%	J3*02	CQVWDSSSDHPWVF
P11475	μ/λ	CLL, #2	V3-21*01	97,85%	J3*02	CQVWDSGSDHPWVF
P326	μ/λ	CLL, #2	V3-21*01	99,70%	J3*02	CQVWDSSSDHPWVF
P6090	μ/λ	CLL, #2	V3-21*01	96.77%	J3*02	CQVWDSGSDHPWVF
P16021	μ/λ	CLL, #2	V3-21*01	97.85%	J3*02	CQVWDSSSDHPWVF
P6540	μ/λ	CLL, #169	V3-21*01	98.21%	J3*02	CQVWDSSSDHPWVF
P907	γ/κ	CLL, #4	V2-30*02	96,94%	J2*01	CMQGTYWPYTF
US240	γ/κ	CLL, #4	V2-30*02	97,62%	J2*01	CMQGTHWPPYTF
US183	γ/κ	CLL, #4	V2-30*02	98,62%	J2*01	CMQGTHWPPYTF
P3916	γ/κ	CLL, #4	V2-30*02	96,94%	J2*01	CMQGTHWPPYTF
P103	γ/κ	CLL, #4	V2-30*02	97,62%	J2*01	CMQGTHWPPYTF
PM013	γ/κ	CLL, #4	V2-30*02	97,28%	J4*01	CMQGTHRPFTF
P10824	μ/κ	CLL, #6	V3-20*01	100.00%	J2*01	CQQYGSSPYTF

KM1PL	μ/κ	CLL, #6	V3-20*01	100.00%	J4*01	CQQYGSSPPLTF
P7150	μ/κ	CLL, #6	V3-20*01	100.00%	J3*01	CQQYGSSPPFTF
P11887	μ/κ	CLL, #6	V3-20*01	100.00%	J1*01	CQQYGSSPPTF
P1430	μ/λ	CLL, #7	V3-9*01	99.64%	J2*01	CQVWDSSTEKVF
P6275	μ/λ	CLL, #7	V3-10*01	99.64%	J2*01	CYSTDSSGNPLF
US845	γ/κ	CLL, #8	V1-39*01	99.64%	J1*01	CQQSYSTPRTF
KA012-10PL	γ/κ	CLL, #8	V1-39*01	100.00%	J1*01	CQQSYSTPRTF
Nov5889	γ/κ	CLL, #8	V1-39*01	100.00%	J1*01	CQQSYSTPGTF
US657	γ/κ	CLL, #8	V1-39*01	99.64%	J1*01	CQQSYSTPRTF
Nov12151	γ/κ	CLL, #8	V1-39*01	100.00%	J4*01	CQQSYSTPPLTF
P5071	μ/λ	CLL, #10	V1-40*01	98,96%	J2*01	CQSYDSSLSVVF
P14438	μ/λ	CLL, #77	V10-54*01	95.79%	J2*01	CSAWDNSLSAQIF
P2959	μ/λ	CLL, #77	V10-54*01	98.60%	J3*02	CSAWDSSLSAWVF
P5623	μ/λ	CLL, #77	V10-54*01	95.79%	J2*01	CSAWDSSLSAQVF
P8407	μ/λ	CLL, #77	V10-54*01	95.79%	J3*02	CSAWDSSLSARVF
P6360	μ/κ	CLL, #148B	V1-5*03	92.11%	J5*01	CQQYTAFSVTF
L7911	μ/λ	CLL #201	V1-44*01	97.89%	J3*02	CAAWDDSLNAWMF
P775	μ/λ	CLL #201	V1-44*01	95.79%	J3*02	CTAWDDSLSSWLF
P22229	μ/λ	CLL #201	V1-44*01	95.44%	J3*02	CAAWDDTLNAWLF
L3961	μ/λ	CLL #201	V1-44*01	96.49%	J3*02	CAAWDDSLDAWLF
F_3687	μ/κ	CLL, fungi	V2-24*01	97.96%	J2*04	CMQATQLCSF
F_725	μ/κ	CLL, fungi	V2-24*01	97.96%	J2*04	CMQATQICSL
P19994	μ/κ	SMZL	V1-39*01	100.00%	J2*01	CQQSYSTPPDTF
P8761	μ/κ	SMZL	V1-8*01	99.64%	J1*01	CQQYYSYPRTF
P23776	μ/λ	SMZL	V1-40*01	98.26%	J3*02	CQSYDSSLSGSVF
P411	μ/κ	SMZL	V3-20*01	97.52%	J3*01	CQQYGNSPPITF
P13836	μ/λ	SMZL	V2-11*01	96.18%	J2*01	CCSYAGFYTSKAF
P5729	μ/κ	SMZL	V3-15*01	100.00%	J1*01	CQQYNNWPPTF
P11388	μ/κ	SMZL	V3D-11*02	99.28%	J1*01	CQQRSNWQWTF
P2120	μ/κ	SMZL	V4-1*01	99.64%	J3*01	CQQYYSTPFTF
P6820	γ/κ	SMZL	V2-24*01	97.28%	J1*01	CMQATHFPRTF
P8257	μ/κ	SMZL	V3-11*01	100.00%	J2*04	CQQRSNWPPVCSF
P11038	μ/λ	SMZL	V2-23*01	100.00%	J3*02	CCSYAGSSTVF
P2301	μ/κ	SMZL	V1-39*01	97.85%	J2*01	CQQSYSTLPYTF

A)

Result summary:	Productive IGH rearranged sequence (no stop codon and in-frame junction)		
V-GENE and allele	Homsap IGHV4-34*01 F	score = 1420	identity = 100.00% (285/285 nt)
J-GENE and allele	Homsap IGHJ4*02 F	score = 240	identity = 100.00% (285/285 nt)
D-GENE and allele by IMGT/JunctionAnalysis	Homsap IGHD3-16*01 F	D-REGION is in reading frame 1	
FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION	[25.17.38.11]	[8.7.13]	CARKCFRAKYYFDYW

B)

Result summary:	Productive IGL rearranged sequence (no stop codon and in-frame junction)		
V-GENE and allele	Homsap IGLV2-23*01 F, or Homsap IGLV2-23*03 F	score = 1435	identity = 100.00% (288/288 nt)
J-GENE and allele	Homsap IGLJ3*02 F	score = 175	identity = 100.00% (288/288 nt)
FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION	[25.17.36.10]	[9.3.9]	CCSYAGSSTVF

Figure 11. The confirmation of the insertion of the variable region of the IG into the plasmids after sequencing. Analysis of the plasmid carrying the (a) IGHV-IGHD-IGHJ and (b) IGK/LV-IGK/LJ gene rearrangements of case P11038 using IMGT/V QUEST tool.

1.1. Production of mAbs

The transfected HEK293T cells with the plasmids which contain the IG genes of heavy and light chains secreted CLL and SMZL mAbs at a concentration range of 1-20 μg/ml as measured by quantitative ELISA. After supernatant concentration using centrifugal filters with appropriate molecular weight cut-off, the mAbs concentration ranged between 70 and 400μg/ml. The integrity of recombinant mAbs was assessed by western blotting, using HRP conjugated anti-Human IgG or IgM antibodies (Figure 12).

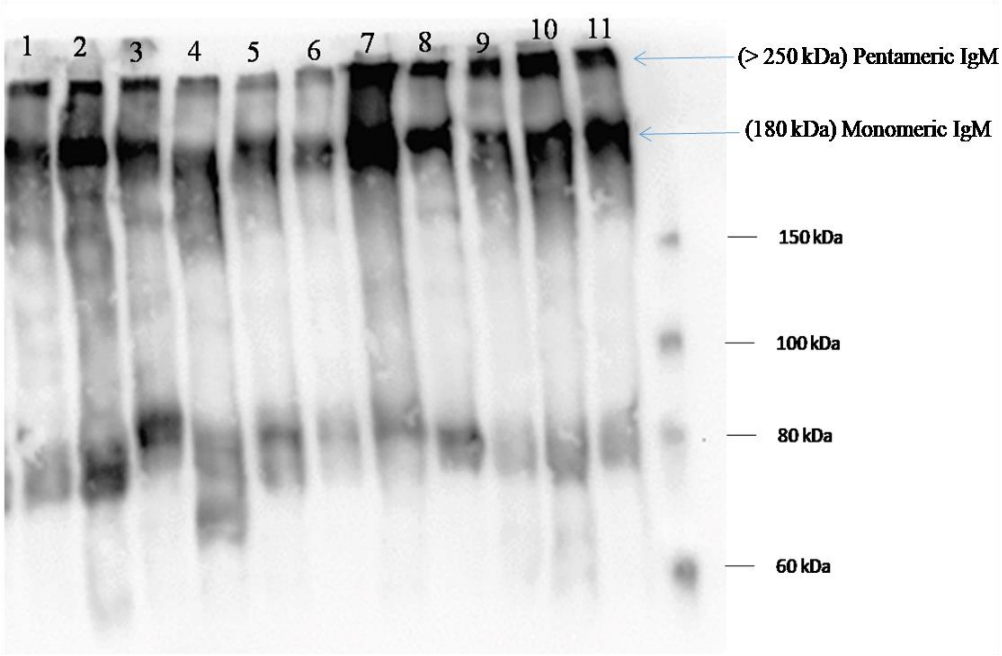

Figure 13. Example of SDS-PAGE of ten CLL and one SMZL recombinant IgM mAbs under non-reducing conditions: 1) P3870, 2) P14197, 3) RS038, 4) CLL412, 5) N4755, 6) P6275, 7) P14438, 8) L7911, 9) P19994, 10) F_725, 11) F_3687. Goat anti-Human IgM - HRP conjugated was used for Western Blotting.

2. Antigen reactivity profiling studies

SMZL and CLL mAbs expressed as either IgM or IgG (depending on the isotype of the authentic IG as assessed by flow cytometry/immunohistochemistry) paired with kappa or lambda light chains were used as primary antibodies in ELISA assays against a variety of autoantigens. Due to the nature of the indirect ELISA assays which were performed using a secondary antibody recognizing either the heavy or the light chain of the primary mAb, direct comparisons of the examined mAbs were possible when comparing mAbs expressing the same type of heavy or light chain.

2.1. Reactivity against dsDNA

The CLL IgM (Figure 13A) and IgG cases (Figure 13B) mAbs were grouped on the basis of BcR stereotypy to test the capability of distinct stereotyped BcRs to recognize dsDNA. Seven of 31 (22,5%) IgM mAbs, assigned to stereotyped subsets #1, #6, #7 and #10 (all U-CLL), were considered positive for dsDNA reactivity with OD values greater than the cut-off (dotted line) (Figure 13A). In particular, 3/6 subset #1, 2/2 subset #7 and 1/1 subset #10 mAbs were positive for dsDNA reactivity. In addition 1/4 subset #6 mAbs exhibited a borderline anti-dsDNA activity. All mAbs from M-CLL cases (subsets #77, #148B, #201 and #fungi) and borderline mutated subset #2/#169 cases were negative for dsDNA reactivity.

IgG mAbs included subset #4 (clinically very indolent and the largest M-CLL subset) [83][82][84] and subset #8 (clinically very aggressive, U-CLL subset) cases [85][133]. Subset #4 mAbs did not bind DNA, since in all 6 cases the OD values were below the cut off, while 2/6 (33%) subset #8 mAbs displayed anti-dsDNA reactivity. (Figure 13B).

IgM mAbs from SMZL cases using the most common IGHV genes (IGHV1-2*04 allele, IGHV4-34 and IGHV3-23)[116] were examined and 6/8 (75%) were positive for dsDNA reactivity, exhibiting values greater than the cut-off (dotted line). In particular, among IGHV1-2*04 mAbs 2/4 were positive: the minimally mutated P23776 case was a strong dsDNA binder and the unmutated P19994 was borderline positive. Two of 2 mAbs encoded by IGHV3-23, both minimally mutated (P2120 and P11388), were also positive. Finally, 2/2 unmutated IGHV4-34 cases (P8257 and P11038) were dsDNA binders (Figure 13C).

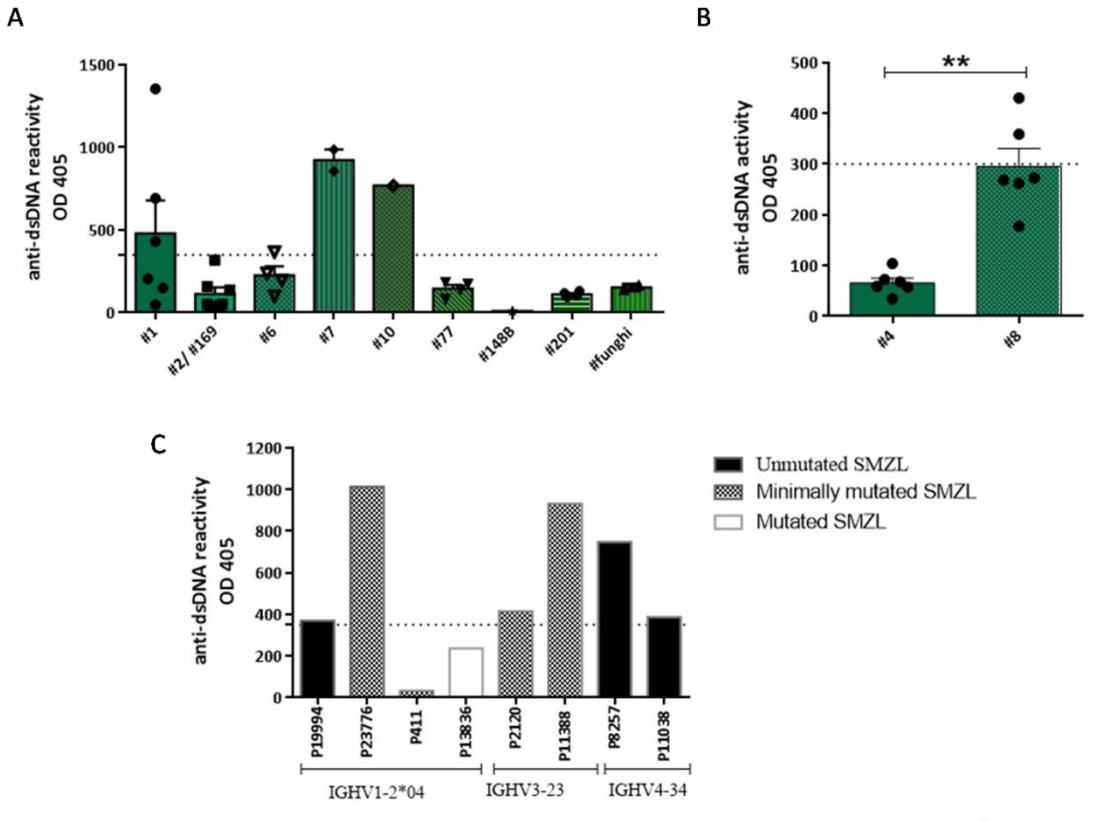

Figure 13. Binding of CLL and SMZL mAbs to dsDNA in ELISA. Anti-dsDNA reactivity of (A) CLL IgM mAbs derived from different CLL subsets, (B) CLL subset #4 and #8 IgG mAbs and (C) IgM SMZL mAbs.P<0.005.**

2.2. Reactivity against cytoskeletal proteins

It has been shown that CLL BcR IGs are able to recognize antigens exposed during cell apoptosis including cytoskeletal antigens [92][93][94][95][96][97][98]. Prompted by this, we examined the reactivity pattern of CLL and SMZL mAbs against the cytoskeletal proteins actin and myosin.

Eight of 31 (26%) IgM CLL mAbs, all derived from U-CLL subsets, displayed anti-actin reactivity. In detail, 4/6 subset #1 mAbs, 2/4 subsets #6 mAbs, 1/2 subset #7 mAbs and 1/1 subset #10 mAbs were positive (Figure 14A).

Regarding IgG expressing CLL, all (6/6, 100%) subset #8 cases were able to bind actin, contrasting with none (0/6, 0%) of the subset #4 mAbs. (Figure 14B).

Five of 10 (50%) SMZL IgM mAbs recognized actin (Figure 14C). In particular, 2/5 IGHV1-2*04 cases were positive (the unmutated P8761 and the minimally mutated P23776). One of 3 mAbs encoded by IGHV3-23 gene, the minimally mutated P11388, was positive too. Finally, P8257 and P11038, both unmutated, IGHV4-34 mAbs were positive for actin binding (Figure 14C).

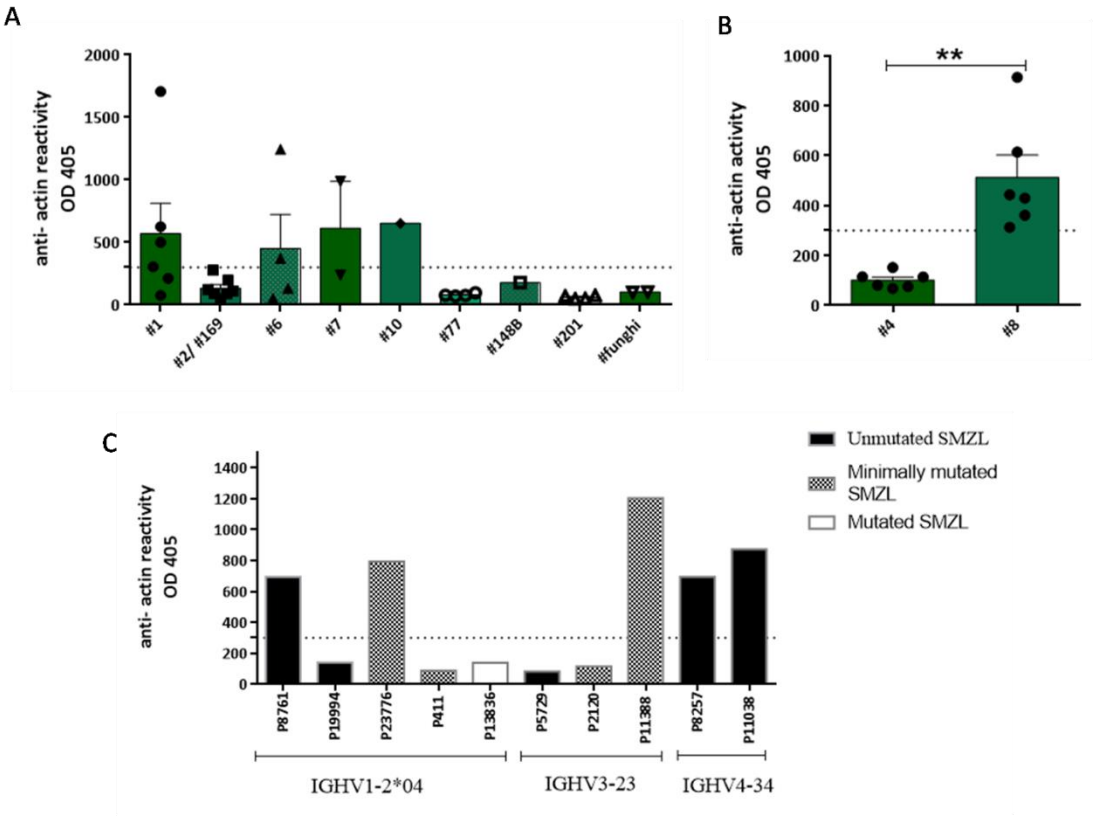

Figure 14. Binding of CLL and SMZL mAbs to actin in ELISA. Binding to actin of (A) IgM mAbs derived from different CLL subsets, (B) CLL subset #4 and #8 IgG mAbs and (C) IgM SMZL mAbs.P<0.005**

The anti-myosin ELISA assays revealed low binding to myosin by a minority of CLL and SMZL mAbs (Figure 15). Only 5/ 31 (16%) IgM CLL mAbs, derived from the unmutated subsets #1, #6, #7 and #10 bound to myosin. In particular, 2/6, 1/4, 1/2 and 1/1 mAbs of CLL subsets #1, #6, #7 and #10, respectively, were considered positive (Figure 15A). None (0/6, 0%) of the IgG mAbs from mutated CLL subset #4 bound to myosin while 3/6 (50%) subset #8 mAbs presented anti-myosin activity. (Figure 15B).

Two of 9 (22%) IgM SMZL mAbs bound to myosin. In particular, only 1/3 (P11388-minimally mutated) and 1/2 (P11038-unmutated) SMZL mAbs, encoded by IGHV3-23 and IGHV4-34 genes respectively, bound myosin, while none of the 4 tested IGHV1-2*04 mAbs were found positive (Figure 15C).

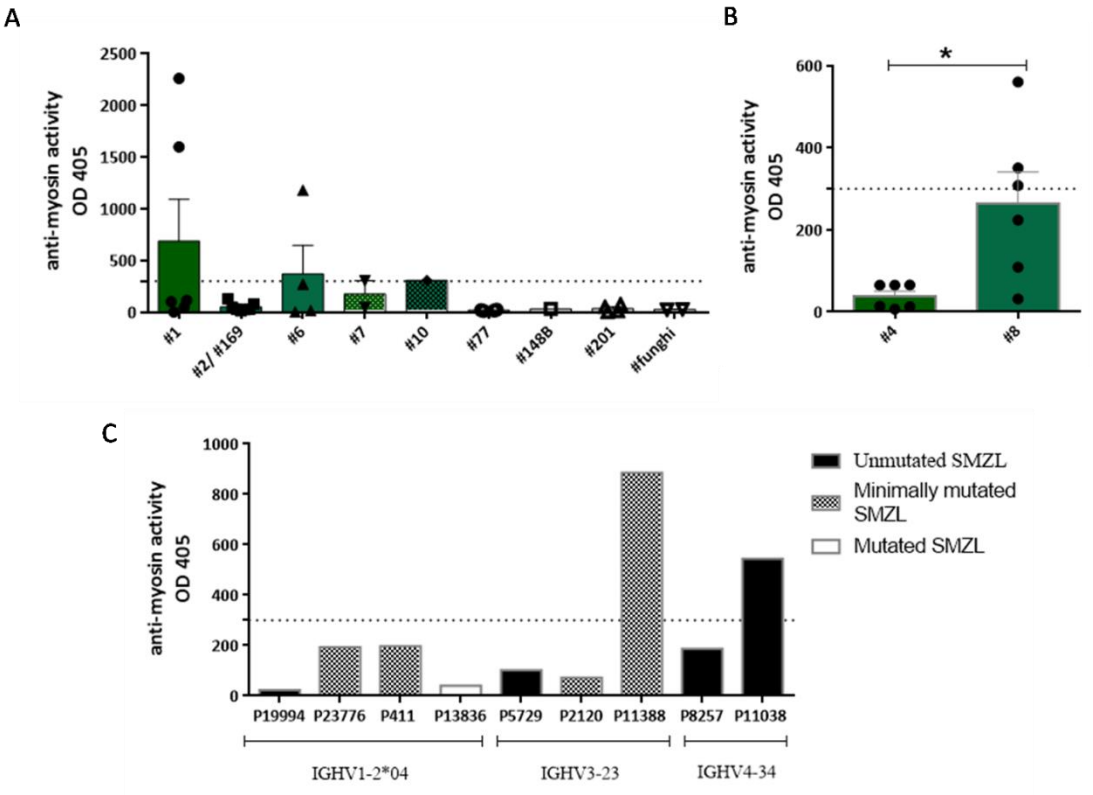

Figure 15. Binding of CLL and SMZL mAbs to myosin in ELISA assays. Anti-myosin reactivity of (A) IgM mAbs from different CLL subsets, (B) CLL subset #4 and #8 IgG mAbs and (C) IgM SMZL mAbs. * P value <0.05

2.3. Reactivity against thyroglobulin

Three of 29 (10%) of IgM CLL mAbs, derived from the unmutated stereotyped subsets #1 and #6, bound to thyroglobulin (TG). In detail, 2/6 subset #1 mAbs and 1/4 subset #6 mAbs were TG binders (Figure 16A).

CLL IgG mAbs did not exhibit anti-TG reactivity, since none of the subset #4 (0/6, 0%) or subset #8 (0/5, 0%) mAbs had OD values greater than the cut off (dotted line) in the anti-TG ELISA assays (Figure 16B). The subset #8 mAbs tended to have greater values than subset #4 mAbs but the difference was not statistically significant.

Two of 5 (40%) analyzed SMZL cases exhibited some binding to TG (Figure 16C). The 2 positive cases (P23776 and P8257) were encoded by IGHV1-2*04 and IGHV3-23 genes with borderline or no SHM load, respectively (Figure 16C).

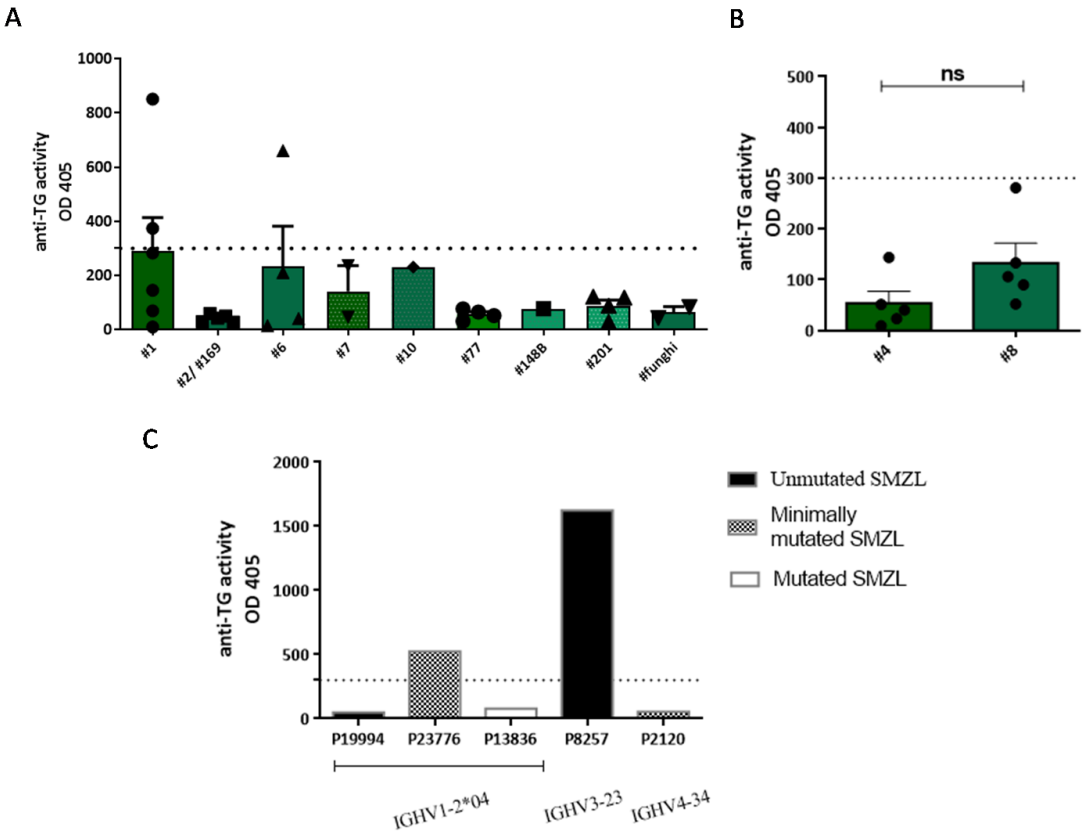

Figure 16. Binding of CLL and SMZL mAbs to TG in ELISA. Anti-TG reactivity of (A) CLL IgM mAbs derived from different CLL subsets, (B) IgG mAbs from CLL subsets #4 and #8 and (C) IgM SMZL mAbs. ns: no significant.

2.4. Reactivity against β-amyloid

Five of 29 (17%) analyzed IgM CLL mAbs exhibited reactivity against β-amyloid protein. In detail, all β-amyloid binder mAbs derived from the U-CLL subsets #1 (2/6 binder mAbs), #6 (2/4 binder mAbs) and #7 (1/2 binder mAbs) (Figure 17A).

None of the 5 (0%) CLL subset #4 IgG mAbs bound β-amyloid, while 3/5 (60%) subset #8 IgG mAbs displayed anti-β-amyloid binding. (Figure 17B).

Two of 5 SMZL (40%) mAbs recognized β-amyloid: the minimally mutated IGHV1-2*04 encoded mAb P23776 and the unmutated IGHV3-23 encoded mAb P8257 (Figure 17C).

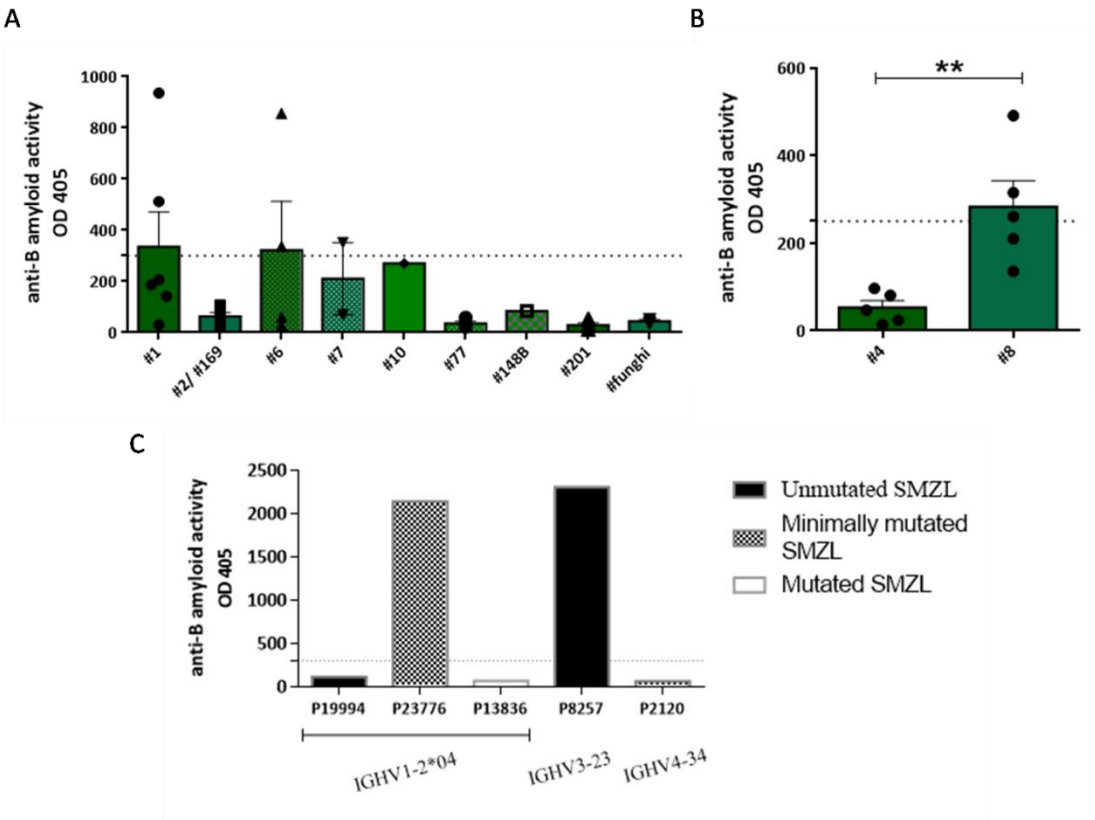

Figure 17. Binding of CLL IgG and SMZL mAbs to β-amyloid in ELISA assays. Anti-β-amyloid reactivity of (A) IgM mAbs from different stereotyped CLL subsets. (B) CLL subset #4 and #8 IgG mAbs and (C) IgM SMZL mAbs. **P<0.005

2.5. Reactivity against Carbonic anhydrase

None of the 5 (0%) IgM CLL tested mAbs derived from subsets #2, #6 and #201 recognized carbonic anhydrase (CA)(Figure 18A). Similarly, all 4 CLL IgG mAbs, 2 each from subset #4 and #8, were negative for anti-CA reactivity (Figure 18B).

Two of 6 (33%) SMZL mAbs were positive for anti-CA reactivity, namely the minimally mutated IGHV3-23 mAb P11388 and the unmutated IGHV4-34 mAb P8257. Another unmutated IGHV4-34 mAb (P11038) exhibited borderline anti-CA reactivity (Figure 18C).

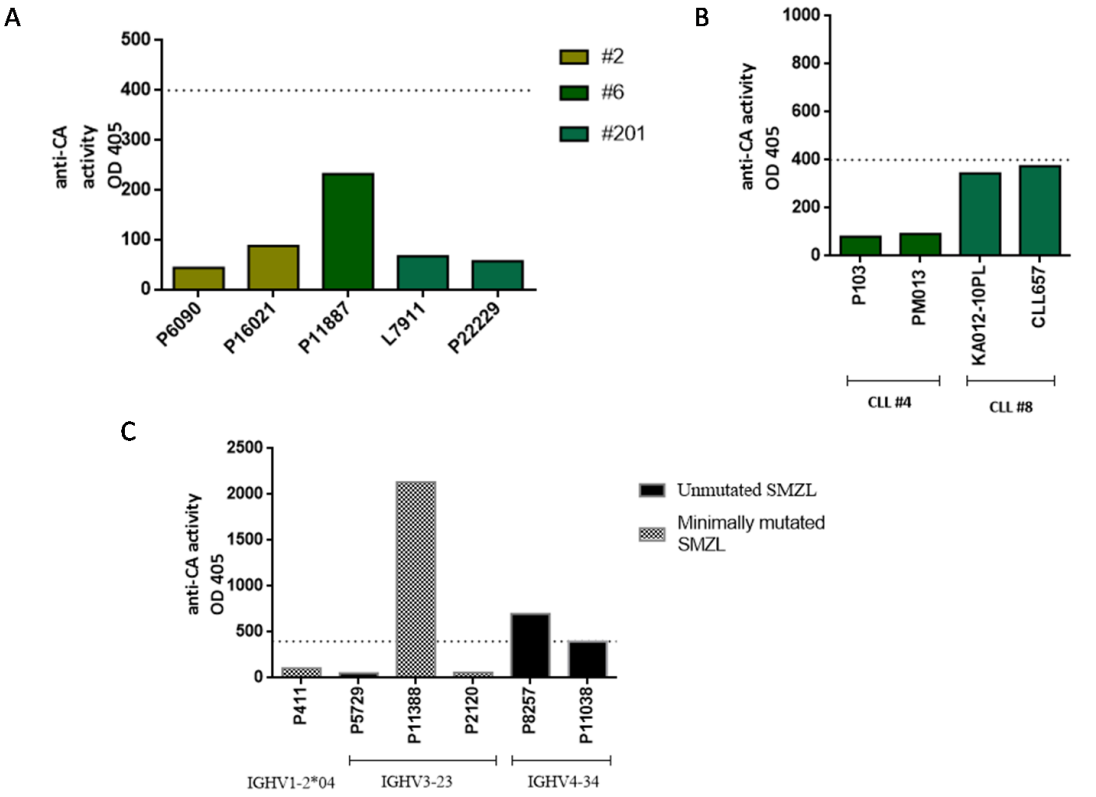

Figure 18. Binding of CLL and SMZL mAbs to CA in ELISA. Binding of (A) IgM mAbs from different CLL subsets, (B) CLL subset #4 and #8 IgG mAbs and (C) IgM SMZL mAbs.

2.6. Reactivity against human Fab'2

Only 1/5 (20%) analyzed IgM CLL mAbs recognized the F(ab')2 fragment of human immunoglobulin, namely the subset #6 P11887 mAb; all remaining cases, namely 2/2 borderline mutated subset #2 mAbs and 2/2 mAbs of the mutated subset #201 were negative for anti-Fab'2 reactivity (Figure 19A).

Two of 2 (100%) subset #8 IgG mAbs were positive for Fab'2 reactivity, contrasting both tested #4 IgG mAbs that were negative (Figure 19B).

Anti-F(ab')2 reactivity was more common among the SMZL mAbs, since 3/6 (50%) tested mAbs bound efficiently to F(ab')2 (Figure 19C): 1/3 IGHV3-23 mAbs (the minimally mutated P11388 mAb) and 2/2 IGHV4-34 mAbs (the unmutated P8257 and P11038 mAbs); the single tested IGHV1-2*04 mAb did not recognize this antigen (Figure 19C).

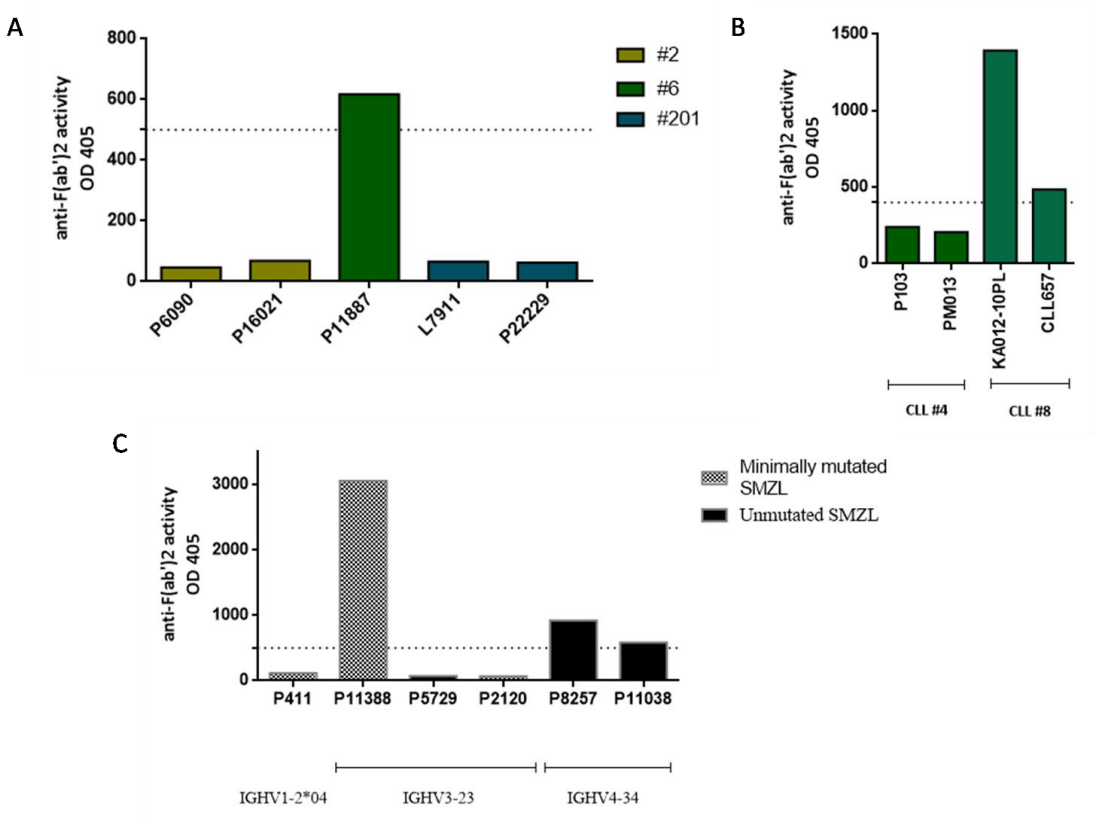

Figure 19. Binding of CLL and SMZL mAbs to F(ab')2 in ELISA assays. Anti-Fab'2 reactivity of (A) CLL IgM mAbs from different CLL subsets, (B) CLL subset #4 and #8 IgG mAbs and (C) SZML IgM mAbs.

2.7. Reactivity against Tri-Nitro-Phenyl (TNP) hapten

Only 3/31 (10%) of IgM CLL mAbs bound to the non-self hapten TNP. In detail, 1/5, 1/4 and 1/1 subset #1, #6 and #10 mAbs respectively were positive (Figure 20A).

Three/6 (50%) of IgG subset #8 mAbs were positive, contrasting 0/6 (0%) of the IgG subset #4 mAbs (Figure 20B).

Four of 9 (44%) IgM SMZL mAbs bound to TNP (Figure 20C): 1/4 IGHV1-2*04 mAbs, (the minimally mutated P23776), 1/3 IGHV3-23 mAbs, (the minimally mutated P11388) and 2/2 IGHV4-34 mAbs (the unmutated P8257 and P11038) were positive for anti-TNP reactivity (Figure 20C).

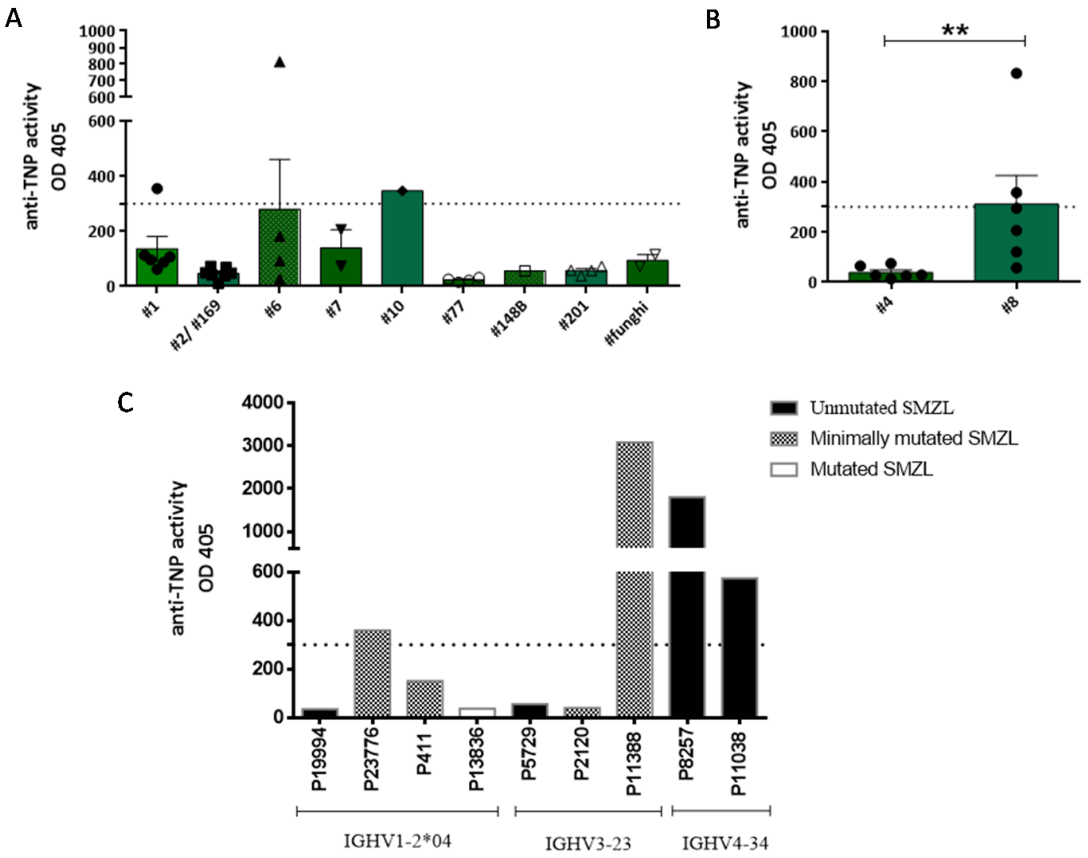

Figure 20. Binding of CLL and SMZL mAbs to TNP in ELISA. Anti-TNP reactivity of (A) CLL IgM mAbs derived from different CLL subsets, (B) IgG CLL mAbs from subsets #4 and #8 and (C) IgM SMZL mAbs. **P value<0.005.

3. Impact of isotype switching on antigen specificity

CLL subset #8 is defined by the expression of stereotyped, unmutated IGHV4-39/V1(D)-39 BcR IGs, exclusively of the G isotype [134]. Prompted by several observations suggesting that the IG constant region may affect the antibody fine specificity [101][135][102][103], we assessed the impact of IgG switching in 4 CLL subset #8 cases. To this end, the clonotypic IGs from cases US845, KA012-10PL, Nov5889 and CLL657 were cloned also as IgMs and the resulting mAbs were named with the suffix.mu (US845.mu, KA012-10PL.mu, Nov5889.mu, CLL657.mu), in order to distinguish them from the authentic CLL #8 IgG mAbs.

Next, the capacity of the wild-type IgG and the manipulated IgM CLL subset #8 mAbs to bind to the antigens actin and CA was tested by ELISA. The reactivity of IgG vs IgM subset #8 mAbs towards these antigens was directly compared in the same ELISA experiment using an anti-kappa light chain secondary antibody. In parallel, as control mAbs for the immunoassays, we used non-subset #8 CLL and SMZL kappa

expressing mAbs, previously used in ELISA assays with anti-IgM secondary antibody (results, chapter 2). The relative OD values of the control kappa expressing mAbs were comparable with the OD values of the ELISA assays with anti-IgM secondary antibody (results, chapter 2), thus confirming the reliability and reproducibility of the ELISA assays. Furthermore, 2 lambda expressing SMZL mAbs were included as technical controls, that although being positive for anti-actin reactivity when tested using anti-IgM secondary antibody (results, chapter 2), exhibited no reactivity when tested using an anti-kappa light chain secondary antibody (Figure 21A and B).

All IgM subset #8 mAbs exhibited higher OD values than their corresponding IgG mAbs for both actin (Figure 21A) and CA (Figure 21 B). In addition, at cohort level, the IgM subset #8 mAbs displayed significantly stronger binding to actin than their corresponding IgG mAbs ($p=0.028$) and also a tendency ($p=0.125$) for stronger recognition of CA (Figure 21C).

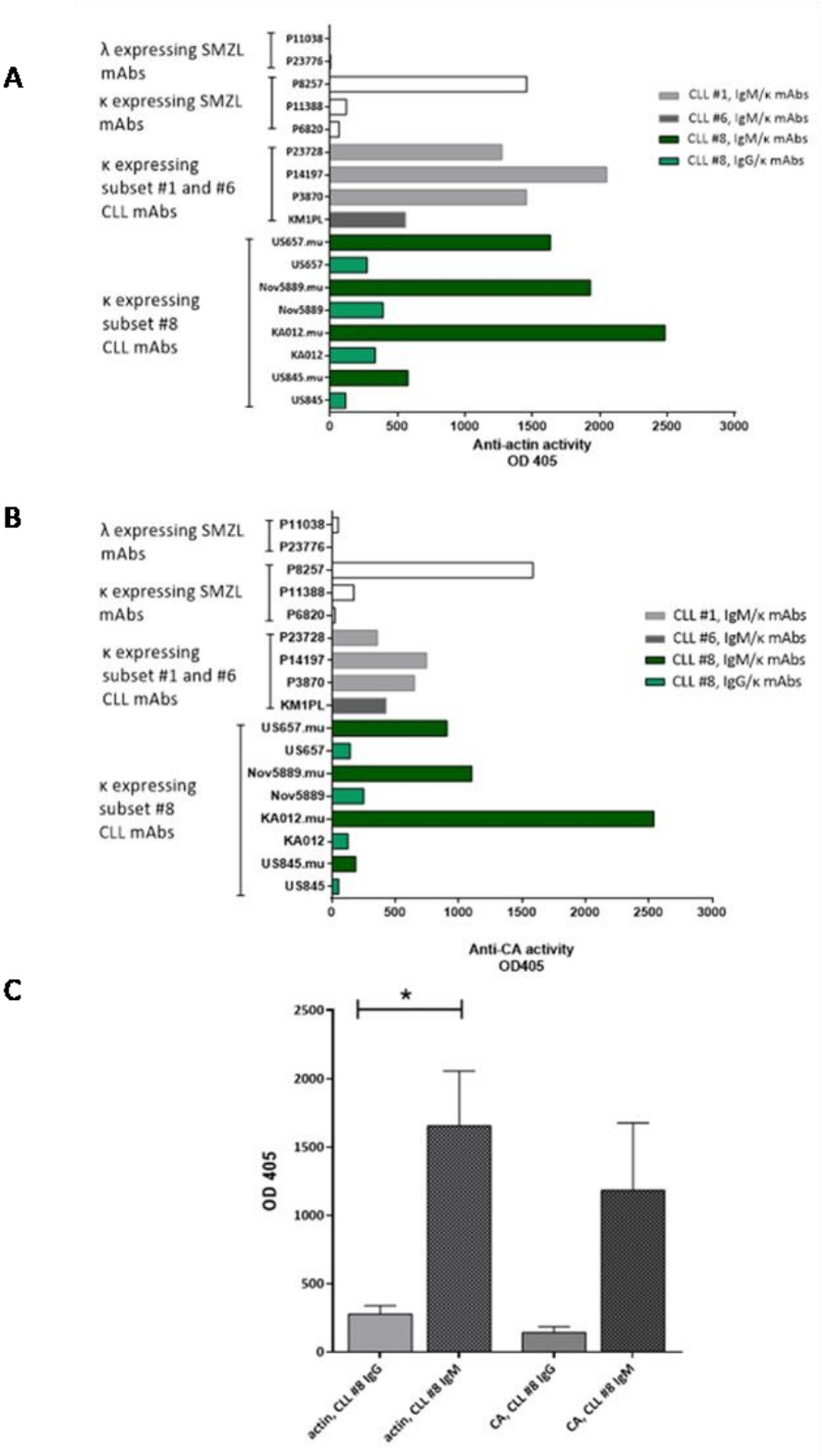

Figure 21. Binding of CLL subset #8 mAbs expressed as IgG or IgM to actin and CA in ELISA assays using anti-kappa light chain secondary antibody. Non-subset #8 CLL and SMZL kappa or lambda expressing mAbs were used as controls. (A) Anti-actin reactivity. (B) Anti-CA reactivity. (A, B) Each bar on the y axis corresponds to a distinct mAb. (C) The subset #8 mAbs were grouped on the basis of isotype to compare anti-actin and anti-CA binding ability of IgG vs IgM CLL subset #8 mAbs (n=4 for each group).

4. Binding of CLL and SMZL mAbs on viable HEK293T cells

Recent studies revealed that many CLL mAbs, mostly deriving from U-CLL cases, are capable of recognizing molecules on the surface of apoptotic cells, including human lymphocytes [92][93]; reactivity also against viable cells has been detected for a M-CLL subset [136]. In addition, SMZL mAbs utilizing the IGHV1-2*04 allele have been shown to recognize peptides on the surface of various human cell lines [124].

Based on the above, we tested by flow cytometry whether the CLL and SMZL mAbs were able to bind molecules on the surface of the HEK293T cell line. To this end, HEK293 cells were incubated with recombinant CLL and SMZL mAbs, followed by incubation with anti-human κ-PE or anti-human λ-PE secondary Ab. Thirty-two CLL and 11 SMZL mAbs were analyzed. The CLL mAbs were grouped on the basis of BcR stereotypy, while SMZL were grouped based on the utilization of different IGHV genes and the SHM status of the IGHV genes. The thresholds of positivity were set at 5% PE-positive HEK293T cells (low positive) and 10% PE-positive HEK293T cells (positive).

Strong interaction with HEK293T cell surfaces was shown mainly for mAbs from U-CLL stereotyped subsets. In detail, 4/6 subset #1 mAbs, 2/2 subset #7 mAbs, 1/2 subset #8 mAbs and 1/1 subset #10 mAb were positive. Interestingly, also 3/4 mAbs of the mutated stereotyped subset #201 were positive. On the other side, all mAbs of the remaining M-CLL subsets (subsets #4, #77 and #fungi) did not show any remarkable reactivity (Figure 22). Finally, 2/6 mAbs of the borderline mutated subset #2 bound to the surface of HEK293 cells (Figure 22).

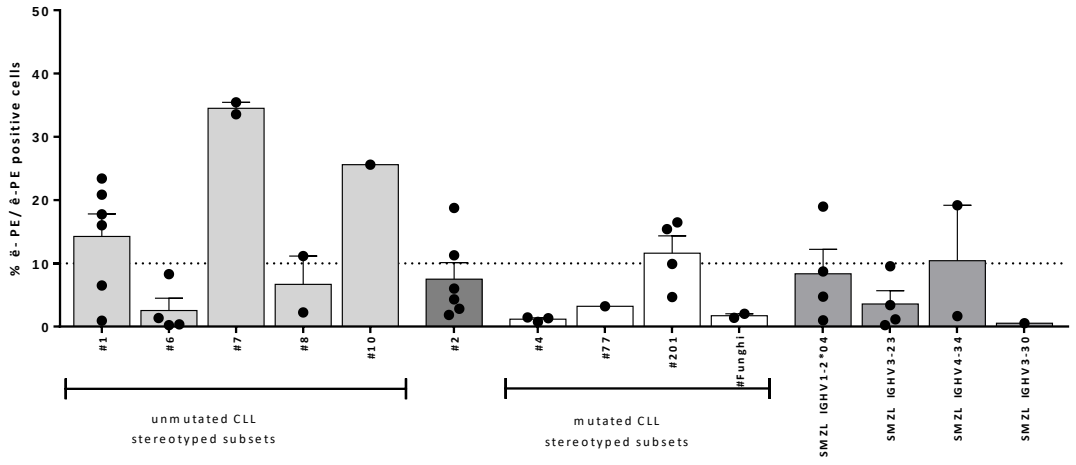

Figure 22. Binding of CLL and SMZL mAbs to HEK293T cell surfaces. The error bars are standard deviations. Dotted line represents the positivity cut off.

Two of 11 (18%) tested SMZL mAbs exhibited strong binding (>10% of HEK293 positive cells), namely the IGHV4-34 mAb P11038 and the IGHV1-2*04 mAb P19994 (Figure 22, Figure 23A,B). In addition, 2/4 IGHV1-2*04 mAbs (P23776, P411) and 1/4 IGHV3-23 mAbs (P11388) recognized HEK293T surface epitopes too, albeit to a lesser extent (≥5% PE-positive HEK293T cells, <10% PE-positive HEK293T cells) (Figure 23A, B). Interestingly, the IGHV mutational status appeared to have a significant impact on HEK293T cell surface binding, since statistically higher binding was observed for unmutated (p=0.0011) or minimally mutated (p=0.016) mAbs compared to mutated mAbs (Figure 23C).

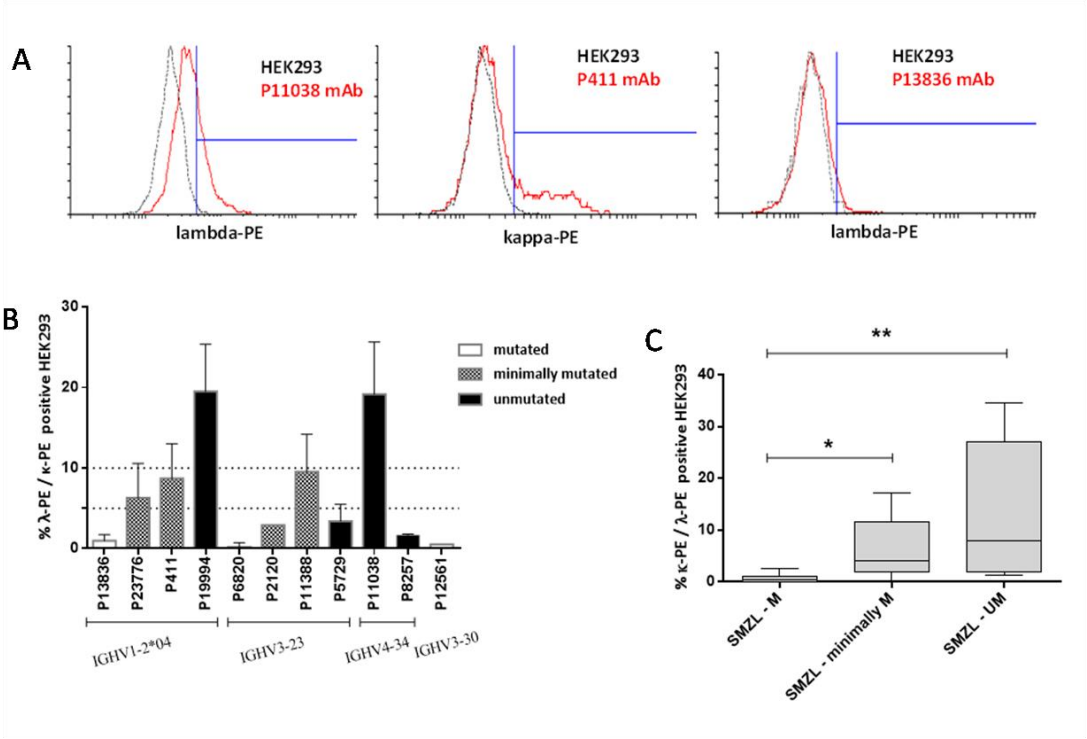

Figure 23. Flow cytometric analysis of the binding of SMZL mAbs to HEK293T cells. A) Examples of flow cytometry analysis of 3 mAbs with different IGHV usage and different IGHV mutation load: P11038 mAb utilized the IGHV4-34 gene with 100% identity to the germline, P411 and P13836 mAbs utilize the IGHV1-2*04 allele with 97,57% and 94,10% identity to the germline respectively. B) Binding of SMZL mAbs encoded by different IGHV genes to HEK293T cells. Dotted lines represent the high (10%) and low (5%) positivity cut off. Values are means from 3-5 separate experiments for each one of the mAbs tested. C) Comparison of binding ability of mutated, minimally mutated and unmutated SMZL mAbs. *P value<0.05, **P value<0.005

Discussion

Accumulating functional, clinical and immunogenetic evidence corroborates the significance of the interactions between the B cell receptor immunoglobulin (BcR IG) and (auto)antigens in the ontogeny and evolution of malignancies of mature B cells, including CLL and SMZL. Nevertheless, open issues abound regarding the antigens recognized by the clonotypic BcR IG. In particular, the nature of the antigens recognized by the M-CLL BcR IGs still remains elusive, while the antigens involved in the natural history of SMZL are mostly unknown.

In the present master degree thesis, we studied the antigen reactivity profile of several M-CLL and U-CLL stereotyped subsets and, for the first time, SMZL mAbs encoded by four of the most frequent IGHV genes in SMZL, namely the IGHV1-2*04, IGHV3-23, IGV4-34 and IGHV5-51 genes [116].

The limited capacity of CLL cells to grow *ex vivo*, combined with the low amount of surface IGs produced by the leukemic cells, hinder investigations into the functional properties of the clonotypic BcR IGs. Various techniques have been employed to overcome these limitations, including stimulation of the malignant clones with phorbol myristate acetate, hybridoma technology and transformation of leukemic cells with the EBV virus [89][90][137]. However, hybridomas as well as EBV transformed CLL cells are often unstable and incapable to produce satisfactory levels of IGs [138][139]. More recently, the technology of recombinant DNA was utilized by Catera et al [92] and Warsame et al [124] for the study of the antigen specificity of CLL and SMZL mAbs, respectively. However, despite the fact that the majority of CLL and SMZL cases express surface IGs of the IgM/D isotype [140][107], in the aforementioned studies the CLL and SMZL mAbs were produced as human IgG1. Although it is generally thought that the isotype determines only the effector functions of IGs [29], many studies indicate that the constant region of IGs is implicated also in antigen specificity [101][135][102]. In addition, a structural and functional study of BcR in CLL showed that the constant region of IGs is directly implicated in homotypic CLL BcR interactions resembling classic antibody-antigen interactions [103][141].

Considering all of the above, in the present thesis we decided to retain the authentic heavy chain isotype on the belief that the respective recombinant mAbs would mirror more faithfully the properties of the clonotypic BcR IGs expressed on the surface of leukemic clones.

Altogether, 31 and 11 CLL cases expressing BcR IGs of the IgM/D or IgG isotype respectively and 11 and 1 SMZL cases expressing BcR IGs of the IgM/D and IgG isotype, respectively, were studied. Prompted by previous findings showing poly- and self-reactivity of CLL and SMZL mAbs [93][94][124][96][95], the produced mAbs were tested against a panel of antigens that are common targets of naturally and disease occurring antibodies, using ELISA assays. Importantly, reactivity against β-amyloid, carbonic anhydrase, hapten TNP and the F(ab')2 fragment of human serum IgG was here examined for the first time for both CLL and SMZL. Furthermore, using flow cytometry, we assessed the recognition of epitopes on the surface of viable HEK293 cells, again not tested previously for CLL mAbs or SMZL mAbs that express other IGHV genes than IGHV1-2*04.

Binding to (auto)antigens

Anti-dsDNA binding was observed for mAbs derived from CLL stereotyped subsets #1, #6 #7, #8 and #10. These stereotyped subsets utilize IGHV genes in unmutated configuration and are generally clinically aggressive [82][133][84][83]. Our results confirm previous studies showing that CLL cases, mostly those derived from U-CLL, have low anti-dsDNA reactivity [91][95]. Anti-DNA binding was more common in SMZL mAbs compared to CLL mAbs (75% of SMZL vs 22.5% of CLL mAbs were positive for anti-dsDNA activity). In contrast to CLL, no association between dsDNA reactivity and the utilization of distinct IGHV genes or the IGHV mutational status was noted in SMZL.

Double stranded DNA is frequently exposed on the cell surface during apoptosis and recognized by auto-antibodies [142]. Indeed, dsDNA is a common target of IGHV4-34 utilizing antibodies in systemic lupus erythematosus [143]. In this study, IGHV4-34 encoded SMZL mAbs bound DNA, whereas IGHV4-34 CLL mAbs (stereotyped subsets #4 and #201) did not recognize this antigen; relevant to mention, previous studies have shown that anti-DNA reactivity was abolished by SHM in subset #4 [133] [153].

Several cytoskeletal proteins such as actin, tubulin, vimentin and nonmuscle myosin heavy chain IIA (MYHIIA) that are also exposed on the cell surface during apoptosis, have been found as common targets of CLL mAbs [144][90][94][96][95], whereas reactivity against cytoskeletal components has been reported for IGHV1-2*04

SMZL mAbs [124]. High levels of antibodies against cytoskeletal antigens are associated with autoimmune conditions such as celiac disease, autoimmune hepatitis and rheumatoid arthritis and, at least in some cases, also have diagnostic value [145][146]. CLL mAbs showed a similar recognition pattern for both cytoskeletal antigens tested in the context of this thesis (actin and myosin). In particular, in accordance with previous studies [96][95][144], only the U-CLL mAbs from stereotyped subsets #1, #6, #7, #8 and #10, recognized actin and/or myosin. A higher incidence of anti-actin and anti-myosin binding was observed in SMZL compared to CLL (50% of SMZL mAbs vs 26% of CLL mAbs were positive for actin and 22% of SMZL mAbs and 16% of CLL mAbs were positive for myosin). In contrast to CLL, the mutational status of IGHV genes did not affect significantly the binding of SMZL mAbs to actin and myosin since both minimally mutated and unmutated SMZL mAbs bound to these antigens.

The produced CLL and SMZL mAbs were also tested against two organ-specific antigens: thyroglobulin (TG) and β-amyloid. TG is protein exclusively located in the thyroid gland and anti-TG antibodies are elevated in autoimmune diseases such as Hashimoto's thyroiditis [147]. On the other hand, the physiological role of β-amyloid is not fully understood [148]. In Alzheimer's disease, β-amyloid is the main component of the amyloid plaques in the brains of the patients [149]. Naturally occurring auto-antibodies in the central nervous system that target, amongst others, β-amyloid, are involved in maintaining homeostasis by removing debris and are known to prevent inflammation [150]. Interestingly, in Alzheimer's patients, the levels of anti-β-amyloid natural antibodies are lower than those of healthy individuals [151].

Reactivity against both antigens was not a common feature among CLL mAbs. Yet, few CLL cases, all derived from the U-CLL stereotyped subsets #1 and #6, #7 and #8, bound efficiently to TG and/or β-amyloid. In contrast, both anti-TG and anti β-amyloid reactivities were more common among SMZL IgM mAbs (40% of positive SMZL mAbs for both reactivities vs 10% and 17% of positivity in CLL mAbs for TG and β-amyloid, respectively). Interestingly, Warsame et al, also showed that the SMZL mAbs encoded by IGHV1-2*04 allele were able to bind TG [124]; in the present study SMZL mAbs utilizing the IGHV3-23 gene were also TG binders.

SMZL mAbs encoded by the IGHV3-23 and IGHV4-34 genes recognized also carbonic anhydrase (CA), a protein involved in the maintenance of acid-base balance.

Interestingly, anti-CA auto-antibodies have recently been demonstrated in various pathological conditions, such as autoimmune diseases (systemic lupus erythematosus, primary biliary cirrhosis, rheumatoid arthritis, and Sjögren's syndrome) and carcinomas (lung, colon, and prostate) [152].

Prompted by the fact that naturally occurring antibodies are frequently polyreactive, recognizing self and non-self structures, including haptens, and are susceptible to idiotype/ anti-idiotype interactions, we also tested the binding of the recombinant mAbs to the hapten TNP and the F(ab')2 fragment of human serum IgG (as a way of assessing idiotype/ anti-idiotype interactions). Similarly to the other tested autoantigens, among the CLL mAbs only those derived from the U-CLL stereotyped subsets #1, #6, #8, #10, were able to recognize TNP and/or F(ab')2. A higher percentage of SMZL mAbs, mostly encoded by the IGHV3-23 and IGHV4-34 genes, bound to both antigens (44% and 50% for TNP and F(ab')2 respectively).

Altogether, polyreactivity and autoreactivity was shown for the mAbs of the unmutated and clinically aggressive CLL stereotyped subsets #1 (clonotypic IG encoded by IGHV genes belonging to clan I) [87][153][82][83], #6, #7 (clonotypic IGs encoded by the IGHV1-69 gene, the most frequent IGHV gene in CLL) [83][82][64] and #8 (clonotypic IG encoded by IGHV4-39 gene) [83][133][85][154] that recognized the majority of the examined antigens and epitopes on the surface of viable human epithelial HEK293 cells.

mAbs of the borderline mutated stereotyped subset #2 that is associated with extremely adverse clinical course did not recognize any of the antigenic elements tested in the present thesis. These results are consistent with previous studies in which subset #2 mAbs failed to bind "classical" CLL antigenic targets such as oxidized LDL or apoptotic cells [93][92]. This lack of reactivity could be attributed to the very short VH CD3 region (only 9 amino acid residues) [82][83] which may result in a limited repertoire of configurations of the CDR3 loop.

mAbs derived from stereotyped subset #4, the largest mutated stereotyped subset defined by the expression of IGHV4-34/V2-30 encoded BcR IGs of the IgG isotype and a prototype of indolent disease [83][82], did not present reactivity against any of the examined antigens, confirming and further extending findings from previous studies [136][155][92]. Interestingly, it has been suggested that the lack of autoreactivity in subset #4 may be due to the SHM serving an editing function

[136][155] for these intrinsically autoreactive antibodies [156]. We speculate that SHM could act in a similar way also in stereotyped subset #201, another M-CLL IGHV4-34 stereotyped subset that did not bind to any antigen in the ELISA assays of the present study. However, unlike most subset #4 and other M-CLL mAbs, subset #201 mAbs bound to viable HEK293 cells. Nevertheless, further experiments should be performed to characterize the exact recognized epitope(s).

SMZL generally exhibited pronounced poly- and auto-reactivity. In particular, 5/10 (50%) SMZL IgM mAbs recognized more than 2 antigens and were characterized as polyreactive. Moreover, each of the polyreactive SMZL mAbs exhibited a distinct antigen polyreactivity profile, while heterogeneity was observed even amongst cases expressing the same gene or cases with similar IGHV gene mutational load.

The role of the IG isotype

The expression of IgG-switched clonotypic BcR IGs is a rarity for CLL [134]. Interestingly, two major CLL stereotyped subsets, namely subsets #4 and #8 [134] are overrepresented in IgG-expressing CLL. Until recently, it was commonnly held that isotype switching changes only the effector functions of IGs without altering the antigen specificity [29]. However, there is emerging evidence that the constant region of IG may affect antibody antigen-recognition [101][102]. Interestingly, it was shown recently that in subset #4 isotype switching to IgG is essential for BcR IG self-association (homotypic CLL BcR interactions that resemble antibody-antigen interactions) [103][141] and recognition of epitopes on the surface of viable human lymphocytes [157].

Prompted by these findings, we sought to assess the importance of IgG switching in subset #8 mAb reactivity. Stereotyped subset #8 is defined by the expression of unmutated IGHV4-39/V1(D)-39 IgG BcRs [83][134]. Subset #8 is associated with extremely aggressive disease [85] and with the highest risk for Richter's syndrome among all CLL [133]. In the present study, we found that subset #8 mAbs recognized most of the tested antigens, hence confirming and further extending the findings of our previous study that demonstrated that subset #8 mAbs are excessively polyreactive and that this antigen polyreactivity may underlie the clinical aggressiveness [95]. Interestingly, the manipulated CLL subset #8 IgM mAbs bound even stronger to the autoantigens than the wild type IgG mAbs, indicating the isotype

switching in subset #8 affects significantly the affinity towards autoantigens, reducing autoreactivity.

Final Conclusions

In the present thesis we demonstrate that many U-CLL mAbs and SMZL mAbs have properties of naturally occurring auto-antibodies i.e polyspecificity/ recognition of conserved components such as dsDNA and actin, reactivity towards the non-self hapten TNP. Nevertheless, differences exist among distinct U-CLL stereotyped subsets, while, notably, cases grouped in the same stereotyped subset showed a similar antigen reactivity profile, highlighting the functional relevance of grouping CLL cases into stereotyped subsets. In addition, we show that the antigen fine specificity is affected not only by the SHM status in CLL but by the isotype switching as well.

When comparing the two entities under study, we observed a pronounced polyreactivity and autoreactivity of SMZL mAbs compared to CLL mAbs, implying differences in the antigen exposure history and/or in the subsequent immune responses to the (auto)antigen(s) of the corresponding malignant B cells and/or the pre-malignant progenitors.

Overall, the study of the antigens recognized by the clonotypic BcR IGs in mature B cell malignancies has important implications for both understanding the pathogenesis of the disease and for improving patient classification according to the type of antigenic stimulation. This can potentially lead to the development of targeted treatment approaches by interfering in the signaling between the antigen and malignant clone.

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