UNIVERSITY OF CRETE
MEDICAL SCHOOL

FUNCTIONAL ANALYSIS OF INNATE IMMUNITY RECEPTORS IN CHRONIC LYMPHOCYTIC LEUKEMIA

Vardi Anna
Medical Doctor

June 2010

CONTENTS

PREFACE

PART I: INTRODUCTION

1. Introduction to the immune system
   1.1. Structure and function of the human immune system
2. Innate immunity
   2.1. Pathogen-associated molecular patterns (PAMPs)
   2.2. Receptors of innate immunity
      2.2.1. Toll-like receptors (TLRs)
      2.2.2 Molecular structure of TLRs
      2.2.3. TLR ligands
      2.2.4. TLR signaling pathway
      2.2.5. MyD88-dependent pathway
      2.2.6. Alternative MyD88-dependent pathway
      2.2.7. TRIF-dependent pathway
      2.2.8. TLR expression
      2.2.9. Nod-like receptors (NLRs)
      2.2.10. NLR signaling
3. Adaptive immunity
   3.1. Antigens
   3.2. Receptors of adaptive immunity
      3.2.1. Immunoglobulin molecular structure and function
      3.2.2. Immunoglobulin gene rearrangement
      3.2.3. Somatic hypermutation
      3.2.4. Class switch recombination
   3.3. Development and survival of B cells
      3.3.1. Development of B cells in the bone marrow

3.3.2. Immune tolerance of B cells

3.3.3. Survival and maturation of B cells in peripheral lymphoid tissues

3.3.4. B-1 cells and marginal zone B cells

3.4. B cell activation and antibody production

3.4.1. T cell-dependent B cell activation

3.4.2. T cell-independent B cell activation

3.5. B cell receptor signaling pathway

3.6. TLRs in B cells

4. Chronic lymphocytic leukemia

4.1. CLL cytogenetics

4.2. MicroRNAs in the pathophysiology of CLL

4.3. The role of antigen stimulation in CLL

4.4. IGHV4-34 CLL subsets

4.5. CLL subset #1

4.6. BCR signaling in CLL cells

4.7. TLRs in CLL cells

4.8 CLL therapy: present and future

PART II: AIM OF STUDY

PART III: STUDY DESIGN

Workpackage:

Functional analysis of TLRs in CLL cells in terms of upregulation of costimulatory molecules. Comparison among CLL subsets.

Materials and methods

PART IV: RESULTS

PART V: DISCUSSION

PART VI: SUMMARY

PARTVII: ABSTRACT

PART VIII: REFERENCES

PART IX: SUPPLEMENTARY

PREFACE

The present master thesis was elaborated in the context of the Graduate Program “The Molecular Basis of Human Disease” of the Medical School of the University of Crete, under the supervision of Dr. Kostas Stamatopoulos, MD in the Hematology Department and HCT Unit, G. Papanicolaou Hospital, and Dr. Helen Papadaki, Professor of Hematology, Medical School, University of Crete. I sincerely thank them for their trust, guidance and valuable help, but most of all for being inspirational.

This study was performed at the Molecular and Cellular Biology Laboratory, as well as at the Flow Cytometry Laboratory of the Hematology Department and HCT Unit, G. Papanikolaou Hospital. I would like to thank the Directors Dr. Athanasios Fassas and Dr. Achilles Anagnostopoulos for giving me the opportunity to become a member of their working team.

The basic training, the experiments and the result analysis were actively directed by Kostas Stamatopoulos, who also provided crucial information from his CLL database. I have been lucky to have such an exceptional and thoughtful teacher of CLL and far beyond that.

This master thesis would not have been fulfilled without the help of the working team of the Molecular and Cellular Biology Laboratory. I would like to particularly thank Stavroula Ntoufa, Biologist and PhD Student, for welcoming me in her project. She has been both a trainer and a friend. I would also like to thank Eva Koravou, Nikos Papakostantinou, Maria Karypidou and Ntina Kotta for their help and patience, and Tassoula Touloumenidou, Head Biologist, for her support on everyday basis. The laboratory team created a warm and pleasant working environment and I am grateful to all for being so welcomed.

PART I: INTRODUCTION

1. Introduction to the immune system

The immune system evolved to protect the body from pathogenic agents, both extrinsic (infectious agents, environmental toxins etc.) and intrinsic (tumor cells, apoptotic cells etc.). Each component of such agents capable of inducing an immune response is called an antigen. Antigen recognition and immune defense rely on multiple mechanisms which act in a parallel and complementary way in order to produce an effective immune response. At the same time, microbial constituents other than antigens are now known to initiate the defense systems of innate immunity. Pattern recognition receptors (PRRs) of phagocytes recognize simple molecules and regular patterns of molecular structure called pathogen-associated molecular patterns (PAMPs) that are present on many microorganisms but not on the body's own cells. The innate immune system is thus able to broadly distinguish self from non-self. By becoming activated through their pattern recognition receptors, antigen presenting cells (APCs) of the innate immune system in turn become able to activate naïve T lymphocytes, thereby initiating the adaptive immune response. The adaptive recognition mechanism used by the lymphocytes has evolved to overcome the constraints faced by the innate immune system. It enables the recognition of an almost infinite diversity of antigens, so that each different pathogen can be specifically targeted.

1.1. Structure and function of the human immune system

At the cellular level, the organization of immune system is based on the coordinated action of various cell populations with distinct roles. All immune cells derive from a common precursor, the hematopoietic stem cell (HSC) of the bone marrow. Various hematopoietic growth factors induce the proliferation and differentiation of the pluripotent HSCs towards different cell types. This differentiation process depends on the expression of certain transcription factors

which control the transcription of lineage-determining genes. The myeloid lineage gives rise to most cells of the innate immune system (monocytes/macrophages, granulocytes, mast cells, dendritic cells). The lymphoid lineage, on the other hand, gives rise to the natural killer (NK) cells of innate immunity and the lymphocytes of the adaptive immune system.

At the tissue and organ level, the immune system comprises an extended network which provides constant surveillance. Cells of innate immunity either reside in tissues or circulate in the bloodstream and migrate to tissues in sites of inflammation. Lymphocytes of adaptive immunity circulate in the blood and the lymph and are also found in large numbers in lymphoid tissues or lymphoid organs, which are organized aggregates of lymphocytes within a scaffold of non-lymphoid cells. Lymphoid organs are broadly divided into central or primary lymphoid organs, where lymphocytes are generated, and peripheral or secondary lymphoid organs, where mature naïve lymphocytes are maintained, antigen is presented and adaptive immune responses are initiated. The primary lymphoid organs are the bone marrow and the thymus, whereas the secondary lymphoid organs are the lymph nodes, the spleen and the mucosa-associated lymphoid tissue (MALT). Lymph nodes are interconnected by a system of lymphatic vessels, which drain extracellular fluid from tissues, through the lymph nodes, and back into the bloodstream. Generally, the distribution and organization of the immune system are designed to increase the possibility of contact between pathogens and immune cells, so that a quick immune response is mounted.

To effectively protect the host against disease, the immune system must fulfill four main tasks. The first is immunological recognition. This is carried out both by the cells of the innate immune system, which provide an immediate response, and by the lymphocytes of the adaptive immune system. The second task is to contain and, if possible, eliminate the pathogen, which depends on immune effector cells and substances (e.g. phagocytes, complement, antibodies etc.). At the same time, the immune response has to be controlled so that the host is not damaged. Immune regulation is therefore a third and important feature of the immune system and failure at this level may lead to conditions such as

autoimmune disorders or allergy. Finally, the fourth task of the immune system is to provide protection against recurring disease due to the same pathogen. A unique feature of the adaptive immune system is the so called immunological memory, yet its exact mechanism remains vaguely understood [1].

2. Innate immunity

The microorganisms encountered daily in the life of a healthy individual only occasionally cause perceptible disease. Most are detected and eliminated within minutes or hours by defense mechanisms that do not rely on the clonal expansion of antigen-specific lymphocytes and thus do not require a prolonged period of induction. These are the mechanisms of innate immunity. Only when the innate host defenses are bypassed, evaded or overwhelmed is an adaptive immune response required. In this context, the innate immune system provides an initial discrimination between self and non-self. However, it is constrained by a rather limited and invariant repertoire of receptors used to recognize invading pathogens. The common pathogen constituents recognized by pattern recognition receptors of the innate immune cells are usually quite different from the pathogen-specific antigens that are recognized by lymphocytes. They are molecular structures that have remained conserved in the microbial taxa, but have disappeared from evolutionary evolved organisms.

2.1. Pathogen-associated molecular patterns (PAMPs)

Perhaps the most tantalizing question in immunology pertains to self/non-self discrimination. Detection of pathogens is a difficult task given their heterogeneity and constant evolution. Several recognition strategies have developed in metazoan species to deal with this issue. The most common is the pattern-recognition strategy, which is based on recognition of molecules that are

broadly conserved among microbial agents yet absent from the host. Such patterns (pathogen-associated molecular patterns, PAMPs) are sensed in the context of innate immunity by specific receptors, called pattern recognition receptors (PRRs), which serve as a first line of defense against invading microbial agents. The cell walls of Gram-positive and Gram-negative bacteria, for example, are composed of a matrix of proteins, carbohydrates and lipids in a repetitive array. The lipoteichoic acids of Gram-positive bacterial cell walls and the lipopolysaccharide of the outer membrane of Gram-negative bacteria play important roles in the recognition of bacteria by the innate immune system. Other microbial components also have a repetitive structure. Bacterial flagella consist of repeated protein subunits, while bacterial DNA contains unmethylated CpG islands. Most viruses express double-stranded RNA during their life cycle. All these repetitive structures that are conserved in the microbial taxa but are absent from the host serve as PAMPs and are recognized by the PRRs of the innate immune system.

2.2. Receptors of innate immunity

The pattern recognition receptors of the innate immune system may have several different functions. Some are phagocytic receptors which stimulate ingestion of the pathogens they recognize. The mannose-binding lectin (MBL), for example, which recognizes a particular orientation of sugar residues found only in microbes, once bound to the pathogen, opsonizes it and facilitates its phagocytosis. Other PRRs are chemotactic receptors, which guide cells of the immune system to sites of infection. The fMet-Leu-Phe (fMLP) receptor which recognizes formylated methionine residues of bacterial polypeptides is a typical example. This receptor is found on macrophages and neutrophils and its ligation guides these cells to sites of infection. Another important function of PRRs is to induce the production of effector molecules of the innate immune response and, moreover, to induce the production of proteins which will contribute in the

initiation and shaping of any subsequent adaptive immune response. Binding of pathogens to some receptors on macrophages or dendritic cells may signal for the upregulation of costimulatory surface molecules and enable them to act as antigen-presenting cells to T lymphocytes, thereby initiating an adaptive immune response. The best-defined activation pathway of this type is triggered through a family of evolutionary conserved transmembrane receptors called Toll-like receptors (TLRs), which will be discussed more extensively below.

2.2.1. Toll-like receptors (TLRs)

The Toll-like receptor (TLR) family is the best characterized class of PRRs in mammals. They belong to an evolutionarily ancient recognition and signaling system, originally discovered as a result of its role in the embryonic development in *Drosophila melanogaster*. It was subsequently found to have a role in the defense against bacterial and fungal infections in the adult insect and is now known to have a key role in the immune response of plants, adult insects and vertebrates, including mammals. The receptor mediating these functions in *Drosophila* is known as Toll, and the homologous proteins in mammals and other animals are therefore known as Toll-like receptors.

2.2.2 Molecular structure of TLRs

TLRs are single-spanning transmembrane proteins with ectodomains containing numerous leucine-rich repeats and a cytoplasmic domain containing a Toll/IL-1R identity region (TIR) motif *(Fig.1).* Ten members of the family have been identified so far in human (TLR1-10) and 13 in mice (TLR1-13) (reviewed in 2-3). Each TLR protein is devoted to recognizing a distinct set of molecular patterns that are not found in normal vertebrates, either as a homodimer or as a heterodimer with other TLRs.

The structure of a representative TLR (TLR3) has recently been solved through X-ray crystallography, revealing that TLR3 is a dimeric protein composed of two horseshoe-shaped subunits that stack together side by side [4]. The cytoplasmic domains of TLR1 and TLR2 have also been crystallized and their structures solved [5]. Each is compact and globular. A structure known as the BB loop is present in each TIR domain and is thought to be part of the signaling interface for interaction with adapter proteins, as will be discussed below. The similarity between TIR domains is such that threading programs can be used to model each TIR domain on those that have been directly analyzed by X-ray crystallography [6].

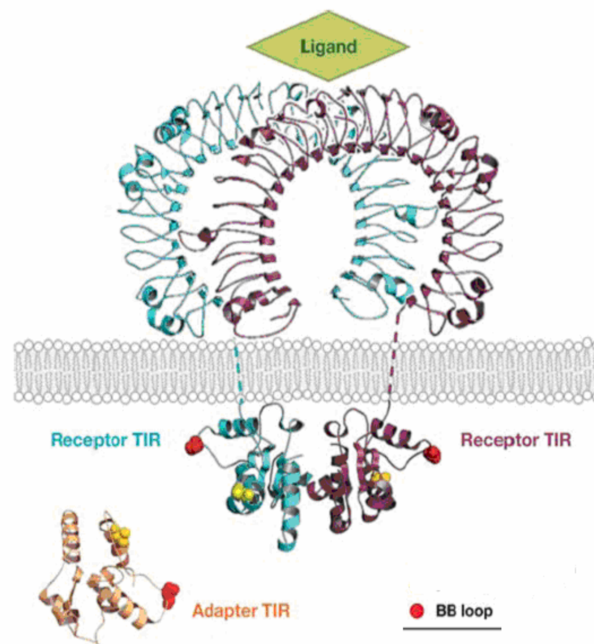

Fig.1. (Adopted and modified from Beutler et.al., Ref.10) Schematic representation of TLR structure, based on the structure of TLR3 ectodomain and the TIR motif of TLR2.

Some TLRs depend on other proteins to signal effectively. For instance, signaling through the TLR2/TLR6 heterodimer depends on both CD36, a double-spanning plasma membrane protein of the class B scavenger receptor family, and CD14 [7]. TLR4 depends even more strongly on CD14 than the TLR2/TLR6 heterodimer

and cannot mobilize all the adapter proteins that are required for full signaling activity without it [8]. In addition, a small protein called MD-2 is known to associate with the TLR4 ectodomain and be required for signal transduction [9].

2.2.3. TLR ligands

Some TLRs are clearly intracellular, residing within the endosomes. These include TLR3, -7, -8, and -9, which sense nucleic acids. Their compartmentalization ensures that they do not come in contact with nucleic acids of the host, but instead recognize nucleic acids released into endosomes after viruses or bacteria are internalized and lysed. TLR1, -2, -4, and -6, on the other hand, are largely expressed on the cell surface, although their presence within the phagosome and later components of the endocytic pathway is not excluded.
Forward genetic studies have revealed some of their respective ligands. As mentioned before, TLRs are thought to function as homodimers, heterodimers or multimers. TLR2 heterodimerizes with TLR6 and TLR1 and recognizes diacetylated and triacetylated bacterial lipoproteins, respectively [11-14]. CD36 and CD14 appear to participate in the detection complex [7]. The TLR2/TLR6 complex has also been reported to recognize lipoteichoic acid (LTA) and β-glucans such as zymosan, yet caution is needed in interpreting these results since these microbial fractions are not always entirely pure. TLR4 recognizes LPS of Gram-negative bacteria by forming a complex with CD14 and MD2, a small LPS-binding protein [8-9,15]. TLR5 is triggered by bacterial flagellin [16]. TLR 3, 7, 8 and 9, on the other hand, recognize nucleic acids and specialize in viral detection. More specifically, TLR3 recognizes double-stranded RNA (dsRNA) [17], TLR7 and TLR8 recognize single-stranded RNA (ssRNA) [18-24] and finally, TLR9 specializes in detection of both bacterial and viral unmethylated CpG DNA motifs [25-28]. TLR7, TLR8 and TLR9 have also been reported to physically and functionally interact with each other, adding further layers of complexity [29]. In the case of these TLRs, self/non-self discrimination is not achieved by the molecular characteristics of the ligands,

since nucleic acids obviously exist in the host, as well. Their localization however in late endosomes-lysosomes prohibits triggering by host nucleic elements which do not normally access these intracellular compartments [19,21,23,27,30-31]. It should be noted that in abnormal conditions, e.g. inefficient clearance of apoptotic cells, host nucleic acids could reach and activate TLRs, thereby breaching tolerance and paving the way to autoimmunity [32].
Synthetic ligands have also been reported to successfully trigger various TLRs. PAM_3CSK_4 and PAM_2CSK_4 signal through TLR1/2 and TLR2/6, respectively. TLR7 and TLR8 recognize nucleoside analogs such as resiquimod or imiquimod, drugs with antineoplastic and antiviral potential which are now believed to mimic the natural ligand ssRNA [18-20, 23].

2.2.4. TLR signaling pathway

After ligand recognition, TLRs trigger a common intracellular signaling pathway that culminates in nuclear factor (NF)-κB and activating protein-1 (AP-1) activation and production of proinflammatory cytokines, such as tumor necrosis factor (TNFα), IL-6, IL-1β and IL-12 (reviewed in 3). TLR3, TLR4, TLR7, TLR8 and TLR9 also activate alternative pathways leading to induction of type I interferons, thereby providing an antiviral response [3]. In the case of TLR3 and TLR4, this appears to depend on activation of interferon regulatory factor 3 (IRF3), while in the case of TLR7, TLR8 and TLR9 activation of IRF7 is required [33-34]. Signaling is initiated by interaction of the TIR-domain of TLRs with TIR-domain-containing cytosolic adapters, such as myeloid differentiation primary response protein 88 (MyD88), TIR-domain-containing adapter protein (TIRAP) or MyD88-adapter like protein (Mal), TIR-domain-containing adapter inducing IFNβ (TRIF), and TRIF-related adapter molecule (TRAM) (reviewed in 2). Another adapter protein is SARM (sterile α and HEAT-armadillo motifs), which, in contrast to the above, appears to negatively regulate TLR3 and TLR4 signaling in terms of NF-κB and IRF3 activation [35].

MyD88 and TRIF are the first adapter molecules to be recruited in the signaling cascade. Therefore, TLR signaling pathways are discriminated into MyD88-dependent and TRIF-dependent and each will be further analyzed below.

2.2.5. MyD88-dependent pathway

MyD88 is a central adapter shared by all TLRs except TLR3. It possesses a TIR domain in the C-terminal end, through which it associates with the TIR domain of TLRs, as well as a death domain in the N-terminal area, through which it is able to recruit members of the interleukin-1 receptor associated kinase (IRAK) family, that carry the same death domain. Until now, four IRAKs have been identified: IRAK1, IRAK2, IRAK3 and IRAK-M. IRAK1 and IRAK4 possess intrinsic Ser/Thr kinase activity, whereas IRAK2 and IRAK-M lack this characteristic, suggesting that they might function as negative regulators of TLR signaling. Indeed, IRAK-M-deficient cells show overproduction of proinflammatory cytokines in response to TLR triggering. On the contrary, IRAK4-deficient mice are unable to produce proinflammatory cytokines in response to TLR ligation. More importantly, IRAK4 mutations have been described in patients with poor inflammatory responses and recurrent infections. These observations underline the importance of IRAK4 in TLR signaling in both mouse and human. After recruitment, IRAK4 and IRAK1 are phosphorylated and disassociated from MyD88. They activate tumor necrosis factor receptor-associated factor 6 (TRAF6), which serves as ubiquitin E3 ligase and activates in turn transforming growth factor-β-activated protein kinase 1 (TAK1) in a ubiquitin-dependent manner. TAK1 forms a complex with TAB1, TAB2 and TAB3 and is further activated. It is then thought to activate the IKK complex that phosphorylates IκBs and ultimately leads to NF-κB activation; however a direct substrate that mediates the IKK complex activation is not yet found. At the same time, activated TAK1 phosphorylates two members of the MAPK kinase family, MKK3 and MKK6, which subsequently activate JNK and p38. TLR signaling has also been shown to activate ERK through the activation of MEK1 and MEK2,

although an upstream kinase phosphorylating MEK1 and MEK2 remains elusive. ERK1 and ERK2 activation has been shown to be reduced in TAK1-deficient B cells, suggesting that TAK1 is implicated in this pathway, as well. MAP kinases such as JNK, p38, and ERK are thought to ultimately activate AP-1. Conclusively, MyD88-dependent TLR signaling culminates in activation of NF-κB and AP-1, which are known to control inflammatory responses.

NF-κB is a dimeric transcription factor which belongs to the Rel-homology domain-containing protein family. The prototypical NF-κB is thought to be a heterodimer composed of the p65 and p50 subunits in most types of cells. In unstimulated conditions, NF-κB is sequestered in the cytoplasm as an inactive complex with inhibitor of NF-κB proteins (IκB). TLR triggering activates the IKK complex, consisting of IKKα and IKKβ protein kinases and a regulatory molecule, IKKγ/Nemo. This complex phosphorylates IκBs and thereby targets them for ubiquitination and degradation, allowing NF-κB to be released, translocate into the nucleus and bind to the κB site.

AP-1 is a dimeric basic region leucine zipper protein composed of members of Jun, Fos, activating transcription factor (ATF) and the Maf subfamily, which bind to TPA- or cAMP-response elements. Among the AP-1 family proteins, c-Jun is thought to play a key role in inflammatory responses.

In the case of TLR2 and TLR4, the MyD88-dependent pathway requires an additional adapter protein, TIRAP/Mal. This adapter is thought to mediate the interaction of MyD88 with the TIR domain of these particular TLRs. Indeed, TIRAP/Mal-deficient mice exhibit defective inflammatory responses when triggered with TLR2/TLR1, TLR2/TLR6 and TLR4 ligands.

2.2.6. Alternative MyD88-dependent pathway

Plasmacytoid dendritic cells (pDCs) are a subset of DCs specialized to produce large amounts of type I interferons (IFN) in response to viral infection and TLR7, -8 and -9 ligation. This ability is completely abolished in MyD88- or IRAK4-deficient pDCs. Unlike other types of DCs, pDCs constitutively express high levels of IRF7.

pDCs from IRF7-deficient mice are incapable of producing type I IFN in response to TLR7,-8 and -9 triggering. In this cell type, IRF7 has been found to interact with MyD88, IRAK1 and TRAF6. This results in the activation and translocation of this transcription factor into the nucleus, where it binds to IFN-stimulated response elements (ISREs) and induces type I IFN production.
IRF5 has also been reported to interact with MyD88, translocate into the nucleus and bind to ISRE motifs. While in mice this leads to inflammatory cytokine gene expression, human IRF5 is suggested to selectively participate in the production of TLR7-mediated type I IFN rather than inflammatory cytokines, implying species-specific function of IRF5.

2.2.7. TRIF-dependent pathway

TRIF is utilized by TLR3 and TLR4. TLR3 signals solely through TRIF, whereas TLR4 also signals through MyD88/Mal. In the case of TLR4, TRIF interaction with the TIR domain is mediated by another adapter, TRAM.
The C-terminal region of TRIF contains a Rip homotypic interaction motif (RHIM), which allows association with members of the receptor interacting protein (RIP) family. It has been shown that RIP-1 is responsible for NF-κB activation, although the exact mechanism is not yet clear. The N-terminal region of TRIF contains three TRAF-binding domains, which mediate interaction with TRAF6. TRAF6 subsequently activates TAK1 and leads to NF-κB and AP-1 activation in a cascade similar to that of the MyD88-dependent pathway.
Importantly, the TRIF-dependent pathway is also mediating the production of type I IFN, especially IFNβ. Transcription of IFNβ is tightly regulated by the cooperation of several transcription factors, including NF-κB, c-Jun, IRF3 and IRF7. It should be noted that the MyD88/Mal pathway, activated by TLR4 triggering, is not capable of inducing IFNβ gene transcription. While NF-κB and c-Jun may be activated not only by TLR ligation but also by a number of several stimuli as well, eg. IL-1β, TNFa and DNA damage, IRF3 and IRF7 are activated following TLR3 and

TLR4 stimulation. More specifically, they become phosphorylated by non-canonical IKKs (TANK-binding kinase 1 and IKKi) and then translocate into the nucleus. It is believed that IRF7 expression is very weak in unstimulated cells, but the initial production of IFNβ through IRF3 induces IRF7 expression in a paracrine way and amplifies type I IFN production via a positive feedback mechanism.

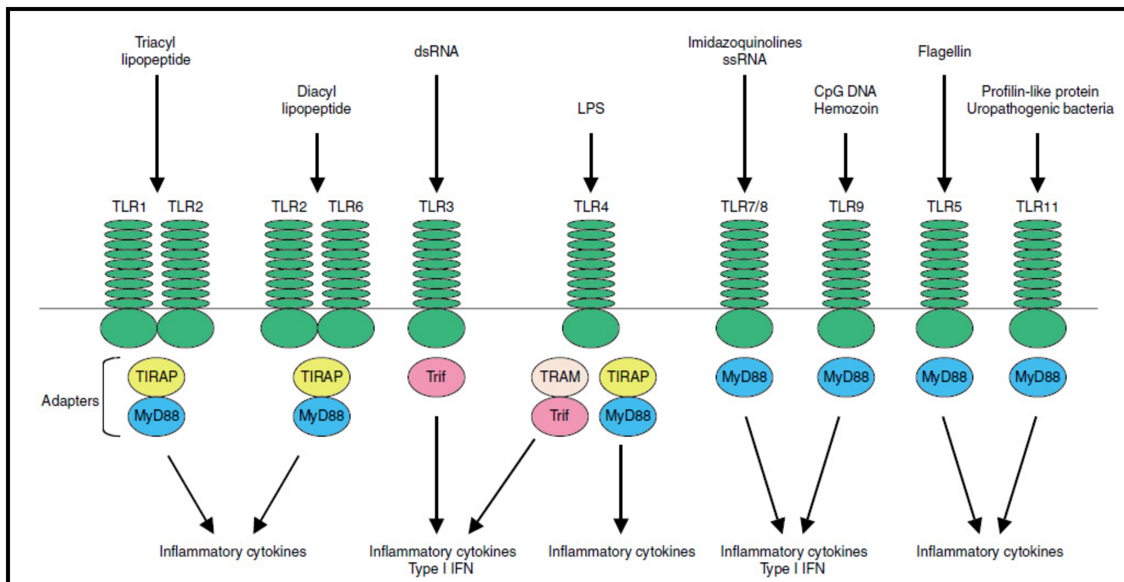

Figure 2 (adapted from Kawai T and Akira S, ref.2). The signaling pathway from MyD88 to NF-kB and AP-1 activation is common to all TLRs except TLR3, which utilizes TRIF. In the case of TLR2 and TLR4, TIRAP is required as an additional adapter. Finally, TLR4 is able to signal through TRIF by utilizing TRAM, an adapter that links TLR4 to TRIF.

2.2.8. TLR expression

In addition to different ligand specificities, cellular localization and downstream signaling cascades, TLRs also differ in their distribution among various cell populations. So far, TLRs are found to be expressed in epithelial and endothelial cells, macrophages, monocytes, mast cells, neutrophils, basophils, NK cells, dendritic cells (DCs), regulatory T cells (Tregs) and B cells, as well as in several types of neoplastic cells [36]. Pathogen recognition by TLRs expressed on epithelial and endothelial cells is thought to induce the production of cytokines,

chemokines and antimicrobial peptides [37-38]. Chemokines regulate cell recruitment to infection sites. At the same time, activation of TLRs on leukocytes appears to augment their ability to migrate towards inflammation and clear pathogenic organisms and infected cells [39-41]. However, the role of TLRs extends from innate host response to adaptive immunity as well. TLR activation in DCs is critical for antigen uptake, upregulation of major histocompatibility (MHC) and costimulatory molecules (CD40, CD80 and CD86), chemokine receptor expression that controls migration to local lymphoid tissue and also secretion of cytokines which drive TH-cell/cytotoxic T-lymphocyte (CTL) response [42] and block Treg immunosuppression [43-44]. Distinct DC subsets, as defined by their anatomical location, appear to be involved in the response to different sets of pathogens through the expression of different sets of TLRs [45-47]. Along this line, one could consider that TLRs function at multiple levels of the host response to pathogens, orchestrating numerous types of immune cells and coordinating innate with adaptive immunity.

2.2.9. Nod-like receptors (NLRs)

TLRs are located in cellular membranes, either on the cell surface or in endosomes. Other receptors, however, localize in the cytosol, where they encounter microbial motifs and "danger signals" and initiate innate immune responses.

The NLR family in mammals consists of more than 20 members, whose common characteristic is a modular domain organization of a C-terminal leucine-rich repeat (LRR) domain, a central nucleotide-binding (NACHT) domain and an N-terminal protein-protein interaction domain composed of a CARD (caspase activation and recruitment domain), a pyrin or a Bir (baculovirus "inhibitor of apoptosis" repeat) domain[48]. NLRs appear to be evolutionary conserved elements, since homologs have been found in plants, where they seem to be

involved in the defense against plant pathogens, and also in the sea urchin, although their functions in this case remain unclear.
The first NLRs shown to function as PRRs were NOD1 and NOD2, encoded by the *Card4* and *Card15 genes*, respectively. Both receptors recognize substructures of bacterial peptidoglucan (PGN) that are released by peptidase and hydrolase processing. More specifically, NOD1 detects peptidoglucan containing meso-diaminopimelic acid (meso-DAP), which is mainly found in Gram-negative bacteria, whereas NOD2 senses muramyl-dipeptide (MDP), which is found in both Gram-negative and Gram-positive bacteria. NOD1 has been found to be expressed in all tissues, while NOD2 expression appears to be restricted to monocytes/macrophages and DCs[(49)]. Later studies reported two additional NLRs, Ipaf and Naip, acting as intracellular sensors of bacterial infection by sensing flagellin and leading to inflammasome activation. Another NLR, NALP3 (NACHT-LRR-PYD-containing protein 3; also called cryopyrin), is believed to detect both microbial components and cellular “danger signals”. There are studies suggesting its function as an intracellular sensor of bacterial RNA, while other studies support that inflammatory signals, such as ATP, depletion of intracellular potassium or even uric acid, can trigger this receptor and subsequently lead to caspase-1 inflammasome activation.

2.2.10. NLR signaling

Until now, the most well-established signaling pathways downstream of NLRs include the NF-κB and the MAPK (mitogen-activated protein kinases) pathway for NOD1 and NOD2, and activation of the caspase-1 inflammasome for NALPs, Ipaf and Naip. Here we will focus on the former.
It is currently believed that detection of peptidoglucan-derived ligands causes NOD1 and NOD2 to rapidly form oligodimers and recruit RIP2 (receptor-interacting protein 2) through CARD-CARD interactions. This complex subsequently activates IKKs, which lead to activation of NF-κB through

phosphorylation and degradation of IκB (inhibitor of NF-κB). It is unclear whether NF-κB activation through NOD signaling initiates innate immune responses that are distinct from the ones elicited by TLR, TNF-receptor or IL-1β-receptor triggering. So far, several molecules that play a regulatory role in NF-κB activation via NOD signaling have been identified. More specifically, TRIP6 (thyroid receptor interacting protein 6) amplifies NOD1 signaling, mainly by interacting with RIP2. Moreover, GRIM19 (gene associated with retinoid-interferon-induced mortality 19) modulates NOD2 signaling and NF-κB activation. In contrast, the kinase TAK1 (TGFβ-activated kinase 1) negatively regulates NF-κB activation by directly interacting with NOD2.

NOD1 and NOD2 interaction with RIP2 also activates the MAPK kinase pathway. JNK, p38, ERK1 and ERK2 appear to be implicated. Finally, the AP-1 complex is formed, translocates into the nucleus and induces the expression of proinflammatory cytokine genes.

There are studies supporting that NOD1 and NOD2 play a role in apoptotic pathways, as well. More specifically, it has been suggested that NOD1 binds to caspase-9 through CARD-CARD interactions and induces apoptosis. NOD2 has also been implicated in this process, although data supporting its direct interaction with caspase-9 are currently lacking. In MCF-7 neoplastic cells, NOD1 was also found to promote apoptosis through caspase-8.

Finally, NOD1 and NOD2 are implicated in IL1-β production, at least in human monocytes. IL1-β production depends on the processing of pro-IL1-β by caspase-1 at the inflammasome, which consists of caspase-1 and -5, the adapter protein ASC, and many members of the NALP family. NOD1 facilitates this processing through CARD-CARD interactions with RIP2 and pro-caspase-1. NOD2, on the other hand, is thought to be implicated in IL1-β secretion, but the mechanism is still unclear.

It should be noted that there are studies supporting a synergistic role between NLRs and TLRs. It has been shown that TLR4 stimulation upregulates the expression of NOD2 and RIP2, and that NOD1 and NOD2 stimulation upregulates the expression of MyD88 and TAK1, respectively. Also, stimulation of macrophages with MDP induces TNFa transcription, but not translation; yet when the same cells

are co-stimulated with LPS, TNFa is effectively synthesized and secreted. However, the molecular background of such cross-talk is not known.

3. Adaptive immunity

The defense systems of innate immunity are effective in combating many pathogens. They provide a rapid response and in many cases are able to contain the attack. Nevertheless, when necessary, the innate immune system is also very efficient in instructing the cellular mediators of adaptive immunity to lead a second, powerful, pathogen-specific strike against the invading organisms. The induction of an adaptive immune response begins by activation of specialized antigen-presenting cells (APCs). DCs are professional APCs that are resident in most tissues at an immature stage and, like macrophages, are long-lived compared to other white blood cells. They detect pathogens through their PRRs, but they also continuously take up extracellular material by macropinocytosis and are thus able to internalize and degrade proteins that their receptors cannot recognize. On taking up pathogens, DCs migrate to peripheral lymphoid tissues, where they mature, present fragments of pathogen antigens on their surface in the context of MHC (major histocompatibility complex) molecules and also express co-stimulatory molecules. These co-stimulatory molecules, together with the antigen presentation and the cytokine milieu produced by activated DCs, stimulate naïve T lymphocytes to proliferate and differentiate into their final functional form. CD8+ T lymphocytes recognize antigens in the context of MHC class I molecules, whereas CD4+ T lymphocytes recognize antigens in the context of MHC class II molecules. Because B lymphocytes require in most cases stimulation from CD4+ T cells in order to proliferate and differentiate into antibody-secreting plasma cells, the activation of naïve T lymphocytes is an essential first step in almost all adaptive immune responses.

The first major characteristic of adaptive immunity is specificity. Each naïve lymphocyte bears antigen receptors specific for a single chemical structure and is distinct from the other lymphocytes in its receptor specificity. This diversity is created by unique genetic mechanisms during lymphocyte development in the bone marrow and the thymus. When lymphocytes are activated by the antigen that corresponds to their specificity, they give rise to clones of antigen-specific effector cells (clonal expansion). During this clonal expansion, a process called "affinity maturation" also takes place, i.e. receptors are edited and the ones with higher affinity for the antigen are selected. On the other hand, lymphocytes bearing receptors specific for self-molecules are deleted at an early stage in lymphoid development (clonal deletion) or inactivated (anergy). Anergy may occur when lymphocytes contact their respective antigen without receiving a second signal from co-stimulatory molecules.

The second major characteristic of adaptive immunity is the so called "immunological memory". After a naïve lymphocyte has been activated, it takes 4-5 days until clonal expansion is complete and differentiation into effector cells has taken place. Most of these effector cells are consumed during the immune response against the pathogen. However, a significant number of activated antigen-specific B and T cells persist after the antigen has been eliminated. These are known as memory cells since they can provide a more rapid and effective response in case the same antigen is encountered. The complete physiological mechanism of immunological memory is not yet fully deciphered. However, it is believed that antigen-specific memory cells are greater in number than naïve cells with the same specificity, that their activation requirements are different and also that they have underwent affinity maturation and therefore are more specific for the particular antigen. It is still a field of debate between immunologists whether antigen re-exposure is required for the preservation of immunological memory or if memory cells have the ability of persisting on their own, either at a resting state or by slow division [(50-51)].

3.1. Antigens

Antigens are considered to be the biochemical structures that can be recognized by the T cell-receptor (TCR) in the context of an MHC molecule or by the B cell-receptor (BCR) or a soluble antibody. They are characterized by four main properties:

i) induction of humoral, cellular or combined immune response after they enter the host (immunogenicity)

ii) ability to specifically bind to the final products of the immune response (antigenicity)

iii) potential induction of allergic reactions (allergenicity)

iv) potential induction of tolerance (tolerogenicity)

Of note is the distinction between the terms antigen-immunogen. Antigen is any substance which, when contacts soluble antibodies, the BCR or the TCR, interacts stereochemically and establishes a binding connection. On the contrary, immunogen is any substance that, when it enters the host, induces the production of specific antibodies and cytotoxic T cells. So, one could say that all immunogens are antigens, but not vice versa.

Immunogenicity is not an intrinsic characteristic of the antigen; instead it is a functional property that depends both on the antigen itself and on the biological system with which it comes in contact. More specifically, the immunogenicity of a given substance depends on its molecular size and chemical structure, its similarity with autologous substances of the host, the dose and route of administration, the ability of phagocytic cell enzymes to degrade it, as well as genetic and environmental factors.

3.1.1. Molecular structure of antigens

Substances that are immunogenic/antigenic are usually macromolecules, i.e. proteins, polysaccharides and nucleic acids. Among them, proteins are

generally more immunogenic, and the more complex their structure, the stronger the immune response they elicit. Polysaccharides most commonly induce humoral, but not cellular immune responses. Finally, nucleic acids usually need to be attached to a protein carrier (hapten) in order to elicit an immune response. Lipids are hardly immunogenic, but when covalently attached to a hapten, they as well might induce an immune response.

An antigen may provide a single but most commonly multiple structures specifically recognized by antibodies, BCRs or TCRs. These specific structures are called antigenic determinants or epitopes.

3.1.2. Antigen classes

Depending on their origin, antigens are divided in exogenous, endogenous and autoantigens. Exogenous antigens invade from the environment. Endogenous antigens are produced within the host as a result of an abnormal process. Finally, autoantigens are autologous molecules that are falsely recognized by the immune system as foreign. Normally, lymphocytes that recognize autologous molecules (autoreactive lymphocytes) are purged early from the immune repertoire by clonal deletion and receptor editing or become anergized.

Depending on the type of immune response they elicit, antigens are also divided in thymus-dependent (TD) and thymus-independent (TI). All thymus-dependent antigens are proteins that require CD4+ T cell help in order to induce antibody production. By contrast, thymus-independent antigens are mostly non-protein and are able to induce antibody production without CD4+ T cell help. Thymus-independent immune responses occur outside germinal centers, e.g. in the marginal zone of the spleen or around lymphoid follicles. Characteristically, TI antigens mostly elicit IgM antibody production. They can be further divided into: i) TI-type 1 antigens which, in high concentrations, can induce polyclonal activation and differentiation of B cells (therefore also called B cell mitogens), e.g. LPS; and, ii) TI-type 2 antigens, highly repetitive structures (dextrans, ficoll,

polysaccharides, D-amino acid polymers) that can most probably activate mature B cells by simultaneously crosslinking a critical number of BCRs on their surface. More about TI antigens will be discussed later, in relation to T cell independent B cell activation.
Finally, a special category of antigens is “superantigens”. Superantigens differ from other antigens in their ability to bind sequences outside the classical binding sites of the BCR or TCR. Their binding allows lymphocyte activation in a non-specific manner. Maybe the best yet characterized superantigens are the toxins of *Staphylococcus aureus*, *Streptococcus pyogenes* and *Peptostreptococcus magnus*. There are studies associating superantigens with autoimmunity, infectious diseases and toxic shock.

3.2. Receptors of adaptive immunity

The antigen-recognition molecules of activated B cells are the immunoglobulins (Ig). These proteins are produced by activated B cells in a vast range of antigen specificities, with each B cell clone producing immunoglobulin of a single specificity. Every day, the human body produces 10^7 B cells, each of a distinct specificity. The total repertoire of antigenic specificities is approximately 10^9, while the total number of B cells in the adult is approximately 10^{11}. Membrane-bound immunoglobulin on the B cell surface serves as the cell’s receptor for antigen, and is known as the B cell receptor. Immunoglobulin of the same antigenic specificity is secreted as antibody by terminally differentiated B cells – the plasma cells. The secretion of antibodies, which bind pathogens or their toxic products in the extracellular spaces of the body, is the main effector function of B cells in adaptive immunity [(51-52)].
The antigen-recognition molecules of T cells exist solely as membrane-bound proteins and their function is only to transmit signals for T cell activation. These T cell receptors (TCRs) differ from BCRs in an important way; they do not recognize and bind antigen directly, but instead recognize peptide fragments which are

bound to MHC molecules on the surface of antigen-presenting cells. TCRs recognize features of both the peptide antigen and the MHC molecule to which it is bound. This is known as MHC restriction. As mentioned above, CD4+ T cells recognize protein antigens only in the context of MHC class II molecules, whereas CD8+ T cells recognize protein antigens in the context of MHC class I molecules.

The following pages will focus on B cell development, generation of BCRs, BCR signaling and effector function of B cells in the human immune system.

3.2.1. Immunoglobulin molecular structure and function

The immunoglobulin molecule comprises four polypeptide chains, two heavy (H) and two light (L) chains. Heavy chains are tethered together through multiple disulfide bonds and each light chain pairs to a heavy chain through a single disulfide bond ***(Fig.3).*** In each immunoglobulin molecule, the two heavy chains and the two light chains are identical. Two types of light chains, termed kappa (κ) and lambda (λ) are found in immunoglobulins. A given immunoglobulin has either κ or λ chains, never one of each. The ratio between the two in the repertoire differs among species. In human the ratio κ:λ is approximately 2:1. No functional difference has been found between antibodies that carry κ or λ chains. Moreover, the heavy chains of an immunoglobulin molecule belong to one of five general classes: μ, γ, δ, ε, α. According to the heavy chain type, five immunoglobulin isotypes – IgM, IgG, IgD, IgE, and IgA – can be distinguished, respectively. Their distinctive functional properties are conferred by the carboxy-terminal part that is not associated with the light chains. IgG is by far the most abundant immunoglobulin and has several subclasses (IgG1, 2, 3, and 4 in human). IgA also has two subclasses (IgA1 and IgA2).

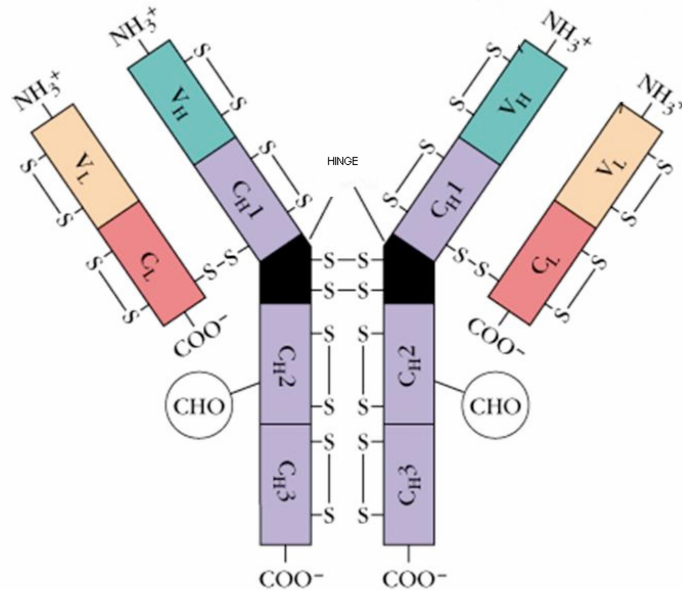

Figure 3. A typical immunoglobulin molecule.

The structure of the B cell receptor is identical to that of its corresponding antibody except for a small region of the carboxy-terminus of the heavy chains. In the B cell receptor the carboxylic terminus is a hydrophobic sequence which anchors the molecule in the membrane, whereas in the antibody it is a hydrophilic sequence which allows secretion.

The amino acid sequences of many immunoglobulin heavy and light chains have been determined and this analysis revealed two important features of antibody molecules. First, each chain consists of a series of similar, although not identical, sequences, each about 110 amino acids long. Each of these repeats corresponds to a discrete, compactly folded protein domain. The light chain comprises two such immunoglobulin domains, whereas the heavy chain contains four. This suggests that the immunoglobulin chains have evolved from repeated duplication of an ancestral gene corresponding to a single domain. The second critical feature is that the amino-terminal sequences of both heavy and light

chains vary greatly between different antibodies. Most importantly, this variability is limited to the first domain, whereas the remaining domains are constant between immunoglobulin chains of the same class. The amino-terminal variable domains (V domains) of the heavy and light chains (VH and VK/VL, respectively), comprise the V region of the antibody which defines antigenic specificity, while the constant domains (C domains) of the heavy and light chains (CH-1,-2,-3 and C_L, respectively) make up the C region. When fully folded and assembled, an antibody molecule comprises three equal-sized parts joined by a flexible stretch of amino acids known as the hinge region. X-ray crystallography actually reveals a Y-shaped structure.

Sequence variability is not distributed evenly throughout the V region, but is concentrated in three certain segments of both the VH and the VK/VL domain, which are called hypervariable regions and are denoted HV1, HV2, and HV3. In the heavy chains they run roughly from residues 30-36, 49-65, and 95-103, respectively, while in the light chains they are located roughly at residues 28-35, 49-59, and 92-103, respectively. HV3 is the most variable region in both heavy and light chains. The sequences between the hypervariable regions, which comprise the rest of the V domain, exhibit less variability and are called framework regions. There are four such regions in each V domain, designated FR1, FR2, FR3, and FR4. The framework regions form β sheets, which serve as the structural framework of the domain, whereas the hypervariable regions correspond to three loops, which are juxtaposed in the folded domain. When the VH and VK/VL domains are paired in the antibody molecule, the hypervariable loops are brought together, so that a single hypervariable site is created at the tip of each arm of the molecule. This is the antigen-binding site. The six hypervariable loops largely determine antigen specificity by forming a docking site complementary to the antigen and are therefore called complementarity-determining regions (CDRs). Because CDRs from both the VH and the VK/VL domains contribute to the antigen-binding site, it is the combination of the heavy and light chain that determines the final antigenic specificity. Thus, by generating different combinations of heavy and light chain V regions, the immune system increases the antibody repertoire. This means of

producing variability of antigenic specificities is called combinatorial diversity. More mechanisms are employed by the immune system for the same purpose and will be discussed below.

3.2.2. Immunoglobulin gene rearrangements

The V domain of an immunoglobulin heavy or light chain is encoded by more than one gene. For the light chain, the V domain is encoded by two separate genes. One encodes the first 95-101 amino acids and is called variable or V gene. The other encodes the remainder of the domain (up to 13 amino acids) and is called joining or J gene. A complete immunoglobulin light chain gene is produced by a process called somatic recombination, as shown in *Fig.4.* Likewise, the V domain of a heavy chain is encoded by three separate genes, V (variable), D (diversity), and J (joining), which are somatically recombined in B cells in a similar fashion.

There are multiple genes of each type in human germline DNA. Not all are functional, as some have accumulated mutations that prevent them from encoding a functional protein. The numbers of functional genes of each type in the human genome, as determined by gene cloning and sequencing, are shown in *Fig.5.* Because there are many V, D and J genes in germline DNA, no single one is essential. This reduces the evolutionary pressure and has resulted in a relatively large number of pseudogenes. Since some of these can undergo rearrangement like a normal gene, a significant proportion of rearrangements incorporates a pseudogene and will thus be nonfunctional.

As discussed previously, there are three sets of immunoglobulin chains, the heavy chains and the two types of light chain, the κ and the λ chains. The immunoglobulin genes forming each of these chains are organized into three clusters or genetic loci – the IGH, IGK and IGL loci – located on chromosomes 14, 2 and 22, respectively.

V(D)J rearrangement is guided by conserved non-coding DNA sequences that are found adjacent to the points at which recombination takes place and are called recognition signal sequences (RSSs). A recombination signal sequence consists of a conserved block of seven nucleotides – the heptamer 5'CACAGTG3' – followed by a non-conserved region known as the spacer, which is

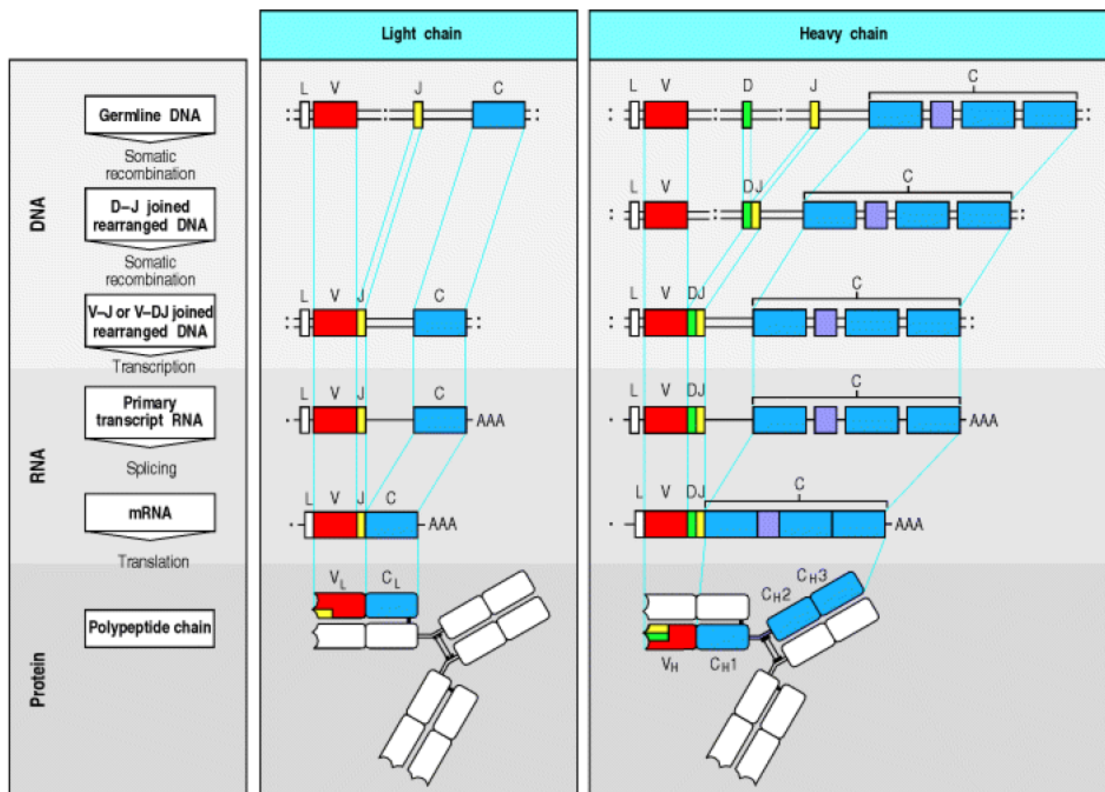

Figure 4. Heavy and light chain gene rearrangement.

either 12 or 23 base pair long, followed by a second conserved block of nine nucleotides – the nonamer 5'ACAAAAACC3'. The spacers vary in sequence but their conserved lengths correspond to one turn (12bp) or two turns (23bp) of the DNA double helix. This brings the heptamer and the nonamer to the same side of the DNA helix, in order to serve as docking sites for the protein complex that catalyzes recombination. The RSS is always found directly adjacent to the coding sequence. A gene flanked by an RSS containing a 12bp spacer can only be

joined with a gene flanked by an RSS with a 23bp spacer. This is known as the 12/23 rule. This mechanism ensures that the rearrangement process will take place in an orderly manner. For example, during the heavy chain rearrangement, an IGHD gene can only be joined to an IGHJ gene, and a IGHV gene segment only to an IGHD gene, because both IGHV and IGHJ genes are flanked by RSSs with 23bp spacers and the IGHD genes have RSSs with 12bp spacers on both sides.

The complex of enzymes that act together to carry out the process of somatic recombination is called the V(D)J recombinase. The lymphoid-specific components of the recombinase are RAG-1 and RAG-2. These genes are expressed in lymphocytes only when they are engaged in assembling their antigen receptors. The other enzymes are mainly ubiquitous DNA modifying proteins, e.g. Ku, DNA-PKc, DNA ligase IV, and XRCC4. It is important that during the formation of the coding joint, sequence variability is introduced as a result of the random addition and subtraction of nucleotides by the recombination process. This source of diversity is called junctional diversity. Of the three hypervariable loops in an immunoglobulin chain, CDR1 and CDR2 are encoded within the V gene. CDR3 coding sequence, however, falls at the joint between the V and the J gene in the light chains, and in the heavy chains it is also partly encoded by the D gene. Therefore, the diversity of CDR3 is greatly increased by this random subtraction and addition of nucleotides at the joining points.

Conclusively, the gene rearrangement that combines gene segments to form a complete V-region exon generates diversity in two ways. Firstly, there are multiple different copies of each type of gene and different combinations can occur in different rearrangement events. Secondly, junctional diversity is introduced at the points where genes are ligated. The combination of different copies of genes, together with the fact that the antigen-binding site is finally created by the combination of a heavy with a light chain, could in theory give rise to approximately $1,9 \times 10^6$ different antibody molecules. By adding junctional diversity, it is estimated that this number rises up to 10^{11}. After the expression of an immunoglobulin molecule with a certain antigenic specificity, contact with the

antigen may initiate a process called somatic hypermutation, which introduces further diversity and will be discussed below.

It should be mentioned that, in reality, diversity is likely to be less than one mathematically calculates. First of all, not all V genes are used at the same frequency; some are common in the antibody repertoire and some are rarely found. Also, not every heavy chain can pair with every light chain; in some cases the produced immunoglobulin molecule is unstable and the cells will either undergo further light chain gene rearrangement until a suitable light chain is produced or they will be eliminated. Finally, the random addition and subtraction of nucleotides during the joining of genes might disrupt the reading frame of the coding sequence beyond the joint; this may lead to a non-functional protein. It is estimated that roughly two out of three rearrangements are non-productive. These B cell progenitors do not manage to express functional immunoglobulin and never become mature B cells. Thus, junctional diversity is achieved only at the expense of considerable cell wastage.

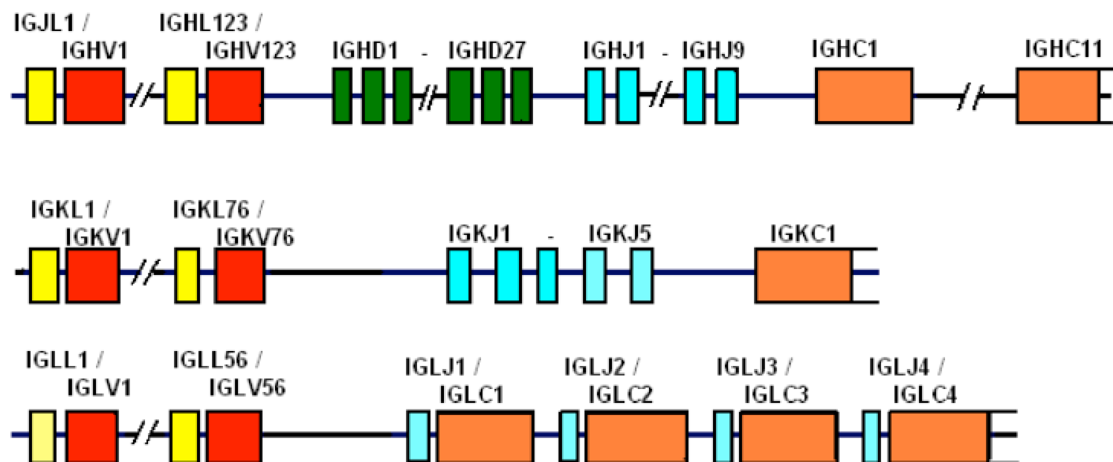

Figure 5. Germline configuration of the immunoglobulin heavy and light chain gene loci in the human.

3.2.3. Somatic hypermutation

After contacting antigen, B cells undergo further diversification through a process known as somatic hypermutation. This takes place in the germinal centers of peripheral lymphoid organs after functional immunoglobulin genes have been assembled. It has also been found to occur outside germinal centers during thymus-independent humoral responses, though less frequently *(Fig.6)*. It introduces point mutations into the rearranged V region exon at a very high rate, resulting to the expression of mutant B cell receptors on the surface of B cells. It is initiated by an enzyme called activation-induced cytidine deaminase (AID), which is specifically expressed in activated B cells. AID possibly acts both as an mRNA cytidine deaminase, converting cytosine to uracil by deamination, and as a DNA cytidine deaminase, directly deaminating cytidine residues in the immunoglobulin genes to uridine. It binds only to single-stranded DNA; the DNA double helix must be temporarily unwound locally and this seems to happen as a result of active transcription of nearby sequences.

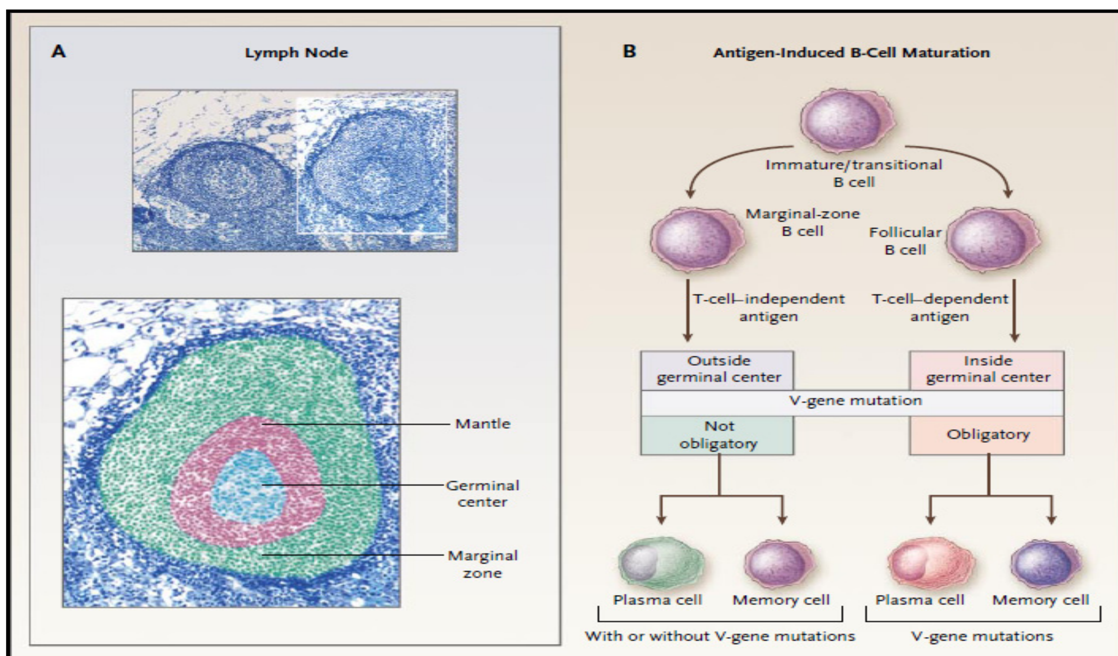

Figure 6 (adapted from Chiorazzi N, Rai KR and Ferrarini M, ref.53). A) A lymphoid follicle. B) B-cell activation in a T-independent (left) or T-dependent (right) manner.

This partially explains why somatic hypermutation is preferentially targeted to rearranged V regions, which are being actively transcribed in B cells, and not to inactive loci. Rearranged V genes are mutated even if the rearrangement is non-productive and the gene is transcribed but not translated into a functional protein. However, it does not explain why other transcribed loci in B cells, like the C genes, are not as affected as the V exon. Moreover, it appears that the base changes are not evenly distributed throughout the V region but rather targeted to certain mutation "hotspots" with distinctive sequence features.
Somatic hypermutation underlies the so called "affinity maturation" of the antibody. Mutations which result in disrupting or reducing the affinity of the receptor for the antigen are selected against. Clones bearing mutations which increase the affinity of the produced receptor for the antigen are preferentially selected to mature into antibody-secreting plasma cells. Collectively, somatic hypermutation results in further increasing the diversity of the peripheral B cell repertoire to approximately 10^{12} different specificities. More importantly, it adjusts the existing clones to the specific antigen that is encountered each time.

3.2.4. Class switch recombination

The first antigen receptors expressed by B cells are IgM and IgD, and the first antibody produced in the course of an immune response is also IgM. Later in the immune response, class switching enables the same assembled VH exon to be associated with different IGHC genes. This process is stimulated by external signals, such as cytokines released by T cells or mitogenic signals delivered by pathogens, and involves an irreversible non-homologous DNA recombination which is guided by stretches of repetitive DNA known as switch regions (S regions). Switch regions lie right upstream each IGHC gene, with the exception of the IGHC-D gene whose expression is not dependent on DNA rearrangement. When a B cell switches from the co-expression of IgM and IgD to the expression

of another subtype, DNA recombination occurs between the S_{μ} and the S region which lies upstream of the gene for the other isotype. The entire intervening DNA is deleted and therefore the process cannot be reversed. All switch recombination events result in the production of a functional protein, because switch regions are located in introns and therefore do not cause any frameshift mutations.
Like in somatic hypermutation, AID appears to play a critical role in class switch recombination, as well. As a matter of fact, deficiency of this particular enzyme in humans is associated with a form of immunodeficiency known as hyper-IgM type 2 syndrome, which is characterized by the absence of immunoglobulins other than IgM. The exact mechanism by which AID is involved in class switching is not entirely clear. It is known however that transcription of the switch regions is required, and this transcription presumably serves to allow AID access to cytidine residues of the switch sequences.
Collectively, class switching provides antibodies of the same antigenic specificity but distinct effector capacities. IgM antibodies are produced early during the primary immune response, before somatic hypermutation begins to take place, therefore tend to have lower affinity for the antigen. Nevertheless, IgM molecules form pentamers and thereby display 10 antigen-binding sites which can bind simultaneously to multivalent antigens, increasing the overall avidity. As a result of their large size, IgM pentamers mainly circulate in the blood and, to a smaller extent, in the lymph. At the same time, their pentameric structure is very efficient in activating the complement. On the other hand, antibodies of the other classes – IgG, IgA, and IgE – are smaller in size and are able to diffuse into the tissues. IgA can form dimers, while IgG and IgE are always monomeric. IgG is the major class in the blood and extracellular fluid, while IgA is the principal class in secretions. IgE exists at very low levels in blood or extracellular fluid; instead it is bound on receptors of mast cells that locate just beneath the skin and mucosa and along blood vessels in connective tissue. IgG efficiently opsonizes pathogens for phagocytosis, activates the complement and neutralizes bacterial toxins. IgA mainly acts as a neutralizing antibody. Finally, IgE triggers mast cells to release chemical substances which induce mechanical reactions, such as coughing,

sneezing or vomiting, and aim to expel infectious agents or environmental allergens from the host.

3.3. Development and survival of B cells

The immense repertoire of B cell receptors is generated during the development of B cells from their uncommitted progenitors. The production and development of new B lymphocytes takes place mainly in the bone marrow, but also in the fetal liver and the neonatal spleen. The mature B cells then migrate to populate the peripheral lymphoid tissues, such as lymph nodes, spleen and mucosal lymphoid tissue. The stages in B lymphocyte development are mainly defined by the various steps in the assembly and expression of functional antigen receptor genes, and by the appearance of features that distinguish the different functional types of B cells. At each step of lymphocyte development, the progress of gene rearrangement is monitored and, if successful, reinforces the cell to proceed to the next step of development. After the expression of a functional BCR, it is the antigen, in coordination with the microenvironment, that drives the B cell clone into further differentiation towards antibody-secreting plasma cells and memory B cells. At the same time, BCRs that are self-reactive are purged from the peripheral repertoire by several mechanisms, all part of the so called B cell immunological tolerance [52].

3.3.1. Development of B cells in the bone marrow

Pluripotent hematopoietic stem cells (HSCs) of the bone marrow give rise to multipotent progenitor cells (MPPs) and then to common lymphoid progenitors (CLPs), from which all cells of the lymphoid lineage – B cells, T cells and NK cells – are derived. MPPs express the transcription factor PU.1 and a cell surface tyrosine kinase known as FLT3; signaling through this kinase is thought to drive their

differentiation towards the next stage. Another essential factor is stem cell factor (SCF), a membrane-bound cytokine expressed on bone marrow stromal cells which stimulates growth by interacting with the receptor tyrosine kinase c-Kit on precursor cells. The chemokine CXCL12 (stromal cell-derived factor 1, SDF-1) is also thought to be essential for the early stages of B cell development.

The common lymphoid progenitor gives rise to the earliest cell of B-lineage, the pro-B cell, in which immunoglobulin gene rearrangement starts to take place. Commitment to B cell fate is sealed by the expression of the transcription factors E2A and EBF. It is thought that IL-7 signaling promotes the expression of E2A, which cooperates with PU.1 to induce the expression of EBF. Together, E2A and EBF drive the expression of the transcription factor Pax-5, which in turn controls the expression of proteins that determine the pro-B cell state.

The stages of B cell development are, in the order they occur: early pro-B cell, late pro-B cell, large pre-B cell, small pre-B cell, and mature B cell. The transcription factors E2A and EBF in the early pro-B cell induce the expression of proteins that initiate the immunoglobulin gene rearrangement process. Only one gene locus is rearranged at a time, in an orderly manner. Although the V(D)J recombinase complex operates in both B- and T-lineage cells utilizing the same core enzymes, rearrangement of T cell receptor genes do no occur in B-lineage cells, nor does complete rearrangement of immunoglobulin genes take place in T-lineage cells. This is possibly regulated by lineage-specific low-level transcription of the genes that are going to be joined.

The first rearrangement event that occurs is the IGHD to IGHJ joining in the immunoglobulin heavy chain locus at the stage of early pro-B cell. This typically involves both alleles of the locus. Of note, human IGHJ genes can be translated in all three reading frames; therefore there is no need at this point for a mechanism that would check the success of D-J joining. The late pro-B cell proceeds with the joining of an IGHV gene to IGHD-J. In contrast to IGHD to IGHJ rearrangement, IGHV to IGHD-J rearrangement occurs first in only one allele. If successful, intact μ heavy chains are produced and the rearrangement process ceases. In at least two out of three cases, rearrangement is nonproductive and the cell either undergoes successive rearrangements of other IGHV genes on the

same chromosome or the other allele is tested, again with the same chances of failure. Late pro-B cells that do not manage to express a functional μ heavy chain are eliminated. The immunoglobulin diversity is at this stage enhanced by the enzyme terminal deoxynucleotidyl transferase (TdT), which randomly adds nontemplated nucleotides at the joints between rearranged gene segments. In adult humans, this enzyme is expressed in pro-B cells, but its expression declines at the pre-B cell stage where light chain rearrangement occurs. In fetal development, TdT is generally expressed at low levels throughout B cell development.

At the next stage, developing B cells need to test for the functionality of their produced heavy chain. Therefore, late pro-B cells produce two invariant "surrogate" proteins (λ5 and VpreB) that are structurally similar to the light chain and together can pair with the μ chain to form the so called pre-B cell receptor (pre-BCR). This complex is transiently expressed on the cell surface of large pre-B cells, together with Igα (CD79α) and Igβ (CD79β). The exact mechanism of signaling through the pre-BCR is not clear; however it stops rearrangement of the heavy chain locus by regulating the expression and function of RAG recombinases and also induces cell proliferation and transition to the next stage of development, where rearrangement of the light chain locus takes place. Other molecules, such as CD19, BLNK and Bruton's tyrosine kinase (Btk), are also required for effective pre-BCR signaling. In humans, deficiency of BLNK blocks B cell development at the pro-B cell stage, and mutations in the Btk gene cause a severe B cell-specific immunodeficiency syndrome called Bruton's X-linked agammaglobulinemia (XLA), in which no mature B cells are produced.

As mentioned above, the transition of late pro-B cells to large pre-B cells is accompanied by several rounds of cell division, expanding the population of large pre-B cells with successfully assembled heavy chain by about 30-60 fold. Rearrangement of the light chain locus begins when RAG proteins are produced again in small pre-B cells. Each of these cells can produce its own differentially rearranged light chain and thus cells with different antigenic specificities are generated from a single pre-B cell. Again, rearrangement at the light chain locus takes place at one allele at a time. Several attempts for productive

rearrangement can be made successively on one chromosome, each utilizing different gene segments, before initiating any rearrangements on the other. Generally, the κ chain locus tends to rearrange before the λ locus in human and that is the reason why the ratio of κ-expressing versus λ-expressing mature B cells is approximately 2:1 in the peripheral repertoire. Cells that manage to assemble a complete surface IgM are classified as immature B cells. Mature B cells produce δ as well as μ heavy chain by a mechanism of alternative mRNA splicing and are characterized by the additional expression of IgD on the cell surface.

3.3.2. Immune tolerance of B cells

Once B cells express sIgM, they need to be tested for autoreactivity. Tolerance at this stage of B cell development is called central tolerance, because it takes place in a central lymphoid organ, the bone marrow. If the newly expressed BCR encounters a strongly cross-linking antigen in the bone marrow, development is arrested. The immature B cell can have at this point four possible fates, depending on the nature of the interaction with its ligand: death by apoptosis (clonal deletion); production of a new receptor by a process known as receptor editing; induction of a state of unresponsiveness or diminished responsiveness to antigen (anergy); and immunological ignorance.
Clonal deletion appears to predominate when the self-antigen is multivalent. Still, there is a window of opportunity for the cell before it commits to programmed cell death, during which it can attempt further gene rearrangements to replace the autoreactive receptor with a new one. This mechanism is known as receptor editing. When an immature B cell first expresses sIgM, RAG proteins are still being produced. If the BCR is not self-reactive, the absence of cross-linking allows the rearrangement process to stop. However, if a self-antigen triggers BCR cross-linking, RAG expression continues and so does the gene rearrangement process, leading to alternative light chain production. This can happen repeatedly until a

non-autoreactive BCR is produced or V and J genes are exhausted. Cells that remain autoreactive will finally undergo apoptosis.
Immature B cells which encounter weakly cross-linking antigens of low valence usually follow a different fate, i.e. they become anergized. Anergic B cells generally retain the IgM within the cytoplasm and allow only little to be expressed on their surface. Moreover, they develop an as yet unclear molecular mechanism which appears to block the BCR signaling pathway. It has been proposed that this signaling defect may involve the inability of BCRs on anergic B cells to access regions of the cell membrane where other important signaling molecules segregate, or the increase in the expression of inhibitors of the BCR signaling pathway. More about anergy in B cells will be discussed later.
Finally, the fourth potential fate of self-reactive immature B cells is to remain in a state of immunological ignorance. This means that they do not sense their respective antigen, either because it is sequestered or present in low concentration. It should be noted that these B cells are not unresponsive and, under certain circumstances such as inflammation, can be activated by self antigens. Still, they are controlled by the so called peripheral tolerance, which can be induced in mature B cells.
Again, the principal mechanisms of peripheral tolerance are clonal deletion and anergy. So, if mature B cells are given a strong signal by an antigen of high and constant concentration, they will most probably become tolerized or induced to die by apoptosis. Moreover, if they bind an antigen without receiving at the same time any costimulatory signals, either from T_H cells or PRRs, they will most probably become permanently inactivated.

3.3.3. Survival and maturation of B cells in peripheral lymphoid tissues

Once B cells complete their development, they migrate through the blood from the bone marrow to the peripheral lymphoid tissues. The homing of B cells to

specific regions of peripheral lymphoid tissues is controlled by chemokines. For example, the chemokine receptor CXCR5, which is constitutively expressed on B cells, plays an important role in guiding them to the lymphoid follicles, where the ligand for this receptor (CXCL13) is expressed on the surface of follicular DCs and other follicular stromal cells.

The daily output of new B cells from the bone marrow is around 5-10% of the total B cell population in the periphery. The size of the peripheral pool remains more or less constant. Yet, B cells of the peripheral repertoire are generally long-lived and only 1-2% of them die every day. The failure of most newly developed B cells to survive appears to be their competition for reaching lymphoid follicles. The follicle seems to provide signals required for B cell survival. Maybe the most well-studied interaction involves the TNF family member BAFF (B cell-activating factor belonging to the TNF family) and its receptor BAFF-R which is expressed on B cells. BAFF-R deficiency is associated with immature B cell peripheral repertoire, which includes few long-lived B cells. Continuous signaling through the B cell receptor also plays an important role in the maturation and survival of peripheral B cells. Mice lacking the tyrosine kinase Syk, which is required for BCR signaling, fail to develop mature B cells but have immature B cells, instead. Thus, a Syk-mediated signal may be required for either B cell maturation or survival of mature B cells. Such signaling through the BCR does not necessarily involve antigen-specific interactions, but could be some sort of "tonic" signaling, e.g. the assembly of the receptor complex which triggers some downstream signaling events.

3.3.4. B-1 cells and marginal zone B cells

B-1 cells are a distinct subset of B cells comprising approximately 5% of all B cells of the peripheral repertoire in human and mice, and are the predominant population in rabbits. They characteristically express CD5, high levels of IgM and low levels of IgD on their surface and are mainly found in the peritoneal and pleural cavity fluid. They first appear during fetal development and were called

B-1 cells because their development was thought to precede that of conventional B cells, which are alternatively called B-2 cells. It is not yet clear if they arise as a distinct lineage from a unique precursor cell or if they derive from a precursor cell that could also give rise to B-2 cells. Most data currently support the idea that differentiation towards the B-1 or B-2 subset is probably driven by antigen selection, rather than resulting from a distinct lineage origin. It is known that certain environmental antigens as well as autoantigens encountered in the periphery drive the survival and expansion of B-1 cells. Some of these antigens, such as phosphocholine, are expressed on the surface of gastrointestinal bacterial flora. B-1 cells indeed exhibit restricted repertoire with high frequency of autoreactive clones and are thought to mediate T-independent immune responses, as will be discussed later. It has been found that B-1 cells hardly contribute to adaptive immune responses against protein antigens; instead they significantly participate in some antibody responses against carbohydrate antigens. Furthermore, they are held responsible for the production of a large proportion of the IgM antibodies that normally circulate in the blood of non-immunized mice. These antibodies are highly cross-reactive, bind with low affinity to both microbial and self antigens and are termed "natural antibodies". This supports the idea that B-1 cells are partly activated because they frequently encounter ubiquitous self and environmental antigens and that they serve to provide a first line of humoral defense within the body cavities.

Marginal zone B cells are another unique subset of B cells and are so called because they reside in the marginal sinus of the white pulp of the spleen. They display different immunophenotypic characteristics compared to follicular B cells, i.e. they express lower levels of CD23, a C-type lectin, and high levels of both the MHC class I-like molecule CD1 and two receptors for the C3 fragment of the complement, CR1 (CD35) and CR2 (CD21). Like B-1 cells, they exhibit restricted antigenic specificities which are biased towards common environmental antigens and self-antigens. They may function independently from T cells and are able to mount a quick immune response if such antigens enter the bloodstream.

3.4. B cell activation and antibody production

Once B cells complete their development in the central lymphoid system, they enter the bloodstream and migrate towards the peripheral lymphoid tissues. Normally, a lymphocyte will leave the peripheral lymphoid tissue and recirculate through lymph and blood, continually reentering lymphoid tissue until it encounters its respective antigen or it dies. When it encounters its antigen, it stops circulating, proliferates and differentiates into antibody-secreting plasma cells, as well as memory cells.

The B cell receptor (BCR) has dual function. Firstly, it recognizes and binds the antigen and thereby initiates a downstream signaling cascade. Secondly, it delivers the bound antigen to the interior of the cell, where it can be degraded into peptides that are in turn presented on the cell surface in the context of MHC class II molecules. Thereby, B cells are able to act as antigen-presenting cells and activate by-stander CD4+ T cells. Activated T cells will then provide signals (e.g. cytokines) which will drive proliferation of activated B cells and differentiation into antibody-producing plasma cells and memory cells.

As mentioned previously, some antigens are able to activate B cells directly, without T cell help, and this provides a rapid response which is very important for the defense against certain pathogens. However, somatic hypermutation which allows affinity maturation and class switch recombination to other isotypes than IgM depends on the interaction of activated B cells with helper T cells and other cells within the germinal centers of peripheral lymphoid organs.

3.4.1. T cell-dependent B cell activation

Naïve B cell activation by a thymus-dependent (TD) antigen requires a costimulatory signal from activated T helper cells (second signal, with first being the antigen binding), as well as accessory signals such as cytokines produced by

the activated T_H cells (third signal). The second signal is delivered by T helper cells which are activated by professional APCs and, at a second phase, recognize degraded fragments of the antigen as peptides presented in the context of MHC class II molecules on the B-cell surface. The interaction between CD40 ligand (CD40L) on the T cell and CD40 on the B cell is maybe the most well-established part of this second signal. In the absence of CD40 or its ligand, virtually all aspects of T cell-dependent B cell activation are blocked: no proliferation and germinal center formation occurs, no SHM and CSR take place and no B cell memory is generated. Although the epitope recognized by the T helper cell must be physically associated with that recognized by the B cell, the two cells need not recognize identical epitopes. The epitopes have to both belong to a common molecule or hapten-carrier system, so that the B cell, after internalization of the antigen through its BCR, will present an appropriate peptide which will be recognized by T cells that have been primed earlier in the infection by DCs. The specific activation of the B cell by its cognate T cell, i.e. by the T cell that has been primed by the same antigen, depends on the B cell's ability to concentrate the appropriate peptide on its surface in the context of MHC class II molecules. Antigen binding to the BCR increases this ability up to 10,000 times.
One interesting question is how a B cell manages to encounter a T_H cell of appropriate antigen specificity, since the frequency of naïve lymphocytes specific for a given antigen is estimated to be between 1 in 10^4 and 1 in 10^6. An additional difficulty should be that T cells and B cells mostly reside in distinct anatomical areas in peripheral lymphoid tissues, the so called T cell areas and the primary lymphoid follicles, respectively. Once B cells have bound antigen, they express adhesion molecules and chemokine receptors, such as LFA-1 and CCR7, respectively, which guide them to the borders of T cell zones; in this way the chance of encountering their cognate T cells is maximized. After this initial encounter, activated B cells and their cognate T cells migrate from the T zone- B zone border to continue their proliferation and differentiation and they establish a primary focus of clonal expansion. In lymph nodes primary foci are formed in the medullary cords, whereas in the spleen they are located at the border of the T zone and the red pulp.

Both B cells and T cells proliferate in the primary focus for several days, and some of the proliferating B cells differentiate into plasmablasts, i.e cells that have begun secreting antibody but are not terminally differentiated and are still dividing. Others migrate into primary lymphoid follicles of lymph nodes, where they form germinal centers. Lymphoid follicles contain large amounts of follicular DCs, which attract both naïve and activated B cells by secreting the chemokine CXCL13. Proliferating germinal center B cells displace the resting B cells towards the periphery of the follicle, creating a “mantle zone” around the center. Follicles containing a germinal center are called secondary lymphoid follicles.
The germinal center is a site of intense B cell proliferation. These rapidly proliferating B cells are called centroblasts. They express markedly low levels of surface immunoglobulin, especially IgD, and are densely packed in the so called “dark zone” of the germinal center. Gradually, they reduce their rate of division, express higher levels of surface immunoglobulin and are then called centrocytes, which migrate to the light zone of the germinal center. In the end, B cells that have undergone affinity maturation and possibly also class switching differentiate into plasma cells under the effect of a regulatory protein, BLIMP-1 (B-lymphocyte-induced maturation protein 1). This is a transcriptional repressor which inhibits the expression of genes controlling proliferation, SHM and CSR. Plasma cells increase the production and secretion of immunoglobulins, downregulate CXCR5, which recognizes CXCL13 and upregulate other adhesion molecules and chemokine receptors. Some of them remain in peripheral lymphoid organs, where they are short-lived, while the majority migrates to the bone marrow, where it survives for a long period and serves as a source of high-affinity long-lasting antibody.
Finally, some B cells that have passed through the germinal center reaction differentiate into memory cells; however the signals that drive B cells towards this direction are not yet clear. Memory B cells inherit the genetic changes that occur within the germinal center, i.e. somatic hypermutation and class switch. They divide very slowly, if at all, are long-lived and express surface immunoglobulin but secrete no antibody. Most importantly, they have significantly lower activation requirements than naïve B cells. Therefore, they are

able to produce a faster and highly specific humoral immune response in case the same antigen is encountered.
Having described T cell dependent B cell activation, one should also mention that it functions as a mechanism of peripheral B cell tolerance, as well. T helper cells that recognize self antigens are very rare, since negative selection at the T_H level is very strict. On the other hand, autoreactive B cell clones may escape central tolerance mechanisms and encounter their respective antigen in the periphery; however they are unable to produce antibodies without cognate T cell help.

3.4.2. T cell-independent B cell activation

As was already mentioned, certain antigens can provoke a humoral response without T cell help. These thymus-independent (TI) antigens are divided into two categories, which activate B cells by distinct mechanisms.
TI-type 1 antigens, e.g. LPS, are able - when used in vitro in high concentrations - to induce a polyclonal activation of B cells, regardless of their antigenic specificity. However, it is unlikely that such high concentrations of antigen exist in vivo during the early phases of infection. In lower concentrations, only those B cells whose BCR also specifically binds the TI antigen become activated. It is possible that, at these low levels, specific binding of the antigen by the B cell receptor manages to achieve sufficient concentration on the cell surface so as to signal its activation. Such responses are important in host defense, as they arise earlier than thymus-dependent responses because they do not require previous priming and clonal expansion of T helper cells. Nevertheless, they are less specific, as they lack the affinity maturation process, and they do not produce memory B cells.
TI-type 2 antigens are molecules of highly repetitive structure, such as bacterial polysaccharides, which activate mature B cells probably by crosslinking a critical number of antigen-specific BCRs on their surface. Responses to TI-type 2 antigens

are prominently produced by B-1 cells and marginal zone B cells. There is evidence supporting that DCs and macrophages are also involved in this type of responses by providing costimulatory signals for initial B cell activation, proliferation and differentiation into plasma cells. Moreover, DCs are thought to assist switching of IgM into certain other antibody classes, e.g. IgG3 in mice, by secreting cytokines, such as BAFF, as well as providing membrane-bound signals to proliferating plasmablasts. B cell responses to TI-type 2 antigens play an important role in host defense against many common extracellular bacteria. These pathogens are often coated by a polysaccharide capsule which helps them avoid phagocytosis; thereby they escape stimulating T cell responses through the presentation of bacterial peptides by macrophages. Antibody that is produced rapidly in response to the bacterial polysaccharide in a T cell-independent manner will opsonize these pathogens for phagocytosis and is therefore of crucial importance for the host. Of note, excessive crosslinking of BCRs renders mature B cells anergic. Thus, the concentration of the TI-type 2 antigen is critical for inducing a proper response.

3.5. B cell receptor signaling pathway

The immunoglobulin molecule has no signaling function on its own. On the cell surface, its heavy chains are non-covalently associated with invariant accessory protein chains, the Igα (CD79a) and Igβ (CD79b), which are required for both its transport to specific areas of the B cell membrane (lipid rafts) and for transduction of intracellular signaling. The fully functional protein complex is also known as the B cell receptor complex. Igα and Igβ are single-chain membrane-spanning proteins and their cytoplasmic tail contains a conserved ITAM sequence (immunoreceptor tyrosine-based activation motif), which is essential for the receptor's signaling ability. ITAMs characteristically contain tyrosine residues that are phosphorylated by associated kinases when the receptor binds its ligand, thereby providing sites for the recruitment of signaling proteins. Such

kinases are Fyn, Blk, Hck, Fgr and Lyn of the Src kinase family; the latter appears to be the most important kinase utilized in this process. The tyrosine kinase Syk is subsequently recruited to the phosphorylated ITAMs and undergoes initial activation by Src kinases, which phosphorylate Syk at Tyr352. After that, Syk kinase domain is released from an autoinhibitory configuration and is trans-autophosphorylated, thereby acquiring full enzymatic activity. Once activated, it associates with several adaptor proteins and phosphorylates downstream signaling molecules. This results in the activation of two key signaling intermediates, PI3K and PLCγ2. PI3K activates Akt, and PLCγ2 leads to the release of intracellular Ca^{++} and activation of protein kinace C (PKC). The signaling cascade culminates in activation of mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 MAPK, and transcription factors, including nuclear factor-κB (NF-κB) and nuclear factor of activated T cells (NF-AT). A complete immunogenic response to antigen requires activation of all these pathways. Suboptimal antigen stimulation can induce tolerogenic signaling, which is thought to be characterized by the sole activation of ERK and NF-AT [(54)].

It would be worth making an additional comment on ZAP-70 (ζ chain-associated protein) and its involvement in BCR signaling pathway. This kinase plays a key role in tranducing signals from the TCR. It was originally believed to be T cell-specific; however it has been recently found to be expressed in normal and malignant B cell subsets, as well. It is structurally homologous to Syk and possibly has a similar role in B cells where both kinases are expressed. Yet, more studies are needed to clarify the molecular mechanism by which it interferes with BCR signaling.

On the cell surface, the BCR signaling complex interacts with molecules that function as co-receptors and modulate its activity in a positive or negative way. The balance between enhancing and inhibiting BCR signaling may possibly play a role in regulating B cell fate. CD19, CD21 and CD81 can participate in the BCR proximal signaling cascade and lower its activation threshold. On the other hand, CD5, CD22, CD72, and FCγRIIb inhibit BCR signal transduction through

their intracellular ITIM sequence (Immunoreceptor tyrosine-based inhibitory motif) which binds SHP-1 or SHIP phosphatases.

Finally, CD38 has also been implicated in BCR signaling. This molecule is widely expressed in leucocytes and is found on the cell surface of B cells at different developmental stages. More specifically, it is highly expressed in plasma cells and in B cells of the germinal center, but is absent from memory B cells. It has been found to augment BCR signaling possibly by inducing Ca^{++} influx.

3.6. TLRs in B cells

B-cells are in the somewhat unusual place to express both clonal receptors of adaptive immunity (BCRs) and non-clonal receptors of innate immunity (TLRs). The TLR expression pattern differs among distinct B-cell subsets and appears to depend on the activation status Human naïve circulating B cells barely express TLRs, unless they are stimulated through their BCR (45,55-56). BCR triggering upregulates TLR1, TLR6, TLR7, TLR9 and TLR10. TLR2 expression is low but sufficient for the formation of functional TLR1/2 and TLR2/6 heterodimers. Memory B-cells, on the other hand, constitutively express these TLRs and respond to TLR stimulation by proliferation and differentiation into antibody-secreting plasma cells (55-57). More specifically, it was shown that CpG stimulation causes IgM^+ memory B-cell proliferation in the absence of cytokines or cognate T-cell help, while isotype-switched memory B-cells also require IL-2 or IL-15. This data actually led Lanzavecchia to propose a model for maintenance of serological memory by polyclonal activation of human IgM^+ memory B-cells through TLRs (57). It should be noted that exceptions have been reported regarding the expression and function of TLRs in naïve human B cells. For instance, type I interferon, which is produced in high amounts by pDCs during viral infections, was found to upregulate TLR7 expression in peripheral naïve B cells (58). Moreover, $CD27^-$ naïve human B cells cultured in a 3-step system using combinations of CpG DNA, cytokines, growth factors and CD40L were shown to efficiently differentiate into

plasma-cells without BCR triggering [59]. Therefore, one could argue that TLR expression and function on B cells may also differ depending on the local microenvironment.

The expression of TLRs on B-cells challenges the line between innate and adaptive immunity. Back in 1980s, long before the discovery of Toll, Janeway had imagined the existence of receptors recognizing conserved pathogen-associated molecular patterns which are common in the microbial taxa but evolutionarily vanished from the metazoan host and proposed that such receptors might provide a second signal necessary for effective lymphocyte activation, thereby offering an additional level of control against autoimmunity [60]. In 2006, a study by Ruprecht and Lanzavecchia argued that TLR stimulation acts as a third signal (second signal being T_H-cell help) required for activation, proliferation, isotype switching and plasma cell differentiation of human naïve B-cells [61]. B-cell response was shown to depend on TLR ligands acting optimally on TLRs expressed by B-cells themselves. Alternatively, supernatants of DCs stimulated by TLR agonists were also capable of potentiating B cell response, but to a much lower level. However, other authors have questioned this finding by demonstrating normal thymus-dependent (TD) immune responses in mice deficient for TLR signaling [62]. Recently, an elegant study utilizing mice in which only B-cells were deficient for TLR signaling showed that B-cell intrinsic TLR signals amplify but are not necessary for humoral responses [63].

In mice, it has been shown that TLR agonists may have differential effects on distinct B-cell subsets. For instance, while TLR agonists promote proliferation in all four murine B-cell subsets (B-1a, B-1b, marginal zone and follicular B-cells), they selectively promote plasma-cell differentiation of those subsets specialized in thymus-independent (TI) antigen responses, i.e. B-1 and marginal zone B-cells [64-65]. A recent study, also in murine systems, demonstrated that TLR9 stimulation directly in B-cells can regulate class switch recombination to IgG2a, which is regarded as the most important antibody isotype against viral and bacterial infections; in human, it corresponds to IgG1 [66]. The molecular background of these effects is not yet understood. However, there is evidence that, besides the TLR signaling cascade, cross-talk with the BCR takes place. Treatment of murine B

cells with TLR agonists, namely LPS and Pam3CSK4, produced an alternate BCR downstream signaling that was different from the classical, signalosome-dependent pathway, still resulting though in ERK phosphorylation and NF-kB activation [67]. Indication that TLR engagement interferes with BCR signaling at the molecular level also exists in human B cells. A recent study supports that TLR7 or TLR9 stimulation in IgM$^+$ B-cells upregulates ZAP70 via sustained Akt phosphorylation[68]. It should be noted that there are several differences between murine and human B-cells in terms of TLR expression and function. Generally, the murine immune system appears to be more sensitive to TLR stimulation, as naïve B-cells constitutively express TLRs and respond to TLR agonists with strong proliferation and plasma-cell differentiation in the absence of BCR triggering [69-70]. One should keep in mind such differences when interpreting results from murine experimental systems.

Dual engagement of IgM and Toll-like receptors in B-cells has been proposed as a mechanism leading to autoimmunity since 2002, when the Rothstein group demonstrated that triggering the BCR and TLR9 of rheumatoid factor (RF)$^+$ autoreactive B-cells in a sequential manner is required for the production of autoantibodies [32]. Soon after that, another study showed that RNA-associated autoantigens effectively activate autoreactive B-cells by combined engagement of BCR/TLR7 [71]. Other evidence that TLRs are implicated in autoimmunity is the fact that MyD88 deficiency in MRL/lpr mice ablates autoantibody production [72] and deficiency of TLR7 or TLR9 abrogates the production of autoantibodies against RNP and dsDNA, respectively [73]. However, in non-autoimmune prone mouse strains, TLR engagement fails to break tolerance. Chronic activation of ERK appears to act as a mechanism of resistance to CpG DNA-induced autoimmunity in anergic B-cells [74]. Uncoupling of the BCR from a calcineurin-dependent pathway is another mechanism for anergic B-cells to evade signals that synergize with CpG DNA for proliferation [74]. Recently, a study suggested endocytic sequestration of the BCR and TLR9 as an additional way to reinforce B-cell anergy [75]. The fact that the engaged BCR and TLR9 arrest together outside late endosomes further implies their synergistic action under common regulation. Along this line, one could propose that

malfunction of the above resistance mechanisms could render otherwise anergic B-cells sensitive to activation by combined BCR/TLR ligation and thereby lead to autoimmunity.

4. Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is a hematologic malignancy characterized by the *in vivo* accumulation of $CD5^+$ monoclonal B-cells in peripheral blood (PB), bone marrow (BM) and lymphoid tissue. Until relatively recently, it was considered to be a homogeneous disease of naive, immune-incompetent, minimally proliferating B- cells which accumulated due to underlying apoptotic defects [(76)]. In the past two decades, however, our view on the pathogenesis of CLL substantially changed. It appears that, besides genetic events, microenvironmental stimuli interfere with the selection and active expansion of the leukemic clone [(77)].
The epidemiology of CLL has first implied a possible genetic predisposition. CLL is more common in men than women. Moreover, it is most common in Caucasians and decreases in frequency in a descending order among blacks, Hispanics, American Indians and Native Alaskans, and Asians and Pacific Islanders. The rarity of CLL among Asians and Pacific Islanders persists even in immigrants to the Western Hemisphere. Furthermore, up to 10% of CLL patients have a first- or second-degree relative with CLL, thus making CLL one of the most common malignancies with familial predisposition. Relatives of patients with CLL also appear to have a higher frequency of other lymphoproliferative disorders and autoimmune diseases. Nevertheless, research for predisposing genes has so far identified only a single germline mutation of death-associated protein kinase (DAPK) to be convincingly linked to CLL. Recent studies suggest that microRNAs are major candidates for the elusive class of CLL predisposing genes and more on this will be discussed in the following section.

The complexity of CLL pathophysiology has become increasingly apparent as basic science started to reveal more on the disease's molecular biology. Several studies have prompted the hypothesis that CLL might represent an antigen-driven disease similar to other B cell malignant disorders, such as gastric MALT lymphoma, and this hypothesis will be further analyzed below. Yet, many questions remain unanswered, including the normal counterpart of CLL cells, the function of the BCR in CLL, and the nature of infectious or other naturally occurring antigens that may drive the expansion of the malignant clone. Basic science has allowed dissecting this heterogeneous disease into subgroups with similar molecular and clinical characteristics and has equipped us with important prognostic markers. Further understanding of the molecular grounds of CLL is anticipated to open new windows for therapeutic exploitation.

4.1. CLL cytogenetics

CLL is characterized by recurrent DNA gains and losses often associated with clinical course and outcome[78] as summarized in ***Table 1.*** Recently, through the use of improved modern protocols to obtain informative metaphases or microarray-based comparative genomic hybridization it has been demonstrated that that chromosomal abnormalities may also exist in >90% of CLL cases; the vast majority are unbalanced translocations which result in losses or gains of genomic material.

13q14.3 deletion

Deletion of chromosome 13q14.3 is the most common cytogenetic lesion in CLL and can be homozygous in up to 15% of the cases. When it represents the sole genetic aberration it is associated with a good clinical outcome. Its pathogenetic role is not yet fully clear, although its high frequency in early stages of CLL suggests that it may be implicated in the transformation process. The deleted region always includes the locus coding for two microRNAs (miRNAs),

mir-16.1 and mir-15a. MicroRNAs represent a class of non-coding small RNA molecules which bind to specific mRNA transcripts and either lead to their degradation or inhibit their translation (RNA silencing). Lack of expression of mir-16.1 and mir-15a has been proposed to release the expression of the anti-apoptotic BCL2 protein, as well as other proteins involved in cell cycle and apoptosis (e.g. MCL1, ETS1 and Jun).

Karyotype	*% of cases (range)*[1]	*Prognosis*	*Known and/or putative involved genes*
13q14.3 deletion	*14-40*	*good*[2]	*mir-16.1*
trisomy 12	*11-18*	*Intermediate*	*CLLU1*
11q22-23 deletion	*10-32*	*Bad*	*ATM*
17p13.1 deletion	*3-27*	*bad*[3]	*TP53*

[1] According to Sheiler et al.(ref.79)

[2]If it is the sole cytogenetic lesion

[3]Also predicts refractoriness to purine analogs

Table 1. Genomic aberrations of prognostic relevance in CLL

Trisomy 12

Trisomy 12 is associated with an intermediate prognosis. The 12q22 region contains the *CLLU1* gene. High CLLU1 protein expression levels have been associated with poor clinical outcome; nevertheless no such difference in patients with or without trisomy 12 has been established.

11q22-q23 deletion

Chromosome 11q22-q23 deletion is associated with aggressive disease. This lesion includes the *ATM* gene, which is known to be involved in DNA repair. This gene is found to be mutated in approximately 15% of CLL cases, even without concomitant 11q deletion. Because most probably loss of heterozygosity (LOH) is

required for ATM to lose its tumor-suppressor function, the poor prognosis of 11q- patients may also involve other genes regulating cell cycle and apoptosis (e.g. NPAT, CUL5, PPP2R1B)

17p13.1 deletion

This deletion which involves the *p53* locus occurs in only a small fraction of CLL patients at diagnosis. It is the most adverse prognostic factor among all cytogenetic lesions. TP53 is a transcription factor activated by DNA damage which arrests cell cycling in order for DNA repair mechanisms to restore genome integrity, thereby preventing clonal progression. It can be inactivated by somatic mutations in the absence of chromosomal deletion, as well. Loss of heterozygosity for p53 is associated with poor response to chemotherapy, including alkylating agents and purine analogs.

4.2. MicroRNAs in the pathophysiology of CLL

CLL was the first malignancy to be linked to miRNAs. More specifically, a cluster of two miRNA genes located at chromosome 13q14.3 (MIRN15a and MIRN16.1) was found to be either deleted or downregulated in the majority of CLL cases, resulting in upregulation of the anti-apoptotic BCL2 protein [(80)]. Data from animal models also support the notion that the altered expression of these two miRNAs may be the molecular lesion driving CLL. The TLC1 (T-cell leukemia 1) transgenic mice who develop a frank leukemia with CLL characteristics exhibit downregulation of both MIRN15a and MIRN16.1 in more than 50% of cases [(81)]. Moreover, mutations in murine Mirn15a/Mirn16.1 have been linked to the development of a CLL-like disease in a mouse model for spontaneous tumors [(82)]. Following this initial discovery, miRNA expression profiling has since identified certain signatures associated with diagnosis of CLL, clinical course and response to treatment. Even though some results have been disparate, it appears that expression of miR-223 and members of the miR-29 family is downregulated in CLL

cells and that this reduced expression is associated with aggressive disease [83-86]. Low expression of miR-181a, let-7a, and miR-30d and increased expression of miR-155 have also been associated with distinct clinical outcomes [84]. Finally, reduced miR-34a expression has been associated with fludarabine-refractory CLL either with or without p53 deletion [87].

4.3. The role of antigen stimulation in CLL

Several lines of evidence support a central role of the BCR in the natural history of the disease. CLL cells express CD19, CD5 and CD23 and have reduced expression levels of IgM, IgD and CD79b, i.e. they display a surface phenotype resembling antigen-experienced B lymphocytes [88]. Their gene expression profile also corresponds to that of memory B cells [89]. Moreover, clinical observations have related the expression of molecules involved in BCR signaling, such as CD38 and ZAP-70, with clinical course and outcome, a fact which implicates BCR in the biological behaviour of the malignant clone [90-91].

In at least 50% of CLL cases, leukemic cells carry somatically mutated IGHV genes (“mutated” CLL cases), which suggests Ag encounter. It should be emphasized that the finding of unmutated IGHV genes does not preclude Ag encounter, as for example in the case of T-cell independent antigens stimulating B-cells outside germinal centers. The IGHV gene mutational status of the malignant clone is considered as one of the most important prognostic factors in CLL. Unmutated IGHV genes are associated with a more aggressive course of the disease, whereas mutated IGHV genes usually predict a more indolent form of CLL [92-93]. More importantly, the IG gene repertoire of CLL cells is characterized by a bias regarding the usage of selected IGHV genes, such as IGHV1-69, IGHV4-34, IGHV3-23 and IGHV3-21, when compared to normal B cell repertoire [94-97]. Recently, several groups reported subsets of CLL cases characterized by distinctive IG heavy and light chain rearrangements culminating in remarkably

homologous complementarity-determining region 3 (CDR3) sequences within their BCRs [98-102].
It was the Chiorazzi group that first coined the term "stereotyped BCRs"; they would consider BCRs to be stereotyped if they utilized the same IGHV/IGHD/IGHJ germline genes, had the same IGHD gene reading frame and showed VH CDR3 amino acid identity >60%. This identity threshold turned out to be in accordance with established bioinformatic algorithms, such as aminoacid substitution matrices. Yet, we now understand that quasi-identical CDR3 regions can also be produced even if different IGHV genes are utilized, especially when the later belong to the same phylogenetic clan and therefore their germline sequences are closely related.
Given that the probability of two separate B cell clones of the peripheral repertoire sharing stereotyped receptors is normally extremely low (10^{-12}), therefore, such striking BCR similarity in unrelated and geographically distant CLL cases supports the existence of a limited set of antigens or classes of structurally related epitopes that are involved in the selection of the leukemic clones. Further evidence for this hypothesis is data suggesting stereotyped somatic hypermutation (SHM) patterns among these CLL subsets [103].
BCR stereotypy is very frequent in CLL. A recent study involving a large series of CLL patients reveals that almost 30% of cases carry stereotyped receptors [102,104]. This percentage exceeds 40% among unmutated cases. CLL cases expressing stereotyped BCRs have also been shown to share common clinical features, so that certain subsets are now associated with distinct prognosis [98,102,105-106]. It would be interesting to mention that, in at least some cases, subset assignment alone seems to define prognosis irrespective of IGHV gene mutational status. For instance, the IGHV3-21/IGLV3-21 subset (no. 2) is an unfavorable prognostic marker regardless the degree of mutation [95,97,107-108]. Taken together, stimulation through the BCR appears to play a crucial role in the pathogenesis of CLL. Therefore, it would be of great importance to determine the selecting antigenic elements.
Sequence analysis cannot provide definitive answers to the question of which are the relevant antigens; however a comparison of CLL BCRs with antibodies of

known specificity has provided interesting hints. CLL cells often express BCRs that bind autoantigens in a polyreactive manner, like "natural antibodies" [109]. These autoantigens are mostly cytoskeletal components that can be exposed on cell surface during apoptosis, injury or normal catabolic processes [110-113], as well as immunoglobulin and single-stranded DNA molecules [114-115]. CLL cells were also found to bind neoantigens, i.e. molecules (haptens) that become immunogenic after chemical modification, e.g. oxidation [116], and also foreign antigens, e.g. components of the bacterial wall [110,117]. Almost ~80% of "unmutated" and ~15% of "mutated" CLL BCRs were shown to react in vitro with a series of self and foreign antigens, as well as fixed and permeabilized cells, in a polyreactive manner. The spectrum of epitopes reacting with CLL mAbs is generally broader in U-CLL clones [118]. Nevertheless, most recombinant mAbs obtained from "mutated" CLL cells that were not polyreactive *per se* acquired polyreactivity when their sequence was reverted to germline [110]. Taken together, these lines of evidence indicate that CLL cells may derive from a population of auto/polyreactive cells which either retain (U-CLL) or lose (M-CLL) polyreactivity as a result of SHM. One could hypothesize that latent viruses, commensal bacteria, neo- or autoantigens promote division of these B-cells, increasing the risk of dangerous DNA mutations. This theory is in agreement with the observation that cytogenetic lesions (eg. del17p13, del11q22-23, trisomy12, del13q14) are rare early in the course of CLL, but may develop later. A similar pathogenetic mechanism has already been described for gastric lymphomas which develop secondary to infection by Helicobacter pylori [119].

CLL subset #4 which is defined by the stereotyped IGHV4-34/IGKV2-30 BCR and represents ~1% of CLL cases constitutes an interesting example with regard to the above [102-103,106,114]. The IGHV4-34 gene encodes antibodies that are intrinsically autoreactive against the N-acetyllactosamine (NAL) antigenic epitope [120-121]. NAL is present on various self-antigens (I/i blood group antigen, CD45 B-cell isoform), as well as foreign pathogens (EBV, CMV, Mycoplasma pneumoniae). In this particular case, recognition involves a hydrophobic patch in framework region 1 (HFR1) instead of the classical antigen binding site [122]. Although the IGHV4-34 gene is frequent among the normal peripheral B-cell repertoire, such

antibodies are rarely detectable in the sera of healthy individuals, suggesting that the inherent polyreactivity of such B-cells has been alleviated by anergy. Nevertheless, IGHV4-34 antibodies have been found in high titers in the sera of patients with systemic lupus erythematosus (SLE), as well as healthy individuals after acute infections with EBV, CMV and M. pneumoniae [123-125].

In CLL subset #4, the stereotyped IGHV4-34/IGKV2-30 BCRs are characterized by long HCDR3s, enriched in positively charged amino acids, similar to anti-DNA autoantibodies [102-104,106,114,126]. The IGHV4-34 gene is most often mutated [94,102-103], possibly implying that SHM is employed in order to censor autoreactivity of the corresponding progenitor cell. Indeed, a stereotyped pattern of somatic hypermutation characterized by the introduction of acidic residues has been reported for this subset [103,127], similarly to anti-DNA autoantidody editing [128-129]. However, SHM does not appear to affect HFR1 [103], therefore in theory the B-cells retain their ability to interact with superantigens. On this basis, it has been proposed that certain auto- or exo- antigens could perhaps provide signals promoting survival, expansion, malignant transformation and/or clonal evolution of these B-cells with distinctive BCRs. In accordance with this notion, a recent retrospective analysis has identified a significant correlation between usage of the IGHV4-34 gene in CLL cases, particularly those belonging to subset #4, and persistence of EBV and CMV [130].

No definite answers exist regarding the timing and duration of antigenic stimuli in the pathogenesis of CLL. A recent study explored the existence of intraclonal diversification (ID) of IGHV genes through ongoing SHM in CLL and showed that, with the notable exception of subset #4 (IGHV4-34/IGKV2-30 BCR), no or low levels of ID occur [131]. In the case of subset #4, the intense ID implies functional interactions with the antigenic element(s) both in the pre-malignant phase but also after leukemic transformation. At the same time, one cannot exclude the possibility of non-specific stimulation through pattern recognition receptors (PRRs) that are expressed on CLL cells, in parallel with or independently of BCR signaling, and more on this will be discussed later.

4.4. IGHV4-34 CLL subsets

The IGHV4-34 gene is detected in approximately 10% of CLL cases. So far, four subsets with distinct, restricted HCDR3s have been identified among CLL cases expressing the IGHV4-34 gene (subsets #4, 11, 16, 29) [102]. The main subset (subset #4), which was described above, is characterized by the concomitant use of IGKV2-30 light chains. Its heavy variable CDR3 (HCDR3) is 20 amino-acid-long and rich in aromatic and positively charged amino acids. Arginine-arginine (RR) or lysine-arginine (KR) dipeptides are characteristically located in the joints between IGHD and IGHJ genes, resulting in the formation of a R(K)RYYY motif at the top of the HCDR3 region. Comparison to public database sequences has revealed that this particular motif is an exclusive characteristic of subset #4. All subset #4 cases are IgG-switched, and the IGHV4-34 gene is mutated. Cases assigned to this subset are relatively young at diagnosis and tend to follow an indolent course of the disease, compared to heterogeneous IGHV4-34 cases.

Subset #16 is characterized by IGHV4-34/IGKV3-20 gene usage and a stereotyped 24 amino-acid-long HCDR3. Little is known about the clinical characteristics of CLL cases belonging to this subset, probably because of the limited number of patients identified so far. A recent study comparing the gene expression profile of subset #4 and subset #16 identified several differentially expressed genes. These genes were mostly involved in cell cycling, proliferation and immune response and were exclusively down-regulated in subset #4, in consistence with the low-proliferative disease observed in subset #4 CLL patients. This distinct gene expression profile could be considered as evidence for biological differences in the pathogenesis of these two subsets.

4.5. CLL subset #1

This subset includes minimally mutated (99-99.9% identity to germline) and completely unmutated (100% identity to germline) IGHV gene sequences. Cases

of this subset express IGHV genes of the same phylogenetic clan (IGHV1-2/IGHV1-3/IGHV1-18/IGHV1-8/IGHV5a/IGHV7-4-1) in combination with stereotyped kappa light chains employing the IGKV1-39/D1-39 genes. This culminates in stereotyped CDR3s with a QWL amino acid motif [(102-103)]. It has been shown that IgM from subset #1 CLL cells binds to oxidized phospholipids [(111)]. Recent work also showed that antigen stimulation alone is not sufficient for induction of proliferation or IgM-secretion. In contrast, combined BCR and TLR9 triggering was able to drive a full proliferative response and differentiation of CLL cells into antibody-secreting cells [(132)].
With regard to the clinical characteristics of this subset, it is associated with progressive disease and poor prognosis, even though age and clinical stage at diagnosis do not appear to differ from heterogeneous HCDR3 cases utilizing the same IGHV genes.

4.6. BCR signaling in CLL cells

In normal B cells, BCR engagement can lead to a number of different outcomes, from activation, survival and proliferation to anergy or deletion by apoptosis. This is generally thought to depend on the affinity/avidity and the duration of antigen binding.
All the data discussed so far point towards a critical role of BCR engagement in the natural history of CLL. However, it appears that only ~50% of CLL cases are able to respond to in vitro cross-linking of their surface immunoglobulin (sIg) with effective activation, as measured by global tyrosine phosphorylation [(133)]. In contrast, the remaining cases are unresponsive to BCR cross-linking, thereby resembling B cells anergized after antigen stimulation. Nevertheless, the BCR signaling pathway in such cases appears to be intact, since this BCR unresponsiveness was shown to be reversible in vitro and dependent on the levels of sIgM [(134)].

The molecular background of this functional difference among CLL cases is not yet clear. This heterogeneous response has been found to correlate with several prognostic markers, such as IGHV gene mutational status, ZAP-70 and CD38 expression. More specifically, cases with unmutated IGHV genes, high expression of ZAP70 and/or CD38 are more frequently found to respond to BCR ligation [133-135]. Given the clinical correlation of such cases with adverse course and outcome, one could speculate that increased signal propagation through the BCR is associated with aggressive CLL clones. It should be noted though that, even in these cases, BCR ligation does not elicit a full immune response, since it does not induce proliferation [134]. However, one should keep in mind that BCR crosslinking with soluble anti-IgM antibodies might not accurately recapitulate the in vivo antigenic stimulation. In favor of this idea are studies showing that immobilized anti-IgM antibodies promote CLL cell survival probably by engaging the BCR for longer [136-137].

Anergy is one of the physiological mechanisms employed to silence autoreactive B-cells. In murine models, where it has been studied extensively, it is characterized by reduced sIgM expression, attenuated BCR signaling and constitutive activation of ERK and NF-AT transcription factor. CLL clones universally express low levels of surface immunoglobulin [96]. An intriguing recent study shows that CLL cases unresponsive to BCR cross-linking exhibit constitutive phosphorylation of MEK1/2 and ERK1/2 together with NF-AT activation, while at the same time lack Akt phosphorylation [138]. This molecular profile matches the biochemical signature of anergy in murine systems [139]. On these grounds, the researchers proposed that these CLL cases could correspond to previously anergized autoreactive B-cells aberrantly expanded as a result of the malignant process. The anergy of B cells is thought to result from sustained exposure to soluble (auto)antigens in the absence of co-stimulatory signals. In this sense, it is interesting to note that BCR unresponsiveness is shown to be reversible when such CLL cells are isolated and cultured in vitro, suggesting chronic stimulation of the BCR by a putative antigen in vivo [134]. It would be challenging to test potential correlations of this molecularly defined anergic state with biological behavior in larger series of patients. If such correlation is confirmed, it would

suggest a possible mechanism underlying the distinct biological behavior of CLL clones and also offer potential targets for pharmacological intervention.

4.7. TLRs in CLL cells

Limited data exist regarding which TLRs are expressed in CLL cells; however one would expect their expression profile to resemble that of Ag-experienced B cells. Several groups reported ubiquitous expression of TLR7 and TLR9 by CLL cells (71,140). Recently, the Caligaris-Cappio group reported additional expression of TLR1, TLR2, TLR6 and TLR10, thus describing an expression pattern which indeed resembles activated B-cells (141). Triggering TLRs with their respective ligands was shown to activate the NF-κB signaling pathway and induce upregulation of surface costimulatory molecules. Still, the effects of TLR stimulation on CLL cells remain unclear, especially as regards survival, proliferation and apoptosis. There are studies suggesting a pleiotropic effect of TLR stimulation, namely TLR9, on leukemic B cell growth and survival (142-143). A recent study showed that TLR9 stimulation induces proliferation mainly in CLL cells from patients with unmutated IGHV genes, whereas apoptosis is induced in CLL cases with mutated IGHV genes (144). This distinct response was attributed to increased Akt signaling in the proliferating cases. Actually, introduction of constitutively active Akt in non-proliferating CLL-cells was sufficient for induction of cyclin A after CpG stimulation. Another report states that TLR7 engagement in CLL cells induces an NF-κB-dependent activation of the nitric oxide (NO) pathway, thereby offering resistance to apoptosis (145).

4.8. CLL therapy: present and future

CLL is extremely heterogeneous in its clinical course; some patients live for decades with no need for treatment, while others have a rapidly progressive clinical course. The goal of current therapeutic protocols has been to maintain

the best quality of life and treat only when patients become symptomatic from their disease. For the majority of patients this means following a “watch and wait” approach to determine the rate of progression of the disease and assess for development of symptoms.

Two widely used staging systems for CLL, Rai and Binet, have established prognostic value for survival and are summarized in *Table 2.*

Stage		Clinical features	Median survival, y
Rai classification			
(low risk)	0	Absolute lymphocytosis >$15x10^9$/L	>10
(intermediate risk)	I	Stage 0 + lymphadenopathy	7
	II	Stage 0 + enlarged liver and/or spleen +/- lymphadenopathy	
(high risk)	III	Stage 0 + anemia (Hb<10 g/dL) +/- lymphadenopathy +/- organomegaly	0.75-4
	IV	Stage 0 + thrombocytopenia (Plts< $100x10^9$/L) +/- lymphadenopathy +/- organomegaly	
Binet classification			
	A	<3 areas of lymphadenopathy; no anemia; no thrombocytopenia	12
	B	≥3 areas of lymphadenopathy; no anemia; no thrombocytopenia	7
	C	Anemia or thrombocytopenia	2-4

Table 2 . Rai and Binet staging systems for CLL

In general practice, newly diagnosed patients with asymptomatic early-stage disease (Rai 0, Binet A) should be monitored without therapy until they present

evidence of disease progression. On the other hand, patients at high risk (Rai III or IV, Binet C) benefit from institution of treatment. As for cases of intermediate risk (Rai I or II, Binet B), they can be monitored without therapy until they have evidence of symptomatic or progressive disease (bone marrow failure, refractory autoimmune anemia/thrombocytopenia, constitutional symptoms, massive or symptomatic lymphadenopathy and/or splenomegaly, lymphocyte doubling time <6 months)[(146)] .

Currently, the cornerstone of CLL treatment is combined chemoimmunotherapy with Rituximab (anti-CD20 antibody), cyclophosphamide (an alkylating agent) and fludarabine (a purine analog), at least for medically fit patients without serious comorbidities. Yet, approximately 20% of patients, especially those carrying a 17p-deletion, are resistant to such type of therapy and survive for less than a year despite high-dose treatment [(147)]. As knowledge on CLL pathogenesis is rapidly increasing, a number of opportunities for therapeutic exploitation emerge. Nevertheless, any alteration to current clinical practice will require demonstration of improved survival with early institution of therapy, or identification of criteria that define "high risk" patients who could benefit from early institution of therapy.

Since BCR signaling appears to play an important role in malignant B-cell pathophysiology, a number of pharmacological agents targeting the BCR itself or downstream molecules of its signaling pathway are currently being tested both in vitro and in clinical trials (reviewed in 148). PKC inhibitors and monoclonal antibodies against CD19 are also being evaluated. It remains to be seen if targeting the BCR pathway is of therapeutic benefit in both BCR-competent and BCR-incompetent CLL cases.

Another therapeutic strategy involves increasing the immunogenicity of the malignant clone. CLL cells are markedly inefficient in antigen presentation, and this is generally attributed to their low expression of costimulatory and adhesion molecules [(149)]. At the same time, T-cell function in CLL patients is also impaired, despite increased absolute numbers, especially as regards the $CD8^+$ T-cell population [(150)]. Interestingly, direct cell-to-cell contact of CLL B-cells with healthy heterologous T-cells appears to induce functional defects to the later [(149)].

Lenalidomide was recently shown to have a positive effect on the impaired immunological synapse between CLL cells and T-cells, however its mechanism of action is only vaguely understood [(149)]. It has already entered a phase II clinical trial and shows clinical efficacy in patients with relapsed or refractory CLL [(151-152)]. Manipulation of CD40/CD40L interaction is also being investigated, but the results are so far contradicting [(153-154)].

Because triggering TLRs induces upregulation of costimulatory molecules on the surface of CLL B-cells, the idea of using TLR ligands as adjuvants for CLL immunotherapy has recently gained momentum. Studies are mainly focusing on TLR7 and TLR9 and their respective agonists have entered clinical trials with the aim to increase immunogenicity and/or augment proliferation and thereby sensitivity to cytotoxic therapies (reviewed in 155). However, it is not clear if the phenotypic changes that TLR signaling induces in CLL cells actually facilitate cytotoxic T-cell responses or lead to inappropriate T-cell activation and thereby anergy and tolerization. In addition, accumulating evidence underlines the growth-promoting impact of exo- or endogenous TLR ligands on leukemic B cells (reviewed in 156). Most recent studies report a pleiotropic effect of TLR stimulation in B-CLL cells as regards apoptosis and proliferation and it appears that this differential effect is related to the cellular and molecular characteristics of the malignant clone [(142-144)]. More research is required to define which subgroups of patients would benefit from TLR stimulation. Additionally, combinations of TLR targeting with other chemotherapeutic or immunomodulating agents need to be investigated.

PART II: AIM OF STUDY

AIM OF STUDY

In the past decade, a wealth of new information has brought chronic lymphocytic leukemia (CLL) in a crossroad between cancer and autoimmunity. The role of antigenic stimulation through the B cell-receptor (BCR) has been intensively investigated and several features of the BCR have emerged as important prognostic factors of the disease. Firstly, the presence of somatic mutations in the immunoglobulin variable region genes was strongly associated with an indolent course. Soon after that, the identification of multiple CLL subsets with "stereotyped" BCRs which relate to common clinical characteristics set the potential of further tailoring disease treatment according to the molecular characteristics of the malignant clone.

Toll-like receptors (TLRs) recognize an array of distinct molecular patterns and are known to bridge innate and adaptive immunity by promoting dendritic cell (DC) maturation and cytokine production [157]. The role of TLRs in B cell activation and antibody production is currently a field of intense research, as it has potential appplications in vaccination, autoimmune diseases and cancer. Human naïve B cells express low to undetectable levels of all TLRs, however BCR crosslinking leads to rapid upregulation of TLR2, TLR6, TLR7, TLR9 and TLR10 [56]. $CD5^{+}$ memory B cells, on the other hand, constitutively express these TLRs [55-56]. Recently, it has been proposed that TLR stimulation acts as a direct third signal amplifying human naïve B cell response to antigen [61,63] and at the same time may maintain serological memory by acting directly on memory B cells. An innovative study by Leadbetter et al showed that DNA-containing chromatin-IgG complexes activate B cells to produce antibodies against self-IgG by engaging the autoreactive BCR and TLR9 in a sequential manner [32]. This new concept was further confirmed by Lau et al who demonstrated that RNA-associated auto-Ags activate B cells by dual BCR/TRL7 engagement, thereby supporting a role of TLRs in breaking tolerance [71].

When it comes to CLL cells, one would expect their TLR expression profile to resemble that of antigen-experienced B cells. Several groups have reported ubiquitous expression of TLR7 and TLR9 by CLL cells [140-141,144] and their respective

agonists have entered clinical trials with the aim to increase immunogenicity by upregulation of costimulatory molecules and/or augment proliferation and thereby sensitivity to cytotoxic therapies (reviewed in 155, 158). A recent article by the Caligaris-Cappio group has demonstrated the expression of TLR1, TLR2, TLR6, TLR10, NOD1 and NOD2 in CLL cells, additionally to TLR7 and TLR9. The researchers also reported that TLR1/2 and TLR2/6 are indeed functional, as their stimulation was found to upregulate the expression of costimulatory molecules (CD25, CD86) and protect cells from spontaneous apoptosis in vitro. Nevertheless, the study involved a small series of patients (n=18), thus precluding definitive conclusions regarding the function of TLRs and NODs among various CLL subsets[141]. The literature already poses the question whether TLR stimulation may signal differently among CLL subgroups. This is underlined by the contradictory findings regarding the effect of TLR9 stimulation in CLL clones of different IGHV gene mutational status. In most "unmutated" cases, TLR9 stimulation resulted in proliferation of the leukemic clone, whereas in most "mutated" cases it resulted in apoptosis [144].
Against this background, it would be obviously relevant to explore the possibility of differential TLR function within CLL subgroups defined by IGHV gene mutational status, IGHV gene usage or BCR stereotypy. Within this frame, the present study aims to investigate the possible existence of distinct patterns of TLR function among different CLL subgroups by analyzing a large series of patients.

Theory: CLL cells have been shown to exhibit a TLR profile similar to that of antigen-experienced cells. This further supports the idea that antigens are involved in the selection and expansion of the leukemic clone. Differentiations in TLR function between different CLL subgroups would provide important evidence of distinct antigen recognition by TLR/BCR and also offer hints about the nature of the relevant antigen(s) for each subgroup. Such type of information regarding different CLL subgroups could also be exploited in order to guide targeted therapeutic interventions.

PART III: STUDY DESIGN

Workpackage:

Functional analysis of TLRs in CLL cells in terms of upregulation of costimulatory molecules. Comparison between CLL subsets.

MATERIALS AND METHODS

Patient group

Blood samples were collected from 67 CLL patients diagnosed according to the recently revised guidelines of the International Workshop Chronic Lymphocytic Leukemia/National Cancer Institute (IWCLL/NCI) (146). All patients were either untreated or off therapy for at least 6 months before the study.
The study group was intentionally biased to cases expressing stereotyped BCRs assigned to subsets #1 and #4 (defined according to Stamatopoulos *et al* (102) and Murray *et al* (103)), which may be considered as prototypes of "unmutated/adverse prognosis" and "mutated/good prognosis" subsets, respectively. Demographic, clinical and biological data for the patients included in the study are given in *Tables 3 and 4.*
In brief, 44 patients (65.7%) carried mutated IGHV genes (<98% identity to germline), whereas the remainder (23 patients, 34.3%) carried unmutated (≥98% identity) IGHV genes. Based on the molecular characteristics of the clonogenic IG receptors, patients were classified in different subgroups, listed in *Table 5.*

Cell purification

CD19+ B cells were purified from whole blood using a Human B cell enrichment kit (RosetteSep; StemCell Technologies, Vancouver, BC, Canada), following the manufacturer's instructions. This system utilizes a cocktail of mouse and rat

1	Lab Identifier	Sex	Age of Diagnosis	Rai	Binet	Disease Progression	Last Follow-Up	Current status
2	P4699	F	76	0	A	YES	12/5/2009	ALIVE
3	P3506	M	71	0	A	YES	15/12/2009	ALIVE
4	P3870	M	68			NO	2/11/2009	ALIVE
5	P4295	F	57	0	A	NO	12/10/2009	ALIVE
6	P2355	M	54	II	A	YES	22/1/2010	ALIVE
7	P2685	F	67	0	A	NO	15/12/2009	ALIVE
8	P3073	F	38	IV	C	YES	15/4/2009	DEAD
9	P8805	F	70	I	A	YES	20/1/2010	ALIVE
10	P9320	M	47	I	A			
11	P1697	M	60	II	A	YES	14/1/2010	ALIVE
12	P571	M	47	0	A	YES	20/7/2007	ALIVE
13	P8762	M	62	I	A	YES	14/1/2010	ALIVE
14	P1618	M	62	0	A	NO	10/11/2009	ALIVE
15	P1156	F	63	II	A	YES	23/2/2010	DEAD
16	P5283	M	61	I	A	NO	23/11/2009	ALIVE
17	P511	M	63	II	B	YES	21/12/2009	ALIVE
18	P2329	F	57	0	A	NO	7/10/2009	ALIVE
19	P7155	M	50	0	A	NO	5/10/2009	ALIVE
20	P2618	F	78	0	A	NO	7/9/2009	ALIVE
21	P8192	M	54	I	A	NO	9/11/2009	ALIVE
22	P7794	F	76	0	A	NO	7/9/2009	ALIVE
23	P6124	M	68	0	A	NO	1/12/2009	ALIVE
24	P9940	M						
25	P4383HA	F	69	0	A	NO	1/12/2009	ALIVE
26	P6460	M	69	0	A	NO	23/11/2009	ALIVE
27	P1894	M	52	0	A	NO	7/9/2009	ALIVE
28	P427	M	53	0	A	NO	7/9/2009	ALIVE
29	P2577	M	37	0	A	NO	27/11/2009	ALIVE
30	P2548	M	69	I	A	YES	10/12/2009	ALIVE
31	P5231	M	57	II	B	YES	25/11/2009	ALIVE
32	P5728	M	53	I	A	NO	15/6/2009	ALIVE
33	P5949	M	50	0	A	NO	11/11/2009	ALIVE
34	P4875	F	72	0	A	NO	20/10/2009	ALIVE
35	P7961	F						
36	P5017	F	70	0	A	YES	7/12/2009	ALIVE
37	P6068	F	76	0	A	NO	29/9/2009	ALIVE
38	P1055	F	48	I	A	NO	25/11/2009	ALIVE
39	P1188	M	58	I	B	NO	1/12/2009	ALIVE
40	P280	M	60	0	A	NO	31/8/2009	ALIVE
41	P2528	F	52	I	A	NO	28/9/2009	ALIVE
42	P8699	F						
43	P3020	F	66	0	A	NO	15/10/2009	ALIVE
44	P5610	F	47	0	A	NO	19/6/2009	ALIVE
45	P4557	F	58	0	A	NO	17/11/2009	ALIVE
46	P103HA	F	45	0	A	NO	20/1/2010	ALIVE
47	P1082	F	67	0	A	NO	11/10/2009	ALIVE
48	P3916	M	43	0	A	NO	18/1/2010	ALIVE
49	P3021	M	44	I	A	NO	7/12/2009	ALIVE
50	P2740	M	47	0	A	NO	12/10/2009	ALIVE
51	P3551HA	F	58	0	A	NO	25/11/2009	ALIVE
52	P1563	M	69	II	B	YES	27/8/2008	ALIVE
53	P6520	M	44	0	A	YES	16/12/2009	ALIVE
54	P9297	M	69	0	A	NO	16/11/2009	ALIVE
55	P711	M	65	II	B	YES	11/12/2009	ALIVE
56	P1626	M	37	0	A	YES	24/10/2009	ALIVE
57	P775	F	42	II	B	YES	14/5/2009	ALIVE
58	P781	F	55	II	A	NO	12/11/2009	ALIVE
59	P4994HA	M	68	0	A	YES	27/5/2009	ALIVE
60	P1615	F	61	0	A	YES	16/11/2008	ALIVE
61	P1050	F	64	II	B	YES	11/2/2009	DEAD
62	P2446	M	72	0	A	NO	25/1/2010	ALIVE
63	P104HB	M	69	0	A	YES	2/12/2009	ALIVE
64	P1522	M	63	0	A	YES	20/5/2010	DEAD
65	P6264	M	54	0	A	NO	18/1/2010	ALIVE
66	P9391	M						
67	P1173	F	62	0	A	YES	17/12/2009	ALIVE
68	P5092	M	77	IV	C	YES	12/11/2009	ALIVE

Table 3. Demographic and clinical data of CLL patients included in the study.

1	Lab Identifier	sIGH	sIGL	sCD38	cZAP-70	Karyotype	FISH
2	P4699	MD	kappa	33,5	73		Trisomy 12
3	P3506	MD	kappa	31,1	49	47,XX,+12	Trisomy 12
4	P3870	MD	kappa			46,XY	
5	P4295	MD	lambda	0,3	33	46,XX	
6	P2355	MD	kappa	4,5	47	46,XY	Del13q
7	P2685		lambda	0,5	12		normal
8	P3073	MD	kappa	40	65	46,XX,del(6)(q21q27)	normal
9	P8805	MD	kappa	7,4	37	46,XX,t(2:13)(p23:q14)[2]/46,XX[24]	Del13q+Del11q
10	P9320	MD	kappa	43,6	24		
11	P1697	MD	kappa	0,3		46,XY	normal
12	P571	MD	kappa	7,8		47,XY,+12	
13	P8762	MD	lambda	67,3	55	46, XY [20]	normal
14	P1618	MD	lambda	1,1	3	46,XY	normal
15	P1156	M	kappa	66,4	68,9	47,XX,inv(9)(p13q22),+12/47,XX,t(1;13)(p12;q12),inv(9)(p13q22),+12	
16	P5283	MD	lambda	91,7	60,5	46,XY	
17	P511	MD	lambda	16,9			
18	P2329	MD	lambda	15	12,5	46,XX	normal
19	P7155	MD	lambda	20,8	54,1	46, XY [25]	Del11q
20	P2618		kappa	1,8			
21	P8192	MD	kappa	1,9	18,3	46,XY[10]	normal
22	P7794	G	kappa	34	0,5	47,XX,+12	Trisomy 12
23	P6124	MD	lambda	2,5	10,3	46,XY	normal
24	P9940	G	kappa	0,2	1,4		
25	P4383HA	G	kappa	0	6,3	46,XX	
26	P6460	MD	lambda	0,9	5,1	46, XY [15]	normal
27	P1894	M	kappa	5,3		46,XY	Del13q+Del13q
28	P427	MD	kappa	0,6			
29	P2577	MD	lambda	1,2	6	46,XY,inv(9)(p13q21)	normal
30	P2548	MD	kappa	28,2		46,X,t(Y;11)(q12;q23),del(13)(q12q14)/46,XY,t(3;5)(q29;q23)	
31	P5231	MD	kappa	91,5	17,8	46,XY	normal
32	P5728	MD	lambda	32,5	2,4	46,XY	normal
33	P5949	MD	kappa	0,5	5,5	46,XY	normal
34	P4875		kappa	1,4	13,4	46,XX	Del13q
35	P7961	MD	kappa	4,4	28,6	46,XX,der(4)t(4;17)(p11;q11),-17+mar[11] / 44,X,-X,der(4)t(4;17)(p11;q11),-13,add(15)(p13),-17,+mar1,+dmin [7] / 46,XY[8]	Del13q+Del17p
36	P5017	MD	kappa	12,2	40		
37	P6068	MD	kappa	0,6	14		
38	P1055	MD	kappa	2,2	28	Not determined	
39	P1188	MD	lambda	4,3	14	46,XY	normal
40	P280	MD	lambda	2,5		46,XY,t(9;12)(p11;q11)	
41	P2528	MD	kappa	1,8	15		
42	P8699	MD	kappa	0,5	24,3		
43	P3020	G	kappa	0,5		46,XX	Del13q
44	P5610	MD	kappa	0,5	5,4	46,XX	normal
45	P4557	MD	kappa	0,2	20	45,X,-X,inv(9)(p11q13)	normal
46	P103HA	G	kappa	1,7		46,XX	normal
47	P1082	G	kappa	0,9		46,XX	normal
48	P3916	G	kappa	2,5	14	46,XY	
49	P3021	G	kappa	0,3	19	46,XY	
50	P2740	MD	kappa	0,7			
51	P3551HA	G	kappa	0,8		46,XX	normal
52	P1563	MD	kappa	0,5	18,6	46,XY	Del13q
53	P6520	G	kappa	0,9	3,1	46, XY [17]	Del13q
54	P9297	G	kappa	0,5	11,9		
55	P711	MD	kappa	4,7	8	47,XY,+12/46,XY,del(13)(q12q14)]/46,XY,del(14)(q24)	
56	P1626	G	kappa	2	9	46,XY	Del13q
57	P775	MD	lambda	1,1		46,XX	normal
58	P781	G	kappa	1,4		46,XX	Del13q
59	P4994HA	M	lambda	0,1	0,4	46,XY	Del13q+Del13q
60	P1615	G	kappa	18	33	47,XX,+12	Trisomy 12
61	P1050	G	kappa	91,3		Not determined	Del13q+Del13q
62	P2446	G	kappa	54		47,XY,+12	Trisomy 12
63	P104HB	MD	lambda	51,3		46,XY	Trisomy 12
64	P1522	MD	lambda	2,1		46,XY	Del13q+Del13q
65	P6264	MD	lambda	0,6	5,2	46, XY [20]	Del13q
66	P9391	MD	kappa	85,6	47,7		
67	P1173	MD	kappa	22,5	30	46,XX	Trisomy 12
68	P5092	MD	kappa	0,7	7,3	46,XY,del(11)(q14)	Del13q+Del11q

Table 4. *Phenotypic and karyotypic characteristics of CLL patients included in the study*

	Lab Identifier	IGHV	IGHD	IGHJ	%	Subset
2	P4699	IGHV1-18*01	IGHD6-19*01	IGHJ4*02	99,70	1
3	P3506	IGHV1-2*02	IGHD6-19*01	IGHJ4*02	99,70	1
4	P3870	IGHV1-2*02	IGHD6-19*01	IGHJ4*02	100,00	1
5	P2355	IGHV1-2*02	IGHD6-19*01	IGHJ4*02	100,00	1
6	P3073	IGHV1-3*01	IGHD6-19*01	IGHJ4*02	100,00	1
7	P1173	IGHV5-a*01	IGHD6-19*01	IGHJ4*02	99,60	1
8	P5092	IGHV5-a*03	IGHD6-19*01	IGHJ4*02	100,00	1
9	P3020	IGHV4-34*01	IGHD3-10*01	IGHJ6*02	90,00	4
10	P103HA	IGHV4-34*01	IGHD3-10*01	IGHJ6*02	95,90	4
11	P3916	IGHV4-34*01	IGHD3-10*01	IGHJ6*02	91,20	4
12	P3551HA	IGHV4-34*01	IGHD5-12*01	IGHJ6*02	93,30	4
13	P6520	IGHV4-34*02	IGHD3-10*01	IGHJ6*02	94,40	4
14	P1626	IGHV4-34*02	IGHD4-17*01	IGHJ6*02	94,80	4
15	P1615	IGHV4-39*01	IGHD6-13*01	IGHJ5*02	100,00	8
16	P1050	IGHV4-39*01	IGHD6-13*01	IGHJ5*02	100,00	8
17	P2446	IGHV4-39*06	IGHD6-13*01	IGHJ5*02	99,70	8
18	P1082	IGHV4-34*01	IGHD2-15*01	IGHJ6*02	96,30	16
19	P781	IGHV4-34*04	IGHD1-26*01	IGHJ6*02	95,00	16
20	P4295	IGHV1-2*02	IGHD2-8*02	IGHJ4*02	93,80	
21	P2685	IGHV1-3*01	IGHD1-26*01	IGHJ5*02	87,80	
22	P8805	IGHV1-69*01	IGHD2-15*01	IGHJ5*02	100,00	
23	P9320	IGHV1-69*01	IGHD3-16*02	IGHJ3*02	100,00	
24	P1697	IGHV1-69*01	IGHD3-22*01	IGHJ4*03	93,90	
25	P571	IGHV1-69*01	IGHD3-10*01	IGHJ6*02	99,40	
26	P8762	IGHV1-69*06	IGHD5-24*01	IGHJ4*02	100,00	
27	P1618	IGHV1-8*01	IGHD2-15*01	IGHJ5*02	91,90	
28	P1156	IGHV3-13*01	IGHD3-3*01	IGHJ3*02	88,70	
29	P5283	IGHV3-20*01	IGHD6-19*01	IGHJ1*01	97,20	
30	P511	IGHV3-20*01	IGHD3-3*01	IGHJ5*02	100,00	
31	P2329	IGHV3-21*01	IGHD4-4*01	IGHJ4*02	96,30	
32	P7155	IGHV3-23*01	IGHD2-15*01	IGHJ3*02	100,00	
33	P2618	IGHV3-23*01	IGHD6-6*01	IGHJ4*02	95,90	
34	P8192	IGHV3-23*01	IGHD3-9*01	IGHJ4*02	91,90	
35	P7794	IGHV3-23*04	IGHD2-15*01	IGHJ4*02	87,85	
36	P6124	IGHV3-30*01	IGHD3-10*01	IGHJ5*02	100,00	
37	P9940	IGHV3-30*03	IGHD3-3*01	IGHJ6*03	94,44	
38	P4383HA	IGHV3-30*03	IGHD3-10*01	IGHJ4*02	95,10	
39	P6460	IGHV3-30*03	IGHD3-10*01	IGHJ6*02	94,10	
40	P1894	IGHV3-30*18	IGHD1-26*01	IGHJ3*02	92,90	
41	P427	IGHV3-30*18	IGHD5-24*01	IGHJ2*01	91,20	
42	P2577	IGHV3-33*01	IGHD6-6*01	IGHJ3*02	91,90	
43	P2548	IGHV3-33*01	IGHD2-15*01	IGHJ5*02	100,00	
44	P5231	IGHV3-43*01	IGHD5-5*01	IGHJ4*02	100,00	
45	P5728	IGHV3-48*01	IGHD3-16*01	IGHJ4*02	97,60	
46	P5949	IGHV3-49*03	IGHD1-1*01	IGHJ6*02	95,20	
47	P4875	IGHV3-53*02	IGHD2-15*01	IGHJ3*02	92,60	
48	P7961	IGHV3-64*05	IGHD3-3*01	IGHJ4*02	99,30	
49	P5017	IGHV3-7*01	IGHD2-2*01	IGHJ6*02	100,00	
50	P6068	IGHV3-7*01	IGHD3-22*01	IGHJ4*02	94,80	
51	P1055	IGHV3-74*02	IGHD4-17*01	IGHJ4*03	92,20	
52	P1188	IGHV3-9*01	ND	IGHJ6*02	96,30	
53	P280	IGHV4-31*01	IGHD4-4*01	IGHJ6*02	94,30	
54	P2528	IGHV4-34*01	IGHD1-14*01	IGHJ4*02	95,60	
55	P8699	IGHV4-34*01	IGHD3-3*01	IGHJ4*02	91,93	
56	P5610	IGHV4-34*01	IGHD4-23*01	IGHJ6*02	94,70	
57	P4557	IGHV4-34*01	IGHD2-2*01	IGHJ4*02	97,20	
58	P3021	IGHV4-34*01	IGHD3-10*01	IGHJ3*02	93,50	
59	P2740	IGHV4-34*01	IGHD4-4*01	IGHJ4*02	95,90	
60	P1563	IGHV4-34*01	IGHD1-26*01	IGHJ6*03	93,20	
61	P9297	IGHV4-34*02	IGHD2-2*01	IGHJ6*02	95,09	
62	P711	IGHV4-34*02	IGHD6-19*01	IGHJ4*03	97,40	
63	P775	IGHV4-34*04	IGHD2-8*01	IGHJ3*01	94,10	
64	P4994HA	IGHV4-34*12	IGHD6-6*01	IGHJ4*02	94,00	
65	P104HB	IGHV4-39*06	IGHD2-2*01	IGHJ6*02	99,60	
66	P1522	IGHV4-61*02	IGHD6-6*01	IGHJ3*02	98,30	
67	P6264	IGHV5-51*01	IGHD2-2*01	IGHJ6*03	92,70	
68	P9391	IGHV5-51*01	IGHD1-26*01	IGHJ4*02	93,40	

Table 5. *BCR molecular characteristics and subset assignment of CLL cases included in the study.*

monoclonal antibodies are directed against cell surface antigens on human blood nucleated cells (CD2, CD3, CD16, CD36, CD56, CD66b) and glycophorin A on red blood cells (RBCs) (*Table 6*). The antibodies crosslink unwanted cells in human whole blood to multiple RBCs, forming immunorosettes; this increases the density of the unwanted (rosetted) cells, so that they pellet along with the free RBCs when centrifuged over a buoyant density medium (Ficoll-Paque). Desired cells (B cells) are not labelled with antibody and easily collected as a highly enriched population at the interface between plasma and ficoll. This procedure ensures that isolated B cells are not artificially stimulated through positive selection.
The purity of all preparations was checked by flow cytometry (BD FACS CANTO) (*Fig.7)* and always exceeded 95% for CD3-CD19+ cells.

Surface markers	Cell type
CD2	T lymphocytes, NK cells, Thymocytes
CD3	T lymphocytes, Thymocytes
CD16	NK cells, Neutrophils, Macrophages
CD36	Mononuclear cells, Platelets
CD56	NK cells
CD66b	Granocytes

Table 6. Positive selection of unwanted white blood cells

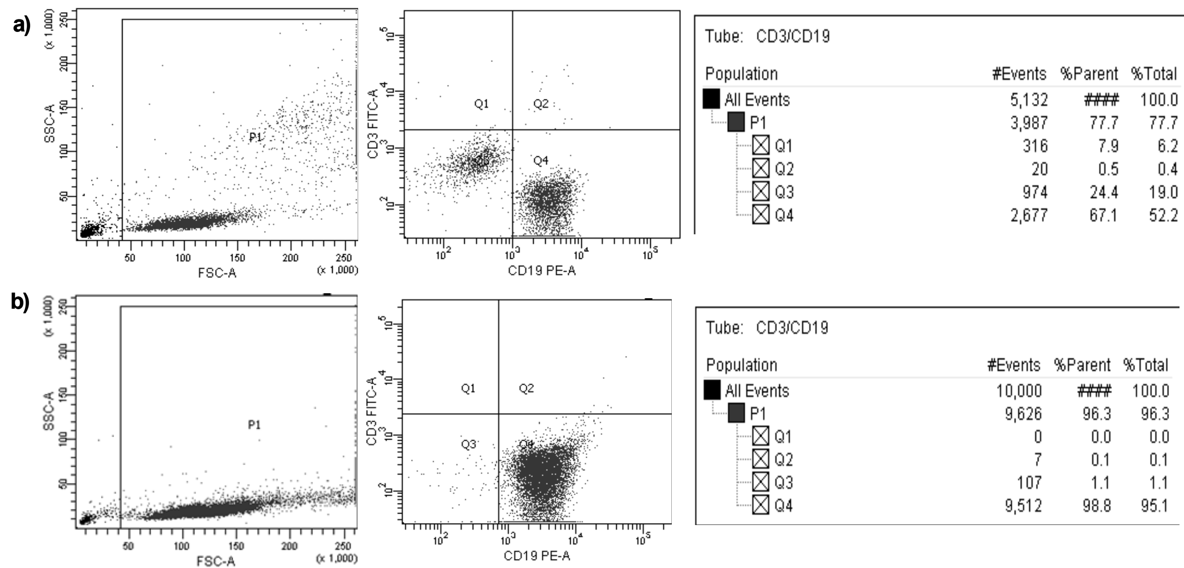

Figure 7*. a) Cell sample before purification. Gating is on viable cells. b) Cell preparation after purification. CD3-/CD19+ cells were always above 95%, in this case 98.8%*

Stimulation of CLL cells

Purified CD19+ B cells (3 x 10^6 cells/ml) were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum, 2 mmol/l l-glutamine and 15 µg/ml gentamicin (SIGMA), in 24 well plates (Costar) in the presence or absence of specific TLRs ligands. Cell cultures were maintained at 37^{0}C in a humidified atmosphere containing 5% CO_2 for 24 h. TLR1/2 heterodimer was stimulated with 1 µg/ml Pam3CSK4 (Synthetic bacterial lipoprotein, Invivogen), TLR2/6 heterodimer with 0,2 µg/ml MALP-2 (Mycoplasmal Macrophage-activating Lipopeptide-2, Alexis, Axxora, San Diego, CA, USA), TLR4 with 200 ng/ml LPS (Ultra Pure E. Coli, Invivogen), TLR9 with 2,5µg/ml ODN 2006 (Type B oligonoucleotide, Invivogen) and TLR8 with 1 µg/ml ORN 06 (GU-rich oligonoucleotide, Invivogen). 10 µg/ml MDP (Muramyldipeptide L isoform, Invivogen) were used for NOD2 stimulation. For TLR7 stimulation, two different ligands were used: Loxoribine, a

guanosine analog (1mM), and Imiquomod – R837, a small synthetic antiviral molecule (0,1 μg/ml, Invivogen) ***(Table 7).***

Receptor	*Ligand*	*Description*	*Concentration*
TLR1/2 heterodimer	Pam3CSK4	Synthetic bacterial lipoprotein, Invivogen	1 μg/ml
TLR2/6 heterodimer	MALP-2	Mycoplasmal Macrophage-activating Lipopeptide-2, Alexis, Axxora	0,2 μg/ml
TLR4	LPS	Ultra Pure E. Coli, Invivogen	200 ng/ml
TLR9	ODN 2006	Type B oligonoucleotide, Invivogen	2,5μg/ml
TLR8	ORN 06	GU-rich oligonoucleotide, Invivogen	1 μg/ml
NOD2	MDP	Muramyldipeptide L isoform, Invivogen	10 μg/ml
TLR7	Loxoribine	Guanosine analog , Invivogen	1mM
TLR7	Imiquomod – R837	Small synthetic antiviral molecule , Invivogen	0,1 μg/ml

Table 7. *Specific ligands used for TLR/NLR stimulation*

Determination of CD25 and CD86 in untreated and stimulated CLL cells by flow cytometry

After 24-hour culture, cells were collected, washed twice and stained for activation markers with anti-CD86-fluorescein isothiocyanate (FITC) (BD Bioscience), anti-CD25-phycoerythrin (PE) (BD Bioscience) and 7-Amino-actinomycin D (7-AAD) vital dye (Beckman Coulter). Isotype controls anti-mouse $IgG1_{k}$-FITC and anti-mouse $IgG1_{k}$-PE were also used. After staining, cells were washed and data acquisition followed on a BD FACS CANTO (Becton Dickinson

Immunocytometry Systems, San Jose, CA). Analysis was performed using the BD FACS DIVA software. Only 7-AAD negative (viable cells) were analysed for CD25 and CD86 expression.

The percentage of positive cells for each marker after stimulation was compared to the unstimulated control and the difference was estimated. A difference >10% for either marker was considered significant. For comparative analysis the median value for each group of patients was calculated. Statistical significance was estimated using the one-sided unpaired Student's t-test. P values <0,05 were considered statistically significant. To investigate possible associations in TLR/NOD functionality we estimated the odds ratio (OR).

PART IV: RESULTS

RESULTS

Functionality of the TLRs in CLL

In a large series of patients we investigated whether the TLR family members and the NLR family member NOD2 are indeed functional in CLL B lymphocytes. To address this question, we stimulated purified CLL cells with ligands for specific TLRs and measured the expression of the B cell activation marker CD25 and the co-stimulatory molecule CD86. As an internal positive control, we used the TLR9 ligand CpG-oligonucleotide (indicated as CpG), which has been consistently found to activate CLL cells in a similar setting.

We stimulated cells from each of the 67 patients included in the study and cultured them for 24 hours. We measured the expression of the co-stimulatory molecules only in viable cells (7AAD^{-}). The percentage of live cells after stimulation differed by <10% over control, indicating that none of the TLR ligands was toxic to the cells after 24 hours of culture at the concentrations used. An increase in the expression of either marker was accounted when the difference between stimulated vs. unstimulated cells was >10%.

In untreated CLL cells we observed expression of CD25 (cut off 20%) in almost half of the cases (35 of 67 cases; 52. 2%). Expression of CD86 in untreated cells was observed in 20 of 67 cases (29.9%). Stimulation with TLR9 ligand increased the expression of CD25 in all cases except one (range 10.7-94.9%; average 56.1%) and CD86 in 44 of 67 cases (range 11.3-82.6%; average 43.4%).

Stimulation of TLR1/2 and TLR2/6 heterodimers with Pam3CSK4 and MALP-2, respectively, upregulated the expression of CD25 and CD86 positive cells in most cases, in accordance with the literature, thus revealing that they are functional in CLL B cells. More specifically, TLR1/2 stimulation induced the expression of CD25 in 57/67 cases (average 48.4%; range 10.4-89.4%), of CD86 in 49/67 cases (average 41.2%; range 10.5-74.6%) and of CD25 and/or CD86 in 58/67 cases (86.6%). TLR2/6 stimulation augmented the percentage of CD25 positive cells in

55/67 cases (average 35%; range 10.8-80.1%), of CD86 in 44/67 cases (average 29.7%; range 11.2-70.9%), and of CD25 and/or CD86 in 59/67 cases (88.1%).
The NLR family member NOD2 has previously been reported to be functional in CLL cells. Our data also show that it can induce the expression of CD25 in most cases (55/67 cases, average 31.3%; range 10.9-72.1%) and the expression of CD86 in almost half of the cases (35/67, average 26.7%; range 10.3-56.3%). Collectively, it upregulated the expression of CD25 and/or CD86 in 57/67 cases (85.1%).
Interestingly, we observed that there was a strong association between TLR1/2, TLR2/6 and NOD2 functionality. More specifically, 91.4% (53/58) of cases responsive to Pam3CSK4 were also found to be responsive to MALP-2 stimulation (odds ratio 5.3). In addition, 98.2% (56/57) of cases responsive to MDP were also found to be responsive to Pam3CSK4 and/or MALP-2 (odds ratio $\rightarrow\infty$). This means that all cases exhibiting TLR1/2 and/or TLR2/6 heterodimer functionality also exhibited functional NOD2. Finally, 70.8% (46/65) of all cases responsive to at least one of the above ligands were found to exhibit functionality of all three of them (odds ratio 13.1)(*Fig.8).*

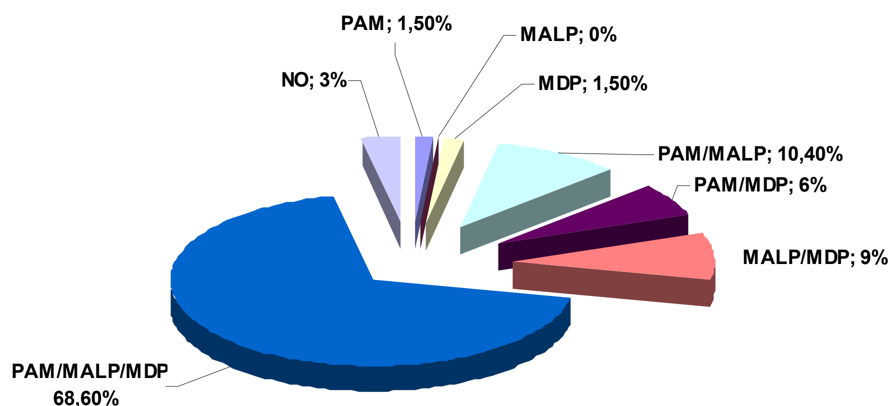

Figure 8. Association pattern of TLR1/2, TLR2/6 and NOD2 functionality

For TLR7 stimulation we used two different ligands. Loxoribine is a naturally occurring small low-molecular-weight nucleoside (guanosine analog) that activates immune cells through TLR7 and leads to proinflammatory cytokine

secretion, whereas Imiquimod is a small synthetic antiviral molecule also known to trigger TLR7. Our data show that these ligands have different effects on CLL cells. Imiquimod induced the expression of CD25 in 39/67 cases (average 41.1%; range 10.1-93.7%); in contrast, Loxoribine had no effect on CD25 expression. Imiquimod also induced the expression of CD86 in 21/67 cases (average 22.4%; range 10.3-45.3%), whereas Loxoribine induced the expression of CD86 in only 4/67 cases (average 31.3%; range 20.2-48.6%).

Stimulation of TLR4 with LPS and TLR8 with ORN 06 had no effect on CD25 and CD86 expression in all cases tested *(Figure 9, Figure 10)*.

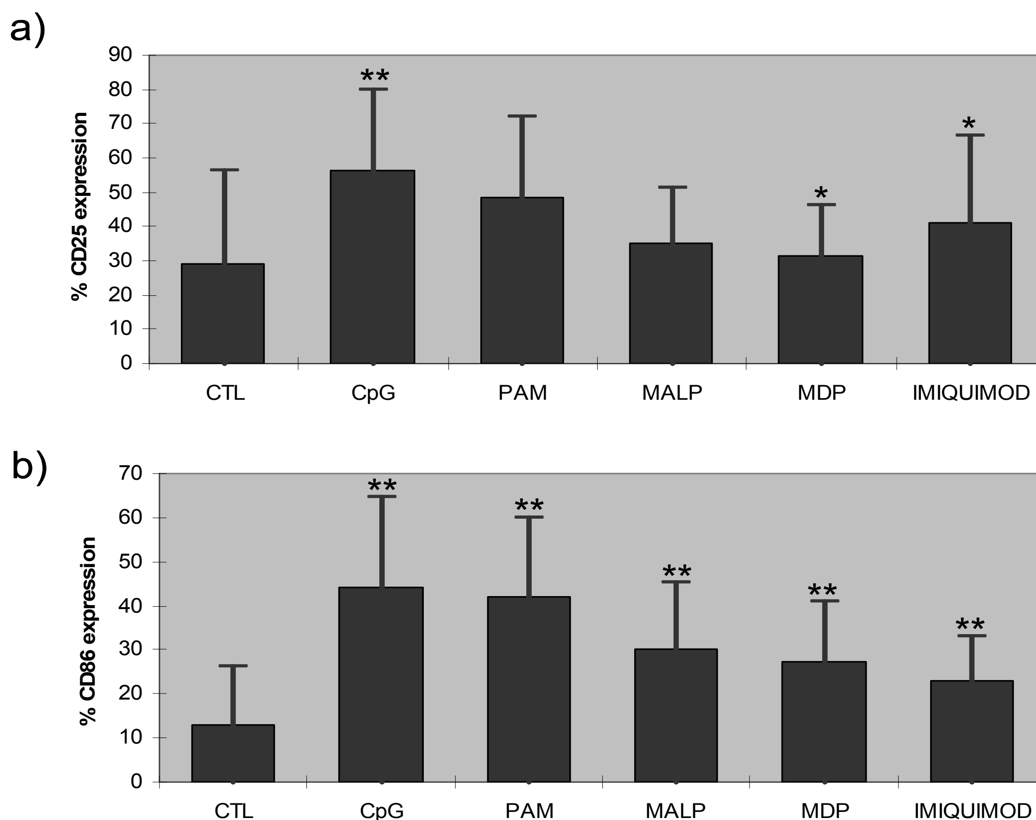

Figure 9. Average upregulation of a) CD25 and b) CD86 expression after stimulation with ligands specific for individual TLRs in comparison to unstimulated control.

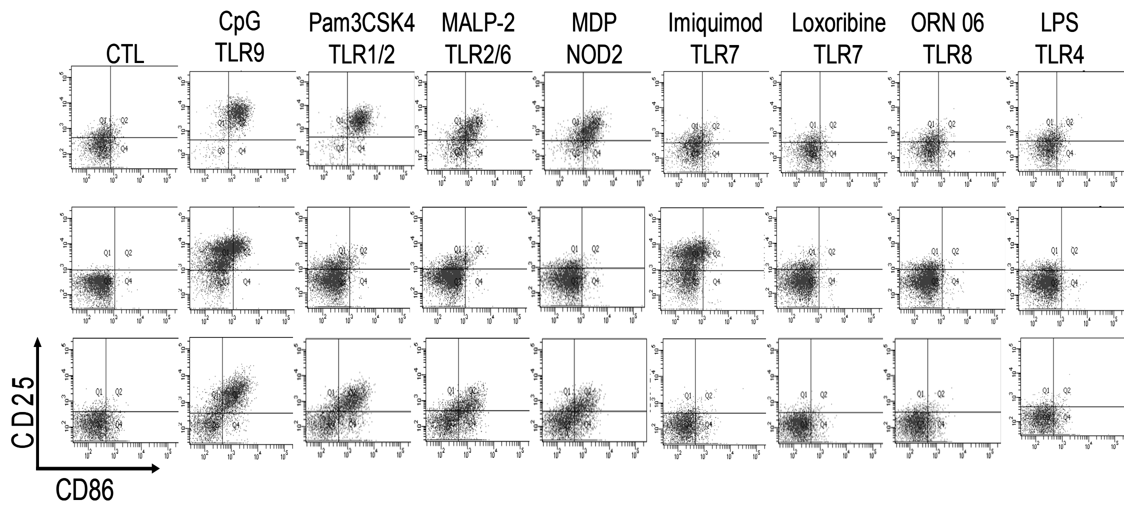

Figure 10. Determination of CD25 and CD86 expression by flow cytometry after stimulation with specific TLR/NLR ligands in comparison to unstimulated control (three distinct cases).

Association of TLR functional profiles with BCR molecular features

I. IGHV gene mutational status

We next investigated whether the observed inter-patient variability could be related to specific molecular characteristics of the B cell receptor. To this end, we first examined the differences between cases carrying mutated vs. unmutated IGHV genes (46 and 21 cases, respectively). Detailed results are shown in ***Supplemental Table 1***. In ***Fig.11*** only statistically significant differences are represented. We found that in cases with mutated IGHV genes TLR9 stimulation with CpG had a strong effect in terms of CD86 induction, whereas in cases with unmutated IGHV genes there was no effect (difference 30.9%; p=0.000). In contrast, TLR7 stimulation with Imiquimod strongly upregulated CD25

expression in unmutated CLL cells, whereas it had no effect in mutated CLL cases (difference 46.4%; p=0.000). Furthermore, a statistically significant difference was identified between the two groups regarding the upregulation of CD25 expression after stimulation of the TLR2/6 heterodimer, in that unmutated CLL cells exhibited a stronger response to TLR2/6 triggering (difference 11.6%; p=0.002).

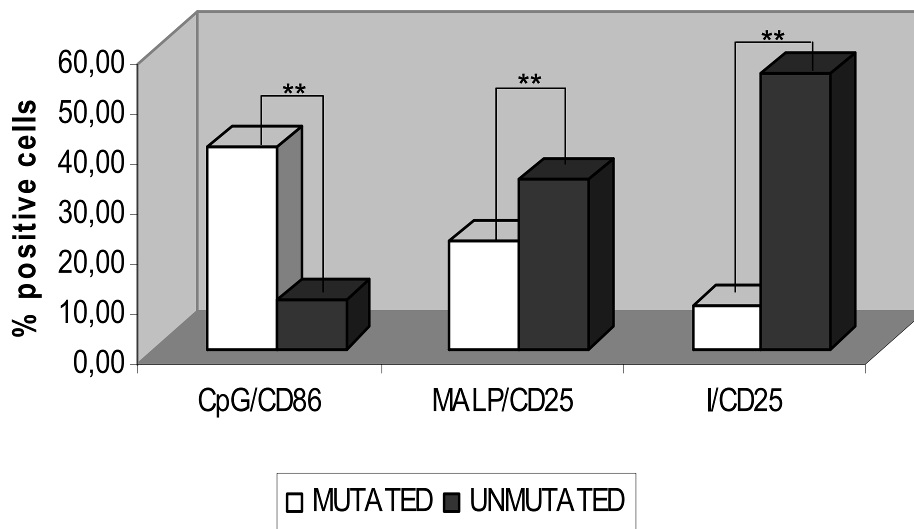

Figure 11. CpG stimulation markedly upregulates CD86 expression in mutated CLL cases, whereas Imiquimod markedly upregulates CD25 expression in unmutated CLL cases. MALP appears to upregulate CD25 expression more strongly in unmutated cases, but the difference is less pronounced (11.6%)

II. BCR stereotypy: focus on subsets #1 and #4

As mentioned earlier, our study group included cases expressing stereotyped B cell receptors. Among cases carrying mutated IGHV genes, subset #4 was over-represented (7/44 cases; 15.9%). Therefore, we evaluated differences in the patterns of functional outcomes after TLR and NOD2 stimulation by comparing subset #4 to all other mutated cases. We found that TLR1/2, TLR2/6 and NOD2

stimulation had a much stronger effect in terms of CD86 induction in subset #4 cases compared to all other mutated cases (difference 30.1%, 25.6%, 17.8%; p=0.048, 0.041, 0.018, respectively). We also noticed a similar trend regarding CD25 upregulation, yet not reaching statistical significance *(Figure 12a)*.

We next investigated if there are differences between subset #4 cases compared to cases expressing the same IGHV gene (IGHV4-34) in heterogeneous rearrangements. We discovered that TLR1/2, TLR2/6 and NOD2 ligands were effective in inducing the expression of both CD25 (difference 49.7%, 23.7%, 16.15%; p=0.006, 0.022, 0.011, respectively) and CD86 (difference 42.6%, 25.8%, 14.5%; p=0.001, 0.011, 0.017, respectively) in subset #4 cases, whereas they had less or no effects in heterogeneous IGHV4-34 cases *(Figure 12b)*.

Interestingly, we also noticed a strong association of TLR1/2, TLR2/6 and NOD2 functionality within subset #4 cases. More specifically, 6/7 cases (85.7%) responded to all three ligands of the above receptors (Pam3CSK4, MALP-2 and MDP, respectively); there was a single case which was not responsive to MALP-2, but still responded to both Pam3CSK4 and MDP.

Among cases carrying unmutated IGHV genes, subset #1 was over-represented (7/21 cases; 33.3%). When comparing subset #1 cases to all other unmutated cases, we noticed that Pam3CSK4 was less potent in inducing CD25 and CD86 expression in subset #1 cases, however this result did not reach statistical significance, likely due to the limited number of cases in subset #1.

Subset #1 and subset #4 can be considered as prototypes of bad or good prognosis CLL subsets; in addition, they are distinguished by several other unique molecular and clinical features. In an attempt to explore whether these distinctions are also reflected in unique functional profiles, we then investigated their differences in terms of TLRs and NOD2 stimulation capacity. Indeed, we found major differences in all TLRs that are functional in CLL B cells, as well as NOD2. As shown in *Fig.13* TLR9, TLR1/2, TLR2/6, and NOD2 stimulation strongly upregulated CD86

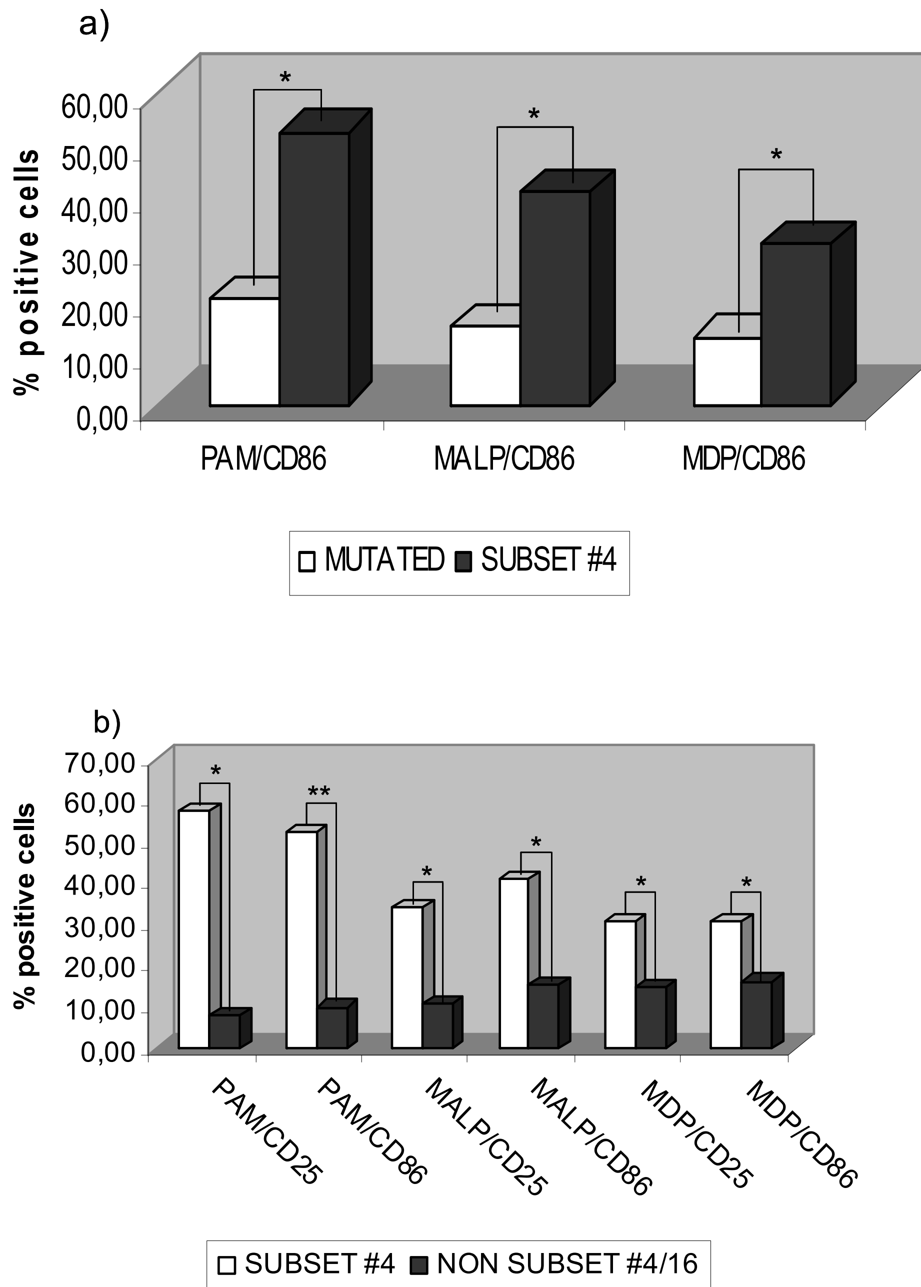

Figure 12. a) TLR1/2, TLR2/6 and NOD2 stimulation has a much stronger effect in CD86 upregulation in subset #4 cases in comparison to all other mutated CLL cases. b) TLR1/2, TLR2/6 and NOD2 stimulation has a much stronger effect in both CD25 and CD86 upregulation in subset #4 cases in comparison to cases carrying IGHV4-34 in heterogeneous rearrangements (non-subset #4/16, i.e. cases not assigned to the two major subsets with stereotyped IGHV4-34 BCRs, #4 and #16).

expression in subset #4 cases whereas it had no effect in subset #1 cases (difference 48.7%, 41.4%, 34.8%, 27.9%; p=0.001, 0.019, 0.029, 0.001, respectively). In contrast, the TLR7 ligand Imiquimod strongly induced CD25 expression in subset #1 cases, whereas it had no effect on subset #4 cases (difference 37.2%; p=0.002).

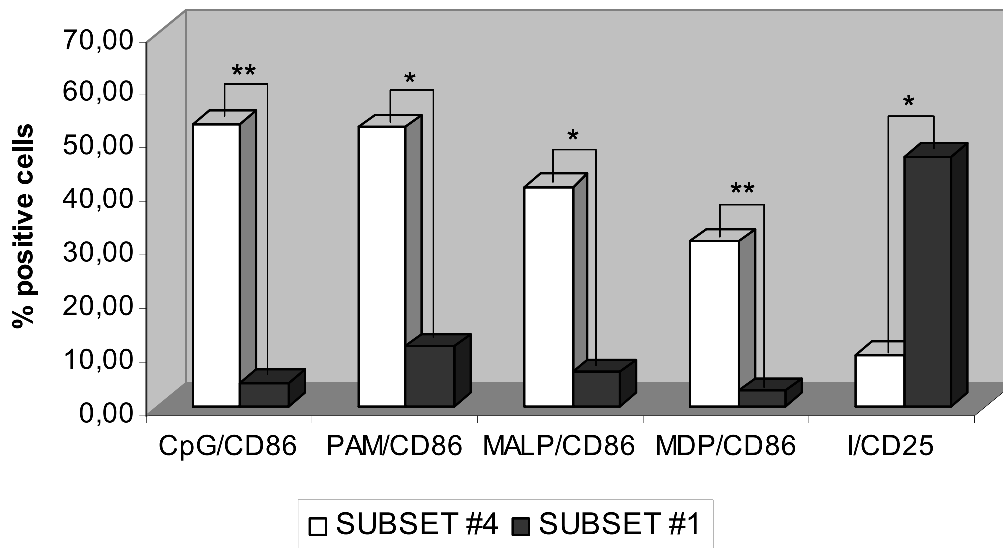

Figure 13. TLR9, TLR1/2, TLR2/6 and NOD2 stimulation strongly upregulates CD86 expression in subset #4 cases, whereas it has little or no effect in subset #1 cases. In contrast, TLR7 stimulation strongly upregulates CD25 expression in subset #1 cases, whereas it has little effect in subset #4 cases.

PART V: DISCUSSION

The new findings concerning the molecular basis of CLL have substantially transformed our perception of its ontogeny. While CLL was thought to arise due to apoptotic defects of naïve B-cells, it now appears to be a complex entity that most probably involves aberrant activation and proliferation of auto/poly reactive B-cells through the BCR. An initial lesion probably occurs in a single auto/polyreactive B-cell which abrogates its tolerance to antigenic stimuli and allows its expansion. In "unmutated" CLL clones, the absence of somatic hypermutation permits persistent interaction with the driving antigen or even a multitude of molecular structures (polyreactivity). In "mutated" CLL clones, on the other hand, the introduction of IGHV mutations either attenuates/neutralizes or further increases the BCR specificity for the driving antigen. This is in accordance with the clinical observation that most "unmutated" cases are associated with aggressive disease, whereas most "mutated" cases follow a more indolent course, possibly due to attenuated signaling through the BCR. It is also in agreement with the finding that "mutated" CLL clones are more frequently found to express a molecular signature of anergy, including decreased BCR signaling, constitutive MEK, ERK and NF-AT activation and absence of Akt phosphorylation. In this context, it would not be unreasonable to speculate that the introduction of IGHV mutations may result in reduced binding affinity for the antigen, thereby activating tolerogenic pathways. Collectively, BCR signaling appears to be a major contributing factor in disease pathogenesis, although the timing, duration and, most importantly, the nature of the antigenic stimuli remain unclear.

TLRs recognize an array of distinct molecular patterns and are known to bridge innate and adaptive immunity by promoting dendritic cell (DC) maturation and cytokine production [(112-113)]. The role of TLRs in B cell activation and antibody production is currently a field of intense research, as it has potential appplications in vaccination, autoimmune diseases and cancer. However, relatively little information is available regarding the TLR repertoire and functionality in CLL cells. Most studies address the expression of only TLR7 and TRR9, involve small series of patients and therefore cannot establish associations of expression and/or function with different subgroups and subsets. The

importance of such associations is underlined by contradictory reports regarding the effect of TLR9 stimulation in CLL cells from different subgroups. In most "unmutated" cases TLR9 stimulation resulted in proliferation of the leukemic clone, whereas in most "mutated" cases it resulted in apoptosis [144]. TLR7 and TLR9 agonists have recently entered clinical trials for B-cell malignancies; therefore it would be of great significance to identify which particular groups could benefit from such treatments and which could potentially be harmed. It is also of clinical importance to know which TLR agonists have the ability to increase the leukemic clone's immunogenicity by upregulating costimulatory molecules and thereby susceptibility to cytotoxic treatment. Again, comparative analysis is needed among CLL subsets. Furthermore, identifying particular TLR expression and/or function patterns in CLL may offer useful hints regarding the nature of the antigenic elements that are involved in the pathogenesis of the disease, which can be then translated into opportunities for prevention or therapeutic exploitation. If distinct TLR expression and/or function patterns exist among CLL subsets, this would offer further evidence of stimulation by restricted antigenic elements. Since BCR specificity is related to disease prognosis then it is possible that such distinct TLR patterns are also associated with the clinical course and outcome of CLL and may serve as prognostic markers.

Our study involved a series of 67 CLL patients, selected with an intentional bias for cases expressing stereotyped BCRs assigned to subsets #1 and #4, since these particular subsets may be considered as prototypes of "unmutated/adverse prognosis" and "mutated/good prognosis" subsets, respectively. We tested the functionality of all TLRs that have been previously shown to be expressed in CLL cells even at very low levels (TLR1/2 heterodimer, TLR2/6 heterodimer, TLR4, TLR7, TRL8 and TLR9). In our study we also investigated the NLR family member NOD2, which has been found to be expressed in CLL cells as well. We evaluated the functionality of the above receptors by triggering negatively isolated CLL cells collected from each of these 67 CLL patients with specific ligands for individual TLRs and measuring by flow cytometry the upregulation of the activation molecules CD25 and CD86. We set a limit of at least 10% increase in the expression of CD25 or CD86 compared to unstimulated

control in order to consider that a given TLR ligand effectively stimulated our cell sample. This limit, though arbitrary, is stricter than the one of 2% which has been used in relevant literature so far (141). One cannot exclude the possibility that TLR or NLR triggering may not lead to upregulation of these two activation markers (CD25 and CD86), yet it signals through other pathways. However, given that TLR and NLR signaling is generally known to culminate in NF-κB activation and upregulation of co-stimulatory molecules, including the ones used in this study, it should be safe to consider these two molecules as reliable markers of TLR and NLR functionality (159).

We found that TLR9 is functional in practically all CLL cases. Stimulation with CpG ODN resulted in the upregulation of CD25 and/or CD86 in 66/67 cases (98.5%). This is in agreement with the literature (141-142,160) and allows to use TLR9 stimulation as internal positive control for validating our findings. The TLR1/2 heterodimer was functional in most CLL cases. Its stimulation with Pam3CSK4 induced the upregulation of CD25 in 57/67 cases (85.1%), upregulation of CD86 in 49/67 cases (73.1%) and upregulation of either marker (or both) in a total of 58/67 cases (86.6%). The TLR2/6 heterodimer was functional in very similar percentages of CLL cases, as well. Its stimulation with MALP-2 resulted in the upregulation of CD25 in 55/67 cases (82.1%), upregulation of CD86 in 49/67 cases (73.1%) and upregulation of either marker in a total of 59/67 cases (88.1%). Indeed, we identified similar patterns of TLR1/2 and TLR2/6 functionality, in that 53/58 cases (91.4%, odds ratio 5.3) which upregulated CD25 and/or CD86 expression after stimulation with Pam3CSK4 also responded to MALP-2 stimulation. These two receptors recognize triacetylated and diacetylated bacterial lipoproteins, respectively (11-14). Both molecular patterns usually co-exist on the same type of pathogens; therefore, one could speculate that both receptors signal together under a common bacterial trigger in certain CLL cases.

NOD2 was also found to be functional in most CLL cases, since its triggering induced the upregulation of CD25 in 55/67 cases (82.1%), upregulation of CD86 in 35/67 cases (52.2%) and upregulation of either marker in 57/67 cases (85.1%). NOD2 senses muramyl-dipeptide (MDP), which is found in both Gram-negative and Gram-positive bacteria; again, there seems to be a correlation of NOD2

and TLR1/2, TLR2/6 functionality: 56/57 cases (98.2%, odds ratio $\rightarrow\infty$) which responded to stimulation with MDP also responded to Pam3CSK4 and/or MALP-2 stimulation. Moreover, among cases that upregulated CD25 and/or CD86 expression after stimulation with either one of the three ligands, 46/65 (70.8%, odds ratio 13.1) cases were found to be responsive to all of them at the same time.

In the case of TLR7, we used two different ligands: loxoribine, a naturally occurring guanosine analog, and imiquimod, a synthetic ligand already used in clinical trials. We found that these two ligands have differential effects in terms of upregulation of costimulatory molecules. More specifically, imiquimod induced the upregulation of CD25 in 39/67 cases (58.2%), upregulation of CD86 in 21/67 cases (31.3%) and upregulation of either marker in a total of 41/67 cases (61.2%). In contrast, loxoribine had no effect in CD25 expression in any CLL case, while it upregulated CD86 expression in only 4/67 cases (6%). This could indicate that loxoribine signals in CLL through different downstream pathways. Indeed, we found that in cases where loxoribine had no effect in terms of CD25/CD86 expression, apoptosis of CLL cells was strongly induced (data not shown).

Finally, stimulation of CLL cells with TLR4 and TLR8 specific ligands had no effect on CD25/CD86 expression in any of the cases tested. This is in agreement with previous findings that these two TLRs are practically not expressed in CLL cells, allowing to use them as internal negative controls. It also validates our limit of 10%, since none of the internal negative controls exceeded or was near this percentage.

We next wanted to investigate whether the variability of TLR functionality among CLL cases was associated with specific molecular features of the BCR. The first comparison was based on IGHV mutational status. We found that TLR9 stimulation with CpG ODN had a strong effect in terms of CD86 upregulation in CLL cases carrying mutated IGHV genes, whereas it had no effect in cases with unmutated IGHV genes. This could mean that CpG is more efficient in increasing immunogenicity of CLL cells in mutated rather than unmutated cases. In contrast, TLR7 stimulation with imiquimod strongly upregulated CD25 expression in unmutated CLL cells, whereas it had no effect in mutated CLL cases.

Therefore, one could assume that imiquimod is more efficient in increasing immunogenicity of unmutated rather than mutated CLL cells. Similarly, MALP was found to more efficiently upregulate CD25 expression in unmutated rather than mutated CLL cells. However, the average difference of CD25 expression after stimulation among the two groups was only 11.6%, so it is not certain that statistical significance translates to biological significance, as well.

We then investigated the existence of possible associations between TLR functionality pattern and BCR stereotypy. As mentioned earlier, our study group included several cases expressing different stereotyped B cell receptors. Among cases carrying mutated IGHV genes, subset #4 was over-represented (7/44 cases; 15.9%). For this reason, we evaluated differences between subset #4 and other mutated cases and found that TLR1/2, TLR2/6 and NOD2 stimulation had a much stronger effect in terms of CD86 upregulation in subset #4 compared to all other mutated cases. A similar trend was noticed regarding CD25 upregulation, although these results did not reach statistical significance. We then narrowed down our comparison group to mutated cases carrying the same IGHV gene (IGHV4-34) in heterogeneous rearrangements, thus excluding cases belonging to two major subsets with stereotyped IGHV4-34 BCRs, namely subset #4 and subset #16. Interestingly, we discovered that TLR1/2, TLR2/6 and NOD2 stimulation significantly upregulated the expression of both CD86 and CD25 in subset #4 cases, whereas it had little or even no such effect in non-subset #4/16 IGHV4-34 cases. So, treating subset #4 CLL cells with Pam3CSK4, MALP-2 and MDP is expected to increase their immunogenicity, likely rendering them more susceptible to CLL immunotherapeutic agents. Maybe more importantly, this finding strongly points towards the idea that the TLR and NOD2 functionality pattern of this particular subset (subset #4) corresponds to a common antigenic stimulation. This concept is further supported by the fact that within this subset there was an even stronger association of TLR1/2, TLR2/6 and NOD2 functionality than previously mentioned. More specifically, 6/7 cases (85.7%) responded to all three ligands of the above receptors (Pam3CSK4, MALP-2 and MDP, respectively); the single case which was not responsive to MALP-2, still responded to both Pam3CSK4 and MDP.

In our study group, subset #1 was intentionally over-represented among cases carrying unmutated IGHV genes (7/21 cases; 33.3%), on the basis that it may be considered a "prototype" subset for unmutated stereotyped CLL cases with poor prognosis. When compared to all other unmutated cases, it was not found to exhibit statistically significant differences in terms of TLRs and NOD2 functionality. However, when compared it to subset #4, which may be considered as a "prototype" subset for mutated stereotyped CLL cases of indolent course, major differences were identified. In particular, TLR9, TLR1/2, TLR2/6 and NOD2 triggering strongly upregulated CD86 expression in subset #4 cases, whereas it had no effect in subset #1 cases. In sharp contrast, TLR7 stimulation with imiquimod strongly upregulated CD25 expression in subset #1 cases, whereas it had no effect in subset #4 cases. These functional profiles need to be considered in the context of the recognition specificities of TLR7 and TLR9: in fact, TLR7 recognizes single-stranded RNA (ssRNA) and therefore is considered to be specialized in viral detection, while TLR9 recognizes both bacterial and viral unmethylated DNA.

The IGHV4-34 gene encodes antibodies that are intrinsically autoreactive because they recognize the N-acetyllactosamine (NAL) antigenic epitope (120-121), which is present on various self-antigens (I/i blood group antigen, CD45 B-cell isoform), as well as foreign pathogens (EBV, CMV, Mycoplasma pneumoniae). Moreover, a recent retrospective analysis has identified a significant correlation between usage of the IGHV4-34 gene in CLL cases, particularly those belonging to subset #4, and persistence of EBV and CMV (130). Both of these pathogens are dsDNA herpesviruses, so they should be able to stimulate TLR9. Mycoplasma pneumoniae, on the other hand, can be the source of ligands for not only TLR9, but TLR2/6 and NOD2, as well. It is not clear whether such pathogens interfere at the initial stages of malignant transformation or whether they are involved in the active expansion of the malignant clone at later stages. Especially for subset #4, intense intraclonal diversification (ID) of IGHV genes through ongoing SHM implies functional interactions with the antigenic element(s) both in the pre-malignant phase but also after leukemic transformation (131). Collectively, one could suggest that an initial lesion in autoreactive IGHV4-34/IGKV2-30 B cells

which are circulating in the peripheral repertoire in an anergic state abrogates their resistance mechanisms to TLR-induced autoimmunity; after that, dual engagement of the BCR and TLR9 (or other functional TLRs and NLRs) by a pathogen whose structure combines both ligands (e.g. EBV, CMV, Mycoplasma pneumoniae) leads to proliferation and active expansion of the B cell clone.

It should be mentioned that other endpoints of TLR and NLR signaling need to be investigated in order to reach full understanding of how these pathways work in CLL cells. Most importantly, the effect of TLR and NLR stimulation in terms of apoptosis and proliferation needs to be analyzed. As mentioned earlier, there is limited and contradictory literature on the subject. Preliminary data from our group indicate that TLR triggering may either induce or actually protect CLL cells from apoptosis, depending on the molecular features of the BCR and more characteristically on BCR stereotypy (data not shown).

It appears that CLL is much more heterogeneous than previously thought. Unraveling its pathogenesis at a molecular level will allow the development of individualized treatments of particular benefit for those patients with aggressive disease and poor prognosis. It may also provide some insight regarding the links between infection, autoimmunity and cancer. Both BCR and TLR signaling in CLL remain a fruitful area for further investigation and it will be interesting to see what future research will bring to our knowledge.

PART VI: SUMMARY

SUMMARY

CLL is a chronic lymphoid malignancy characterized by the *in vivo* accumulation of CD5+ monoclonal B-cells in peripheral blood (PB), bone marrow (BM) and lymphoid tissue. Not so long ago, it was considered as a homogeneous disease of naive, immune-incompetent, minimally proliferating B cells which accumulated due to underlying apoptotic defects [76]. Over the last two decades, however, our view on the pathogenesis of CLL has been fully revisited. It appears that a crosstalk between genetic events and microenvironmental stimuli critically affects the onset and progression of the disease, which is much more heterogeneous than previously thought [77].

Several lines of evidence support a central role of the B-cell receptor (BCR) in the natural history of the disease and molecular characteristics of the BCR are now considered as significant independent prognostic markers for disease course and outcome. In at least 50% of CLL cases, leukemic cells carry somatically mutated IGHV genes ("mutated" CLL cases), and this is generally associated with better prognosis. "Unmutated" CLL cases, on the other hand, are expected to follow a more aggressive course. More importantly, the immunoglobulin (Ig) repertoire of CLL cells is characterized by a bias regarding the usage of selected IGHV genes when compared to normal B cell repertoire [94-97]. Recently, several groups reported subsets of CLL cases characterized by distinctive Ig heavy and light chain rearrangements culminating in remarkably homologous ("stereotyped") complementarity-determining region 3 (CDR3) sequences within their BCRs [98-102]. Such striking BCR similarity in unrelated CLL cases implies a role for a limited set of antigens or structurally related epitopes in leukemogenesis. Further evidence for this hypothesis is the existence of stereotyped somatic hypermutations (SHM) among these CLL subsets [103].

Until now, more than 150 CLL subsets have been defined. Recent studies report that almost 30% of CLL patients carry stereotyped receptors [102,104]. CLL cases expressing stereotyped BCRs have also been shown to share common clinical features, so that certain subsets are now associated with distinct prognosis [98,102,105-106]. Taken together, stimulation through the BCR appears to play a

crucial role in the pathogenesis of CLL. Therefore, it would be of great importance to determine the selecting antigenic elements.

CLL cells often express BCRs that bind autoantigens in a polyreactive manner. CLL cells were also found to bind neoantigens, as well as foreign antigens [(110-113,116-117)]. Almost 80% of "unmutated" and ~15% of "mutated" CLL BCRs were shown to react in vitro with a series of self and foreign antigens, as well as fixed and permeabilized cells, in a polyreactive manner [(118)]. Furthermore, most recombinant mAbs obtained from "unmutated" CLL cells that were not polyreactive *per se* acquired polyreactivity when their sequence was reverted to germline [(110)]. Taken together, these lines of evidence indicate that CLL cells may derive from a population of auto/polyreactive cells.

TLRs recognize an array of distinct molecular patterns and are known to bridge innate and adaptive immunity. Human naïve B cells express low to undetectable levels of all TLRs, however BCR crosslinking leads to rapid upregulation of TLR2, TLR6, TLR7, TLR9 and TLR10 [(56)]. $CD5^+$ memory B cells, on the other hand, constitutively express these TLRs [(55-56)]. Recently, it has been proposed that TLR stimulation acts as a direct third signal amplifying human naïve B cell response to antigen [(61,63)]. An innovative study by Leadbetter et al showed that DNA-containing chromatin-IgG complexes activate B cells to produce antibodies against self-IgG by engaging the autoreactive BCR and TLR9 in a sequential manner [(32)]. This new concept was further confirmed by Lau et al who demonstrated that RNA-associated auto-Ags activate B cells by dual BCR/TRL7 engagement, thereby supporting a role of TLRs in breaking tolerance [(71)]. It would be intriquing to test whether this model applies to the pathogenesis of CLL, at least in the cases with BCR auto/polyreactivity.

Limited data exist regarding which TLRs are expressed in CLL cells. So far, their expression profile seems to resemble that of antigen-experienced B cells. Several groups reported ubiquitous expression of TLR7 and TLR9 by CLL cells [(140-141,144)] and the respective agonists have entered clinical trials with the aim to increase immunogenicity by upregulation of costimulatory molecules and/or augment proliferation and thereby sensitivity to cytotoxic therapies (reviewed in 155, 158). Still, the effects of TLR stimulation on CLL cells remain unclear. There are also

reports suggesting a pleiotropic effect of TLR stimulation, especially though TLR9, depending on IGHV mutational status [144].

The present study aimed to investigate TLR and NLR function within CLL subgroups defined by IGHV gene mutational status, IGHV gene usage or BCR stereotypy. To this end, we investigated a series of 67 CLL patients, selected with an intentional bias for cases with stereotyped BCRs assigned to subset #4 and #1, since these two subsets may be considered as prototypes of "mutated/good prognosis" and "unmutated/bad prognosis" subsets, respectively. Untouched purified CLL cells from each case were stimulated with ligands for all TLRs that have been detected in CLL cells plus NOD2 of the NLR family, and upregulation of CD25 and CD86 expression was tested by flow cytometry as a marker of activation.

We report that TLR9 was functional in practically all cases (98.5% of cases upregulated CD25 and/or CD86 after CpG stimulation). Moreover, TLR1/2, TLR2/6 and NOD2 were functional in most cases (86.6%, 89.6% and 86.6%, respectively) and there was actually a correlation regarding their functionality. Approximately two-thirds of cases exhibited functionality of all three receptors. Given that TLR1/2 and TLR2/6 recognize triacetylated and diacetylated bacterial lipoproteins, respectively, and NOD2 senses MDP which is found on both Gram positive and Gram negative bacteria, one could hypothesize that these three receptors signal together under a common bacterial trigger in certain CLL cases. TLR7 was found to be functional in fewer cases (61.2%) and only when triggered with its synthetic ligand, imiquimod. Loxoribine, the natural ligand of TLR7, had practically no effect on CD25/CD86 upregulation. Finally, TLR4 and TLR8 were not found to be functional in any CLL case.

We next investigated possible associations of TLR/NLR functional profiles with molecular features of the BCR, i.e. IGHV mutational status, IGHV gene usage and BCR stereotypy. The first comparison concerned "mutated" versus "unmutated" CLL cases. TLR9 stimulation was found to have a strong effect in terms of CD86 induction in "mutated" CLL cases, whereas it has no such effect in "unmutated" CLL cases. In contrast, TLR7 stimulation with imiquimod strongly upregulated

CD25 expression in “unmutated” CLL cells, whereas it had no such effect in “mutated” cases.

We then focused on subsets #4 and #1. By comparing subset #4 cases to all other CLL cases carrying mutated IGHV genes as well as to CLL cases carrying the IGHV4-34 gene in heterogeneous rearrangements, we discovered that TLR1/2, TLR2/6 and NOD2 stimulation has a much stronger effect in CD25/CD86 upregulation in subset #4; noticeably, 6/7 subset #4 cases were responsive to all three ligands - Pam3CSK4, MALP-2 and MDP. Therefore, one could hypothesize that this TLR/NLR functionality pattern of subset #4 corresponds to a common antigenic stimulation. When subset #4 was compared to subset #1, we found that TLR9, TLR1/2, TLR 2/6 and NOD2 stimulation had no effect in CD86 expression in subset #1. Instead, TLR7 stimulation strongly induced CD25 upregulation, in sharp contrast to subset #4. One could speculate that, behind the distinct TLR and NLR functional patterns of subsets #4 and #1, hide different microbial pathogens.

All of the above may be considered as further evidence of how heterogeneous CLL is at both the molecular and functional level. Could TLR ligation act as a non-specific stimulation to CLL cells, and if yes, how non- specific is it? Different patterns of TLR function between subsets may correspond to different antigenic elements and may require distinct therapeutic approaches when it comes to TLR agonists or inhibitors. Other endpoints of TLR and NLR signaling, such as apoptosis and proliferation, need to be investigated in order to better understand how these pathways work in CLL and how they can be therapeutically exploited.

PART VII: ABSTRACT

Background: Mature B cells recognize antigens through the B cell receptor (BCR) in a specific way and through Toll-like receptors (TLRs) and Nod-like receptors (NLRs) in a costimulatory manner. Plenty of evidence supports a role for antigenic stimulation in the natural history of chronic lymphocytic leukemia (CLL). While BCR signaling has extensively been studied, little is known regarding the function of TLRs and NLRs in CLL cells.

Design and methods: We used a series of 67 patients, selected with an intentional bias for cases assigned to two different subsets with stereotyped BCRs, namely subsets #1 and #4. We stimulated negatively isolated CLL cells with specific ligands for all TLRs/NLRs found to be expressed in B cells and measured by flow cytometry the upregulation of CD25 and CD86 expression as a marker of NF-κB activation. We compared our findings among CLL subgroups based on IGHV mutational status, IGHV gene usage and BCR stereotypy.

Results: TLR9 was functional in practically all cases. TLR1/2, TLR2/6 and NOD2 were functional in most cases with concordant functionality in different cases. TLR7 was functional in fewer cases and responded only to imiquimod, but not loxoribine. Moreover, TLR9 upregulated CD86 expression only in "mutated" CLL cases while TLR7 upregulated CD25 expression only in "unmutated" CLL cases. Finally, TLR1/2, TLR2/6 and NOD2 stimulation had a much stronger effect in CD25/86 upregulation in subset #4 cases when compared to all other "mutated" cases or cases carrying the IGHV4-34 gene in heterogeneous rearrangements. When subset #4 was compared to subset #1, we found that TLR9, TLR1/2, TLR 2/6 and NOD2 stimulation had no effect in CD86 expression in subset #1. Instead, TLR7 stimulation strongly induced CD25 upregulation, in sharp contrast with subset #4.

Conclusions: TLRs and NLRs are functional in CLL cells, yet in a heterogeneous fashion. Their stimulation has a pleiotropic effect on the upregulation of costimulatory molecules, depending on the IGHV mutational status. Patterns of TLR/NLR functionality can be recognized among CLL subsets with distinct stereotyped BCRs, offering hints for the nature of the antigenic drive.

PART VIII: REFERENCES

REFERENCES

[Papers published after 2007 are highlighted **in bold**]

1. Janeway's Immunobiology, 7th edition, 2008. Part I: An Introduction to immunobiology and Innate Immunity.

2. Kawai T, Akira S. TLR signalling. Cell Death and Diff. 2006; 13:816-825

3. Akira S and Takeda K. Toll-like receptor signalling. Nat. Rev. Immunol. 2004; 4:499–511

4. Choe J, Kelker MS, Wilson IA. Crystal structure of human Toll-like receptor 3 (TLR3) ectodomain. Science 2005; 309:581–85

5. Xu Y, Tao X, Shen B, Horng T, Medzhitov R, et al. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. Nature 2000; 408:111–15

6. Dunne A, Ejdeback M, Ludidi PL, O'Neill LA, Gay NJ. Structural complementarity of Toll/interleukin-1 receptor identity regions in Toll-like receptors and the adaptors Mal and MyD88. J. Biol. Chem. 2003; 278:41443–51

7. Hoebe K, Georgel P, Rutschmann S, Du X, Mudd S, et al. CD36 is a sensor of diacylglycerides. Nature 2005; 433:523–27

8. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990; 249:1431–33

9. Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, et al. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. Nat. Immunol. 2002; 3:667–72

10. Beutler B, Jiang Z, Georgel P, et al. Genetic Analysis of Host Resistance: Toll-Like Receptor Signaling and Immunity at Large. Annu. Rev. Immunol. 2006; 24:353–89

11. Takeuchi O, Kaufmann A, Grote K, Kawai T, Hoshino K, Morr M, Muhlradt PF and Akira S. Preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. J. Immunol. 2000; 164: 554–557

12. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL and Akira S. Role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J. Immunol. 2002; 169: 10–14
13. Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, Takeda K and Akira S. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int. Immunol. 2001; 13: 933–940
14. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L and Aderem A. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. PNAS 2000; 97: 13766–13771
15. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J. Immunol. 2009; 162: 3749–3752
16. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2002; 410: 1099–1103
17. Alexopoulou L, Holt AC, Medzhitov R and Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001; 413: 732–738
18. Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K and Akira S. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat. Immunol. 2002; 3: 196–200
19. Diebold SS, Kaisho T, Hemmi H, Akira S and Reis e Sousa C. Innate antiviral responses by means of TLR7 mediated recognition of single-stranded RNA. Science 2004; 303: 1529–1531
20. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H and Bauer S. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004; 303: 1526–1529
21. Heil F, Ahmad-Nejad P, Hemmi H, Hochrein H, Ampenberger F, Gellert T, Dietrich H, Lipford G, Takeda K, Akira S, Wagner H and Bauer S. The Toll-like

receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7 8 and 9 subfamily. Eur. J. Immunol. 2003; 33: 2987–2997
22. Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, Lipford G and Bauer S. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat. Immunol. 2002; 3: 499
23. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A and Flavell RA. Recognition of single-stranded RNA viruses by Toll-like receptor 7. PNAS 2004; 101: 5598–5603
24. Hornung V, Guenthner-Biller M, Bourquin C, Ablasser A, Schlee M, Uematsu S, Noronha A, Manoharan M, Akira S, de Fougerolles A, Endres S and Hartmann G. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat. Med. 2005; 11: 263–270
25. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K and Akira S. A Toll-like receptor recognizes bacterial DNA. Nature 2000; 408: 740–745
26. Krug A, French AR, Barchet W, Fischer JA, Dzionek A, Pingel JT, Orihuela MM, Akira S, Yokoyama WM and Colonna M. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. Immunity 2004; 21: 107–119
27. Lund J, Sato A, Akira S, Medzhitov R and Iwasaki A. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J. Exp. Med. 2003; 198: 513–520
28. Coban C, Ishii KJ, Kawai T, Hemmi H, Sato S, Uematsu S, Yamamoto M, Takeuchi O, Itagaki S, Kumar N, Horii T and Akira S. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. J. Exp. Med. 2005; 201: 19–25
29. Wang, J., Shao, Y., Bennett, T.A., Shankar, R.A., Wightman, P.D. & Reddy, L.G. The functional effects of physical interactions among Toll-like receptors 8 and 9. J. Biol. Chem. 2006; 7: 281.
30. Lee, J. et al. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. PNAS 2003; 100: 6646−6651.

31. Matsumoto, M. et al. Subcellular localization of toll-like receptor 3 in human dendritic cells. J. Immunol. 2003; 171:3154–3162.
32. Leadbetter EA, Rifkin IR, et al. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature 2002; 416:603-607
33. Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, Shimada N, Ohba Y, Takaoka A, Yoshida N, Taniguchi T. IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature 2005; 434: 772–777
34. Kawai T, Sato S, Ishii K.J, Coban C, Hemmi H, Yamamoto M, Terai K, Matsuda M, Inoue J, Uematsu S, Takeuchi O, Akira S. Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. Nat. Immunol. 2004; 5: 1061–1068
35. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nature Immunol. 2001; 2:947-950
36. Hopkins PA, Sriskandan S. Mammalian Toll-like receptors: to immunity and beyond. Clin. Exp. Immunol. 2005; 140: 395–407.
37. Kagnoff, M.F. & Eckmann, L. Epithelial cells as sensors for microbial infection. J. Clin. Invest. 1997; 100:6–10.
38. Hertz, C.J. et al. Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. J. Immunol. 2003; 171: 6820–6826.
39. Nagase, H. et al. Expression and function of Toll-like receptors in eosinophils: activation by Toll-like receptor 7 ligand. J. Immunol. 2003; 171: 3977–3982.
40. McCurdy, J.D., Olynych, T.J., Maher, L.H. & Marshall, J.S. Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. J. Immunol. 2003; 170: 1625–1629.
41. Fan, J. & Malik, A.B. Toll-like receptor-4 (TLR4) signaling augments chemokine-induced neutrophil migration by modulating cell surface expression of chemokine receptors. Nat. Med. 2003; 9: 315–321.
42. Banchereau, J. & Steinman, R.M. Dendritic cells and the control of immunity. Nature 1998; 392: 245–252.
43. Pasare, C. & Medzhitov, R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 2003; 299: 1033–1036.

44. Yang, Y., Huang, C.T., Huang, X. & Pardoll, D.M. Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. Nat. Immunol. 2004; 5:508−515.
45. Hornung, V. et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. 2002; 168: 4531−4537.
46. Jarrossay, D., Napolitani, G., Colonna, M., Sallusto, F. & Lanzavecchia, A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. Eur. J. Immunol. 2001; 31: 3388−3393
47. Kadowaki, N. et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J. Exp. Med. 2001; 194: 863-869
48. Fritz JH, Ferrero R, Philpott DJ, & Girardin SE. Nod-like proteins in immunity, inflammation and disease. Nat. Immunol. Rev. 2006; 7(12): 1250-1257
49. Inohara N, Chamaillard M, McDonald C and Nunez G. NOD–LRR proteins: role in host–microbial interactions and inflammatory disease. Annu. Rev. Biochem.2005; 74:355–383.
50. Janeway's Immunobiology, 7th edition, 2008.Part IV: The adaptive immune response.
51. Janeway's Immunobiology, 7th edition, 2008.Part II: The recognition of antigen
52. Janeway's Immunobiology, 7th edition, 2008.Part III: The development of mature lymphocyte receptor repertoires
53. Chiorazzi N, Rai KR and Ferrarini M. Chronic Lymphocytic Leukemia. N Engl J Med. 2005; 352:804-815
54. Efremov DG, Gobessi S, Longo PG. Signaling pathways activated by antigen-receptor engagement in chronic lymphocytic leukemia B-cells. Autoimm Rev. 2007; 7:102-108
55. Bourke, E., Bosisio, D., Golay, J., Polentarutti, N. and Mantovani, A. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood 2003; 102: 956–963.

56. Bernasconi, N. L., Onai, N. and Lanzavecchia, A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood 2003; 101: 4500–4504

57. Bernasconi NL, Traggiai E, Lanzavecchia A: Maintenance of serological memory by polyclonal activation of human memory B cells. Science 2002; 298:2199-2202.

58. Bekeredjian-Ding IB, Wagner M, Hornung V, et al. Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. J Immunol. 2005; 174:4043-4050.

59. Huggins J, Pellegrin T, Felgar RE et.al. CpG DNAactivation and plasma-cell differentiation of CD27$^-$ naïve human B cells. Blood 2007; 109:1611-1619

60. Janeway, C. A. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb. Symp. Quant. Biol. 1989; 54 Pt 1:1–13.

61 . Ruprecht C., Lanzavecchia A. Toll-like receptor stimulation as a third signal required for activation of human naive B cells. Eur.J.Immunol. 2006; 36:810-16

62. Nemazee , D. , A. Gavin , K. Hoebe , and B. Beutler. Immunology: Toll-like receptors and antibody responses. Nature 2006; 441:E4 .

63. Meyer-Bahlburg A., Khim S., Rawlings DJ. B cell – intrinsic TLR signals amplify but are not required for humoral immunity. JEM 2007; 204(13):3095-3101

64. Genestier L, Taillardet M, Mondiere P et.al. TLR Agonists Selectively Promote Terminal Plasma Cell Differentiation of B Cell Subsets Specialized in Thymus-Independent Responses. J Immunol. 2007; 178:7779-7786

65. Rubtsov AV, Swanson CL, Troy S et.al. TLR Agonists Promote Marginal Zone B Cell Activation and Facilitate T-Dependent IgM Responses. J Immunol. 2008; 180:3882-3888

66. Jegerlehner A, Maurer P, Hinton H, Kopf M, Bachman MF. TLR9 Signaling in B Cells Determines Class Switch Recombination to IgG2a. J Immunol. 2006; 178:2415-2420

67. Dye JR, Palvanov A, Guo B, Rothstein TL. B Cell Receptor Cross-Talk: Exposure to Lipopolysaccharide Induces an Alternate Pathway for B Cell Receptor-Induced ERK Phosphorylation and NF-κB Activation. J Immunol. 2007; 179:229-235

68. Bekeredjian-Ding I, Doster A, Schiller M et.al. TLR9-Activating DNA Up-Regulates ZAP70 via Sustained PKB Induction in IgM+ B Cells. J Immunol 2008; 181(12): 8267-77

69. Genestier L, Taillardet M, Mondiere P, Gheit H, Bella C, Defrance T. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. J Immunol. 2007; 178:7779-7786.

70. Gururajan M, Jacob J, Pulendran B. Toll-like receptor expression and responsiveness of distinct murine splenic and mucosal B-cell subsets. PLoS ONE 2007; 2:e863.

71. Lau CM, Broughton C, et al. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll like receptor 7 engagement. J Exp Med 2005; 202:1171-1177

72. Sadanaga A, et al. Protection against autoimmune nephritis in MyD88-deficient MRL/lpr mice. Arthritis Rheum. 2007; 56:1618–1628.

73. Christensen SR, et al. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. Immunity 2006; 25:417–428.

74. Rui L, Vinuesa CG, Blasioli J, Goodnow CC. Resistance to CpG DNA-induced autoimmunity through tolerogenic B cell antigen receptor ERK signaling. Nat Immunol. 2003; 4:594–600.

75. O'Neill SK, Veselits ML, Zhang M et.al. Endocytic sequestration of the B cell antigen receptor and toll-like receptor 9 in anergic cells. PNAS 2009; 106:6262-6267

76. Rozman C, Montserrat E. Chronic lymphocytic leukemia. N Engl J Med 1995; 333:1052-1057.[Erratum]

77. Ghia P., Chiorazzi N., Stamatopoulos K. Microenvironmental influences in chronic lymphocytic leukaemia: the role of antigen stimulation. J.Int.Med. 2008; 264:549-562

78. Dal-Bo M, Bertoni F, Forconi F et.al. Intrinsic and extrinsic factors influencing the clinical course of B-cell chronic lymphocytic leukemia: prognostic markers with pathogenetic relevance. J Transl Med. 2009; 7(76):1-14

79. Seiler T, Dohner H, Stilgenbauer S: Risk stratification in chronic lymphocytic leukemia. *Semin Oncol* 2006; 33:186-194.

80. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. PNAS 2002; 99: 15524–15529.

81. Nicoloso MS, Kipps TJ, Croce CM, Calin GA. MicroRNAs in the pathogeny of chronic lymphocytic leukemia. BJH 2007; 139:709-716

82. Raveche ES, Salerno E, Scaglione BJ, et al. Abnormal microRNA-16 locus with synteny to human 13q14 linked to CLL in NZB mice. Blood 2007; 109:5079–5086.

83. Calin GA, Ferracin M, Cimmino A, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med*. 2005; 353(17):1793-1801.

84. Marton S, Garcia MR, Robello C, et al. Small RNAs analysis in CLL reveals a deregulation of miRNA expression and novel miRNA candidates of putative relevance in CLL pathogenesis. Leukemia 2008; 22(2): 330-338.

85. Fulci V, Chiaretti S, Goldoni M, et al. Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. Blood 2007; 109(11): 4944-4951.

86. Stamatopoulos B, Meuleman N, Haibe-Kains B, et al. MicroRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukemia and improves disease risk stratification. Blood 2009; 113(21):5237-5245.

87. Zenz T, Mohr J, Eldering E, et al. miR-34a as part of the resistance network in chronic lymphocytic leukemia. Blood 2009; 113(16):3801-3808.

88. Damle RN, Ghiotto F, Valetto A et al. B-cell chronic lymphocytic leukemia cells express a surface membrane phenotype of activated, antigen-experienced B lymphocytes. Blood 2002; 99: 4087–93.

89. Klein U, Tu Y, Stolovitzky GA et al. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med 2001; 194: 1625–38.

90. Moreno C, Montserrat E. New prognostic markers in chronic lymphocytic leukemia. Blood Rev. 2008; 22: 211–9.

91. Rassenti LZ, Jain S, Keating MJ et al. Relative value of ZAP-70, CD38, and immunoglobulin mutation status in predicting aggressive disease in chronic lymphocytic leukemia. Blood 2008; 112:1923-30.
92. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999; 94:1848-54.
93. Krober A, Seiler T, Benner A, et al. V(H) mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia. Blood 2002; 100: 1410-6.
94. Fais F, Ghiotto F, Hashimoto S, et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. J Clin Invest. 1998; 102:1515-1525.
95. Tobin G, Thunberg U, Johnson A, et al. Somatically mutated IgV(H)3-21 genes characterize a new subset of chronic lymphocytic leukemia. Blood 2002; 99:2262-2264.
96. Chiorazzi N, Ferrarini M. B cell chronic lymphocytic leukemia: lessons learned from studies of the B cell antigen receptor. Annu Rev Immunol. 2003; 21:841-894.
97. Ghia P, Stamatopoulos K, Belessi C, et al. Geographic patterns and pathogenetic implications of IGHV gene usage in chronic lymphocytic leukemia: the lesson of the IGHV3-21 gene. Blood 2005; 105:1678-1685
98. Ghiotto F, Fais F, Valetto A, et al. Remarkably similar antigen receptors among a subset of patients with chronic lymphocytic leukemia. J Clin Invest. 2004; 113:1008-1016.
99. Widhopf GF 2nd, Rassenti LZ, Toy TL, Gribben JG, Wierda WG, Kipps TJ. Chronic lymphocytic leukemia B cells of more than 1% of patients express virtually identical immunoglobulins. Blood 2004; 104:2499-2504.
100. Messmer BT, Albesiano E, Efremov DG, et al. Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. J Exp Med. 2004; 200:519-525.
101. Tobin G, Thunberg U, Karlsson K, et al. Subsets with restricted immunoglobulin gene rearrangement features indicate a role for antigen selection in the development of chronic lymphocytic leukemia. Blood 2004; 104:2879-2885.

102. Stamatopoulos K, Belessi C, Moreno C, et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: pathogenetic implications and clinical correlations. Blood 2007; 109:259-270.
103. Murray F, Darzentas N, et al. Stereotyped patterns of somatic hypermutation in subsets of patients with chronic lymphocytic leukemia: implications for the role of antigen selection in leukemogenesis. Blood 2008; 111:1524-1533
104. Darzentas N, Hadzidimitriou A, Murray F et al. A different ontogenesis for chronic lymphocytic leukemia cases carrying stereotyped antigen receptors: molecular and computational evidence. Leukemia 2010; 24:125-32.
105. Thorselius M, Krober A, Murray F, et al. Strikingly homologous immunoglobulin gene rearrangements and poor outcome in VH3-21-utilizing chronic lymphocytic leukemia independent of geographical origin and mutational status. Blood 2006; 107:2889-2894.
106. Potter KN, Mockridge CI, Neville L, et al. Structural and functional features of the B-cell receptor in IgG-positive chronic lymphocytic leukemia. Clin Cancer Res. 2006; 12:1672-1679.
107. Tobin G, Thunberg U, Johnson A, et al. Chronic lymphocytic leukemias utilizing the VH3–21 gene display highly restricted Vlambda2–14 gene use and homologous CDR3s: implicating recognition of a common antigen epitope. Blood 2003; 101: 4952-4957
108. Lin K, Manocha S, Harris RJ, Matrai Z, Sherrington PD, Pettitt AR. High frequency of p53 dysfunction and low level of VH mutation in chronic lymphocytic leukemia patients using the VH3–21 gene segment. Blood 2003; 102:1145-1146.
109. Borche L, Lim A, Binet JL, Dighiero G. Evidence that chronic lymphocytic leukemia B lymphocytes are frequently committed to production of natural autoantibodies. Blood 1990; 76:562-9.
110. Herve M, Xu K, Ng YS et al. Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. J Clin Invest 2005; 115: 1636–43.

111. Lanemo Myhrinder A, Hellqvist E, Sidorova E et al. A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. Blood 2008; 111: 3838–48.
112. Chu C, Catera R, Hatzi K et al. Chronic lymphocytic leukemia antibodies with a common stereotypic rearrangement recognize non-muscle myosin heavy chain IIA. Blood 2008; 112(13):4122-5129
113. Chu C, Catera R, Zhang L et al. Elevated Binding of Chronic Lymphocytic Leukemia Antibody to a Subset of Apoptotic Cells with Exposed Non-Muscle Myosin Heavy Chain IIA Correlates with Poor Patient Outcome. Blood 2009; 114:330a.
114. Messmer BT, Albesiano E, Efremov DG, et al. Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. J Exp Med. 2004; 200:519-25
115. Bomben R, Dal Bo M, Capello D et al. Molecular and clinical features of chronic lymphocytic leukaemia with stereotyped B cell receptors: results from an Italian multicentre study. Br J Haematol. 2009; 144:492-506.
116. Catera R, Silverman GJ, Hatzi K et al. Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation. Mol Med 2008; 14(11-12):665-674
117. Hatzi K, Catera R, Ferrarini M et al. B-cell chronic lymphocytic leukemia (B-CLL) cells expresss antibodies reactive with antigenic epitopes expressed on the surface of common bacteria. Blood 2006; 108:12a.
118. Seiler T, Woelfle M, Yancopoulos S et al. Characterization of structurally defined epitopes recognized by monoclonal antibodies produced by chronic lymphocytic leukemia B cells. Blood 2009; 114:3615-24
119. Cavalli F, Isaacson PG, Gascoyne RD, Zucca E. MALT lymphomas. Hematology (Am Soc Hematol Educ Program) 2001: 241-58.
120. Pugh-Bernard AE, Silverman GJ, Cappione AJ, et al. Regulation of inherently autoreactive VH4–34 B cells in the maintenance of human B cell tolerance. J Clin Invest. 2001; 108:1061-1070.

121. Silberstein LE, George A, Durdik JM, Kipps TJ. The V4–34 encoded anti-i autoantibodies recognize a large subset of human and mouse B-cells. Blood Cells Mol Dis. 1996; 22:126-138.
122. Potter KN, Hobby P, Klijn S et al. Evidence for involvement of a hydrophobic patch in framework region 1 of human V4-34-encoded Igs in recognition of the red blood cell I antigen. J Immunol. 2002; 169: 3777-82
123. Mockridge CI, Rahman A, Buchan S, et al. Common patterns of B cell perturbation and expanded V4-34 immunoglobulin gene usage in autoimmunity and infection. Autoimmunity 2004; 37:9-15
124. Bhat NM, Bieber MM, Yang YC et al. B cell lymphoproliferative disorders and IGHV4-34 gene encoded antibodies. Hum Antibodies. 2004; 13:63-8
125. Cappione AJ, Pugh-Bernard AE, Anolik JH et al. Lupus IgG VH4.34 antibodies bind to a 220-kDa glycoform of CD45/B220 on the surface of human B lymphocytes. J Immunol. 2004 ;172:4298-307
126. Jang YJ, Stollar BD. Anti-DNA antibodies: aspects of structure and pathogenicity. Cell Mol Life Sci. 2003; 60:309-20.
127. Hadzidimitriou A, Darzentas N, Murray F et al. Evidence for the significant role of immunoglobulin light chains in antigen recognition and selection in chronic lymphocytic leukemia. Blood 2009; 113:403-11.
128. Li H, Jiang Y, Prak EL, Radic M, Weigert M. Editors and editing of anti-DNA receptors. Immunity 2001; 15:947-57.
129. Bhat NM, Bieber MM, Spellerberg MB, Stevenson FK, Teng NN. Recognition of auto- and exoantigens by V4-34 gene encoded antibodies. Scand J Immunol. 2000; 51:134-40.
130. Kostareli E, Hadzidimitriou A, Stavroyianni N, et al. Molecular evidence for EBV and CMV persistence in a subset of patients with chronic lymphocytic leukemia expressing stereotyped IGHV4-34 B-cell receptors. Leukemia 2009; 23:919-24.
131. Sutton LA, Kostareli E, Hadzidimitriou A et al. Extensive intraclonal diversification in a subgroup of chronic lymphocytic leukemia patients with stereotyped IGHV4-34 receptors: implications for ongoing interactions with antigen. Blood 2009; 114:4460-8.

132. Bergh A, Stamatopoulos K, Rosenquist R, and Rosén A. Strict control of stereotyped subset-1 B cell proliferation in CLL: Antigen (oxLDL) alone is silencing – TLR9 is required. (manuscript in preparation)
133. Lanham S, Hamblin T, Oscier D, Ibbotson R, Stevenson F, Packham G. Differential signaling via surface IgM is associated with VH gene mutational status and CD38 expression in chronic lymphocytic leukemia. Blood 2003; 101: 1087–93.
134. Mockridge CI, Potter KN, Wheatley I, Neville LA, Packham G, Stevenson FK. Reversible anergy of sIgM mediated signaling in the two subsets of CLL defined by VH-gene mutational status. Blood 2007; 109: 4424–31.
135. Chen L,Widhopf G, Huynh L, Rassenti L, Rai KR,Weiss A, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. Blood 2002; 100:4609–14.
136. Petlickovski A, Laurenti L, Li X, Marietti S, Chiusolo P, Sica S, et al. Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B-cells. Blood 2005; 105: 4820–7.
137. Deglesne PA, Chevallier N, Letestu R, Baran-Marszak F, Beitar T, Salanoubat C, et al. Survival response to B-cell receptor ligation is restricted to progressive chronic lymphocytic leukemia cells irrespective of Zap70 expression. Cancer Res. 2006; 15(66):7158–66.
138. Muzio M, Apollonio B, Scielzo C et.al. Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. Blood 2008; 112:188-195
139. Merrell KT, Benschop RJ, Gauld SB, et al. Identification of anergic B cells within a wild-type repertoire. Immunity 2006; 25:953-962.
140. Spaner DE, Shi Y, Mena J, Hammond C, Tomic J, He L et al. Immunomodulatory effects of TLR7 activation on CLL cells. Leukemia 2006; 20: 286–295.
141. Muzio M, Scielzo C, Bertilaccio M, et al. Expression and function of toll like receptors in chronic lymphocytic leukaemia cells. Br J Haematol. 2009; 144:507-16
142. Jahrsdorfer B, Muhlenhoff L, et al. B-cell lymphomas differ in their responsiveness to CpG oligonucleotides. Clin Cancer Res 2005; 11:1490-1499

143. Jahrsdorfer B, Wooldridge JE, et al. Immunostimulatory oligonucleotides induce apoptosis of B cell chronic lymphocytic leukemia cells. J Leukoc Biol 2005; 77:378-387

144. Longo P, Laurenti L, Gobessi S, Petlickovski A, Pelosi M, Chiusolo P et al. The Akt signaling pathway determines the proliferative capacity of CLL-B cells from patients with progressive and stable disease. Leukemia 2007; 21:110-120

145. Hammadi A, Billard C, Faussat AM, Kolb JP. Stimulation of iNOS expression and apoptosis resistance in B-cell chronic lymphocytic leukemia (B-CLL) cells through engagement of Toll-like receptor 7 (TLR-7) and NF-κB activation. Nitric Oxide 2008; 19:138-145

146. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood 2008; 111: 5446-5456

147. Bosch, F. et al. Fludarabine, cyclophosphamide, and mitoxantrone as initial therapy of chronic lymphocytic leukemia: high response rate and disease eradication. Clin. Cancer Res. 2008; 14:155–161.

148. Pleyer L, Egle A, et.al. Molecular and cellular mechanisms of CLL: novel therapeutic approaches. Nature Rev. Clin. Oncol. 2009; 6:405-418

149. Ramsay, A. G. et al. Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. J. Clin. Invest. 2008; 118:2427–2437.

150. Serrano, D. et al. Clonal expansion within the $CD4^{+}CD57^{+}$ and $CD8^{+}CD57^{+}$ T cell subsets in chronic lymphocytic leukemia. J. Immunol. 1997; 158:1482–1489.

151. Chanan-Khan, A. et al. Clinical efficacy of lenalidomide in patients with relapsed or refractory chronic lymphocytic leukemia: results of a phase II study. J. Clin. Oncol. 2006; 24: 5343–5349.

152. Ferrajoli, A. et al. Lenalidomide is active in patients with relapsed/refractory chronic lymphocytic leukemia (CLL) carrying unfavorable chromosomal abnormalities [abstract]. Blood 2007; 110: 754A.

153. Trojan, A. et al. Immunoglobulin framework-derived peptides function as cytotoxic T-cell epitopes commonly expressed in B-cell malignancies. Nat. Med. 2000; 6: 667–672.

154. Luqman, M. et al. The antileukemia activity of a human anti-CD40 antagonist antibody, HCD122, on human chronic lymphocytic leukemia cells. Blood 2008; 112: 711–720.

155. Spaner DE, Masellis A. Toll-like receptor agonists in the treatment of chronic lymphocytic leukemia. Leukemia 2007; 21; 53 -60

156. Chiron D, Bekerdjian-Ding I, et al. Toll-like receptors: lessons to learn from normal and malignant human B cells. Blood 2008; 112: 2205-2212

157. Reis e SC. Dendritic cells in a mature age. Nat.Rev.Immunology 2006; 6:476-483

158. Lisa P, Alexander E, et.al. Molecular and cellular mechanisms of CLL: novel therapeutic approaches. Nature Rev. Clin. Oncol. 2009; 6, 405-418

159. Decker T, Schneller F, Kronschnabl M, et al. Immunostimulatory CpG-oligonucleotides induce functional high affinity IL-2 receptors on B-CLL cells: costimulation with IL-2 results in a highly immunogenic phenotype. Experimental Hematology 2000; 28: 558–568.

160. Jahrsdorfer B, Hartmann G, Racila E, et al. CpG DNA increases primary malignant B cell expression of costimulatory molecules and target antigens. Journal of Leukocyte Biology 2001; 69: 81–88.

PART IX: SUPPLEMENTARY

1	A/A	CpG/CD25	CpG/CD86	PAM/CD25	PAM/CD86	MALP/CD25	MALP/CD86	MDP/CD25	MDP/CD86	I/CD25	I/CD86	LOX/CD25	LOX/CD86	ORN/CD25	ORN/CD86	LPS/CD25	LPS/CD86
2	P3551	61,1	76,3	19,7	14,5	1,3	0,8	33,1	32,2	23,1	10,3	-4,6	-1,4	1,4	0,6	6,4	3
3	P1626	62,6	37,1	58,1	39,6	34,3	28,8	21,5	12,7	4,7	4,7			0,2	1,1	1,3	-2,4
4	P103	71,2	69,9	66	73,7	35,8	38,9	46,1	50,9	2,1	3	-8,4	-2,8	1	-0,1	3,1	2,5
5	P6520	31,4	60,5	27,6	56,4	28,5	56,5	20,6	52,6	12,8	30,6	-9,2	5,4	-0,4	-1,2	1,3	4,9
6	P3916	70,6	53	57,6	52,4	44	43,8	22,5	29,5	12,4	4,2	-4,4	-4,7	-1,4	-3,2	-1,7	-3,2
7	P3020	49,6	36,7	59,8	49,5	44,7	44,3	35,7	30,7	9,6	29,9	-12,8	-17,7	-1,7	-1,8	0,5	3,6
8	P511	62,6	15,3	55,7	49,4	26,8	30,1	24,3	16,1	23,2	5,9	-0,3	3,2	-0,1	2,7	0,3	2,5
9	P7155	62,2	14,9	65	17,5	55,6	14,6	54,5	6,7	54,8	4,2			1,6	0,1	2,2	0,1
10	P3870	55,9	4,3	10,5	0,7	40,3	1,1	43	0,1	46,8	0,6	4,3	0	2,2	-0,2	2,3	-0,1
11	P104	91,6	11,3	89,4	46,3	32	5	72,1	2,9	88,4	10,7	2	-1,1	-0,3	-2,6	0,8	-1,3
12	P6068	31,8	0,6	4,1	0,4	0,4	1,1	-1,4	-1,1	-0,8	2,4			-0,2	2,2	-0,1	2,6
13	P1618	48,9	40,3	69,5	58,5	16,8	13	31,4	27,7	21,3	6,5			1,6	0,8	1,3	0,3
14	P5949	59,9	40,6	65,2	45,9	30,1	15,5	26	11,9	10,3	2,4			1,7	0,4	1,9	0,7
15	P280	84	32,8	34,6	22,3	1,9	1	6,2	1,9	1,3	0,8			0,1	0	0,3	-0,2
16	P4875	90,5	8,4	68,7	7	17,4	1,1	2	-0,5	8,2	0,3			0,1	-0,3	1	0,1
17	P1188	45,8	58,6	45,3	62	44,1	58,2	44,5	56,3	21,7	27,6	-16,7	-6,5	0,2	0,8	8,8	3
18	P6460	20,7	60,4	20,6	74,6	8,3	12,8	9,2	13,2	9,5	21,3	5,7	-5,5	1	0,4	-4,3	4,1
19	P1615	83	26,8	82,8	36,5	80,1	25,9	64,6	13,4	75,6	21,6	-3,2	48,6	1	2,8	1,8	-0,1
20	P9391	69,7	20,8	68,7	54,4	50,3	25,9	32,2	7,4	33,9	1,1	-5,8	-0,9	-0,7	-0,3	2,8	0,3
21	P2577	76,3	47,8	51,8	51,6	35,7	38,4	10,9	16,4	0,6	-2	-0,5	-5,4	0,1	-0,7	0,6	0,3
22	P781	51,5	40,4	11,4	11,5	47	39,4	11,9	12,9	18,7	18,6	-11,7	-10,5	-2,8	-3,6	27,9	24,4
23	P6124	46,2	26,1	14,6	8,7	40,1	34,4	39,2	18,9	44,6	13,7	3,6	10	0,7	-0,7	1,7	0,4
24	P2446	94,9	31,9	86,7	56,7	18,3	16,4	6,6	8,1	93,7	18,3	2,7	-1,8	0,1	-0,3	48,1	40,3
25	P711	12,8	48,1	2,5	3,4	2,3	4	2,4	7,8	2,8	-2,5	-5,2	-3	-1,5	0,7	3,6	5
26	P8805	76,3	4,8	60,3	24,3	34,6	11,8	22,6	3,3	16,2	0,5	-0,3	5,9	0	0,1	0,6	0
27	P2548	74,1	25,3	68,6	46,3	57,7	38,5	55,3	21,9	68,2	12	1,1	3,8	-0,3	1	4	1,8
28	P5017	64,9	2,1	50,8	6,7	53,8	8,1	5,3	0,2	55,4	0,1	2,9	-0,2	0,6	0	0,5	-0,2
29	P4383	74	82,6	6,7	4,8	28	25,3	52,1	44,9	37,4	16,4	-2,3	2,6	0,4	-0,1	2,5	2,3
30	P1055	51,1	59,5	12,7	18,1	47,5	59,7	35	42,5	10,6	35,5	-6,9	3,4	3,2	2,7	9,2	8,9
31	P1894	78,5	47,8	22,8	16,8	7,3	6,3	46,5	28,4	46,3	29,6	-0,1	27,6	5,2	10,9	11	7,9
32	P4699	80,4	19,1	16,5	2,2	13,6	3,3	9,6	2,8	64,4	6,1	9,6	0,8	11	1,5	5,8	1,4
33	P2685	36	3,1	15,5	20,1	5,7	7,7	23,8	15	3,8	4			3	5,2	6,5	9,7
34	P2329	71,8	1,8	1,7	0	21,3	2,2	12,1	1,8	3,9	0,5			0,4	1,5	0,2	0,5

1	A/A	CpG/CD25	CpG/CD86	PAM/CD25	PAM/CD86	MALP/CD25	MALP/CD86	MDP/CD25	MDP/CD86	I/CD25	I/CD86	LOX/CD25	LOX/CD86	ORN/CD25	ORN/CD86	LPS/CD25	LPS/CD86
2	P3551	61,1	76,3	19,7	14,5	1,3	0,8	33,1	32,2	23,1	10,3	-4,6	-1,4	1,4	0,6	6,4	3
3	P1626	62,6	37,1	58,1	39,6	34,3	28,8	21,5	12,7	4,7	4,7			0,2	1,1	1,3	-2,4
4	P103	71,2	69,9	66	73,7	35,8	38,9	46,1	50,9	2,1	3	-8,4	-2,8	1	-0,1	3,1	2,5
5	P6520	31,4	60,5	27,6	56,4	28,5	56,5	20,6	52,6	12,8	30,6	-9,2	5,4	-0,4	-1,2	1,3	4,9
6	P3916	70,6	53	57,6	52,4	44	43,8	22,5	29,5	12,4	4,2	-4,4	-4,7	-1,4	-3,2	-1,7	-3,2
7	P3020	49,6	36,7	59,8	49,5	44,7	44,3	35,7	30,7	9,6	29,9	-12,8	-17,7	-1,7	-1,8	0,5	3,6
8	P511	62,6	15,3	55,7	49,4	26,8	30,1	24,3	16,1	23,2	5,9	-0,3	3,2	-0,1	2,7	0,3	2,5
9	P7155	62,2	14,9	65	17,5	55,6	14,6	54,5	6,7	54,8	4,2			1,6	0,1	2,2	0,1
10	P3870	55,9	4,3	10,5	0,7	40,3	1,1	43	0,1	46,8	0,6	4,3	0	2,2	-0,2	2,3	-0,1
11	P104	91,6	11,3	89,4	46,3	32	5	72,1	2,9	88,4	10,7	2	-1,1	-0,3	-2,6	0,8	-1,3
12	P6068	31,8	0,6	4,1	0,4	0,4	1,1	-1,4	-1,1	-0,8	2,4			-0,2	2,2	-0,1	2,6
13	P1618	48,9	40,3	69,5	58,5	16,8	13	31,4	27,7	21,3	6,5			1,6	0,8	1,3	0,3
14	P5949	59,9	40,6	65,2	45,9	30,1	15,5	26	11,9	10,3	2,4			1,7	0,4	1,9	0,7
15	P280	84	32,8	34,6	22,3	1,9	1	6,2	1,9	1,3	0,8			0,1	0	0,3	-0,2
16	P4875	90,5	8,4	68,7	7	17,4	1,1	2	-0,5	8,2	0,3			0,1	-0,3	1	0,1
17	P1188	45,8	58,6	45,3	62	44,1	58,2	44,5	56,3	21,7	27,6	-16,7	-6,5	0,2	0,8	8,8	3
18	P6460	20,7	60,4	20,6	74,6	8,3	12,8	9,2	13,2	9,5	21,3	5,7	-5,5	1	0,4	-4,3	4,1
19	P1615	83	26,8	82,8	36,5	80,1	25,9	64,6	13,4	75,6	21,6	-3,2	48,6	1	2,8	1,8	-0,1
20	P9391	69,7	20,8	68,7	54,4	50,3	25,9	32,2	7,4	33,9	1,1	-5,8	-0,9	-0,7	-0,3	2,8	0,3
21	P2577	76,3	47,8	51,8	51,6	35,7	38,4	10,9	16,4	0,6	-2	-0,5	-5,4	0,1	-0,7	0,6	0,3
22	P781	51,5	40,4	11,4	11,5	47	39,4	11,9	12,9	18,7	18,6	-11,7	-10,5	-2,8	-3,6	27,9	24,4
23	P6124	46,2	26,1	14,6	8,7	40,1	34,4	39,2	18,9	44,6	13,7	3,6	10	0,7	-0,7	1,7	0,4
24	P2446	94,9	31,9	86,7	56,7	18,3	16,4	6,6	8,1	93,7	18,3	2,7	-1,8	0,1	-0,3	48,1	40,3
25	P711	12,8	48,1	2,5	3,4	2,3	4	2,4	7,8	2,8	-2,5	-5,2	-3	-1,5	0,7	3,6	5
26	P8805	76,3	4,8	60,3	24,3	34,6	11,8	22,6	3,3	16,2	0,5	-0,3	5,9	0	0,1	0,6	0
27	P2548	74,1	25,3	68,6	46,3	57,7	38,5	55,3	21,9	68,2	12	1,1	3,8	-0,3	1	4	1,8
28	P5017	64,9	2,1	50,8	6,7	53,8	8,1	5,3	0,2	55,4	0,1	2,9	-0,2	0,6	0	0,5	-0,2
29	P4383	74	82,6	6,7	4,8	28	25,3	52,1	44,9	37,4	16,4	-2,3	2,6	0,4	-0,1	2,5	2,3
30	P1055	51,1	59,5	12,7	18,1	47,5	59,7	35	42,5	10,6	35,5	-6,9	3,4	3,2	2,7	9,2	8,9
31	P1894	78,5	47,8	22,8	16,8	7,3	6,3	46,5	28,4	46,3	29,6	-0,1	27,6	5,2	10,9	11	7,9
32	P4699	80,4	19,1	16,5	2,2	13,6	3,3	9,6	2,8	64,4	6,1	9,6	0,8	11	1,5	5,8	1,4
33	P2685	36	3,1	15,5	20,1	5,7	7,7	23,8	15	3,8	4			3	5,2	6,5	9,7
34	P2329	71,8	1,8	1,7	0	21,3	2,2	12,1	1,8	3,9	0,5			0,4	1,5	0,2	0,5

ΠΕΡΙΛΗΨΗ

Η χρόνια λεμφοκυτταρική λευχαιμία (ΧΛΛ) είναι μια αιματολογική κακοήθεια που χαρακτηρίζεται από την in vivo συσσώρευση CD5+ μονοκλωνικών Β λεμφοκυττάρων στο περιφερικό αίμα, στο μυελό των οστών και στο λεμφικό ιστό. Μέχρι σχετικά πρόσφατα θεωρούνταν ως μια ομοιογενής πάθηση ανώριμων Β λεμφοκυττάρων που αθροίζονται λόγω βλάβης μηχανισμών της απόπτωσης(76). Μέσα στις δυο τελευταίες δεκαετίες ωστόσο η αντίληψή μας για την παθογένεια της ΧΛΛ έχει πλήρως αναθεωρηθεί. Φαίνεται πως συνδυασμός τόσο γενετικών συμβαμάτων όσο και ερεθισμάτων από το μικροπεριβάλλον επηρεάζουν σημαντικά την εμφάνιση και πρόοδο της νόσου, η οποία είναι πολύ περισσότερο ετερογενής από ό,τι αρχικά θεωρούνταν (77).
Αρκετά δεδομένα υποστηρίζουν ότι ο Β κυτταρικός υποδοχέας παίζει κεντρικό ρόλο στη φυσική ιστορία της νόσου και μοριακά χαρακτηριστικά του θεωρούνται πλέον σημαντικοί ανεξάρτητοι προγνωστικοί παράγοντες για την πορεία και έκβασή της. Σε τουλάχιστον 50% των περιπτώσεων ΧΛΛ τα λευχαιμικά κύτταρα φέρουν σωματικές μεταλλάξεις των IGHV γονιδίων και αυτό γενικά συσχετίζεται με καλύτερη πρόγνωση. Αντιθέτως, «μη μεταλλαγμένες» περιπτώσεις ΧΛΛ αναμένονται να ακολουθήσουν πιο επιθετική πορεία. Επιπλέον, το ρεπερτόριο των ανοσοσφαιρινών που απαντώνται στα Β λευχαιμικά κύτταρα από ασθενείς με ΧΛΛ παρουσιάζει μια προτίμηση σε επιλεγμένα IGHV γονίδια σε σύγκριση με το φυσιολογικό ρεπερτόριο των Β λεμφοκυττάρων (94-97). Πρόσφατα, αρκετές ερευνητικές ομάδες ανακοίνωσαν την ύπαρξη υποομάδων περιπτώσεων ΧΛΛ (υποσύνολα) που χαρακτηρίζονται από ξεχωριστές αναδιατάξεις βαρέων και ελαφρών αλύσων ανοσοσφαιρινών, οι οποίες καταλήγουν στη δημιουργία αξιοσημείωτα ομόλογων («στερεότυπων») CDR3 αλληλουχιών στους Β κυτταρικούς υποδοχείς τους (98-102). Τέτοια εντυπωσιακή ομοιότητα Β κυτταρικών υποδοχέων σε ανεξάρτητες περιπτώσεις ΧΛΛ υποδεικνύει την ύπαρξη περιορισμένου αριθμού αντιγόνων ή δομικά σχετιζόμενων επιτόπων που παίζουν ρόλο στη λευχαιμογένεση. Επιπρόσθετη απόδειξη για αυτήν την υπόθεση αποτελεί

η ανάδειξη στερεότυπης σωματικής υπερμεταλλαξιγένεσης σε αυτά ακριβώς τα υποσύνολα ΧΛΛ [103].

Μέχρι τώρα έχουν ταυτοποιηθεί περισσότερα από 150 υποσύνολα ΧΛΛ. Πρόσφατες έρευνες αναφέρουν ότι περίπου 30% των ασθενών με ΧΛΛ φέρουν στερεότυπους Β κυτταρικούς υποδοχείς [102-104]. Επιπλέον, περιπτώσεις ΧΛΛ με ταυτόσημους, στερεότυπους Β κυτταρικούς υποδοχείς έχει δειχθεί ότι παρουσιάζουν και κοινά κλινικά χαρακτηριστικά, ούτως ώστε συγκεκριμένα υποσύνολα έχουν πλέον συσχετιστεί με συγκεκριμένη πρόγνωση [98,102,105-106]. Συνολικά λοιπόν, διέγερση των Β λεμφοκυττάρων μέσω του Β κυτταρικού υποδοχέα φαίνεται να παίζει κρίσιμο ρόλο στην παθογένεση της ΧΛΛ. Συνεπώς, θα είχε μεγάλη σημασία να διευκρινιστούν τα εμπλεκόμενα αντιγονικά στοιχεία.

Τα λευχαιμικά κύτταρα στη ΧΛΛ συχνά εκφράζουν Β κυτταρικούς υποδοχείς που προσδένουν αυτοαντιγόνα με πολυαντιδραστικό τρόπο. Επίσης, έχουν βρεθεί να προσδένουν νεοαντιγόνα, καθώς και αλλοαντιγόνα [110-113,116-117]. Περίπου 80% των «αμετάλλακτων» και ~15% των «μεταλλαγμένων» Β κυτταρικών υποδοχέων στη ΧΛΛ έχουν βρεθεί να αντιδρούν in vitro με μια σειρά από αυτο- και αλλοαντιγόνα, όπως επίσης και με κύτταρα που έχουν καταστεί διαπερατά, με πολυαντιδραστικό τρόπο [118]. Αξίζει να αναφερθεί ότι τα περισσότερα ανασυνδυασμένα μονοκλωνικά αντισώματα από «αμετάλλακτα» ΧΛΛ κύτταρα που δεν εμφάνιζαν πολυαντιδραστικότητα *per se* την απέκτησαν όταν η αλληλουχία τους αναστράφηκε στην αρχική, πριν την εισαγωγή σωματικών μεταλλάξεων [110].. Συνοψίζοντας τα παραπάνω προκύπτει η υπόθεση ότι τα ΧΛΛ κύτταρα μπορεί να προέρχονται από έναν πληθυσμό αυτό/πολυαντιδραστικών Β λεμφοκυττάρων.

Οι Toll-like υποδοχείς (TLR) αναγνωρίζουν μια σειρά από διακριτά μοριακά μοτίβα και είναι γνωστό ότι γεφυρώνουν έμφυτη και προσαρμοστική ανοσία. Τα ανθρώπινα ανώριμα Β λεμφοκύτταρα εκφράζουν χαμηλά έως μη ανιχνεύσιμα επίπεδα όλων των TLR, ωστόσο η διέγερση του Β κυτταρικού υποδοχέα οδηγεί σε ταχύτατη έκφραση των TLR2, TLR6, TLR7, TLR9 and TLR10 [56]. Αντιθέτως, τα $CD5^+$ Β λεμφοκύτταρα μνήμης εκφράζουν ιδιοσυστασιακά αυτούς τους TLR[55-56]. Πρόσφατα διατυπώθηκε η πρόταση ότι η διέγερση των TLR λειτουργεί ως απευθείας σήμα που ενισχύει την απάντηση των ανώριμων Β λεμφοκυττάρων του ανθρώπου στο αντιγόνο [61,63]. Μια πρωτοποριακή μελέτη από τον Leadbetter και

συν. έδειξε πως συμπλέγματα IgG ανοσοσφαιρίνης και χρωματίνης τα οποία περιέχουν DNA είναι ικανά να ενεργοποιήσουν αυτοαντιδραστικά Β λεμφοκύτταρα έτσι ώστε να παράγουν αντισώματα κατά αυτο-IgG διεγείροντας τον Β κυτταρικό υποδοχέα και τον TLR9 με διαδοχική σειρά [32]. Αυτή η ιδέα επιβεβαιώθηκε περαιτέρω από τον Lau και συν., οι οποίοι έδειξαν ότι συμπλέγματα αυτοαντιγόνων με RNA ενεργοποιούν Β λεμφοκύτταρα διεγείροντας ταυτόχρονα τον Β κυτταρικό υποδοχέα και τον TLR7, υποστηρίζοντας έτσι τον ρόλο των TLR στην κατάργηση της ανοσολογικής ανοχής [71]. Θα ήταν ενδιαφέρον να ελέγξει κανείς αν το μοντέλο αυτό μπορεί να έχει εφαρμογή και στην παθογένεια της ΧΛΛ, τουλάχιστον όσον αφορά τις περιπτώσεις με αυτό/πολυαντιδραστικό Β κυτταρικό υποδοχέα.

Περιορισμένα δεδομένα υπάρχουν αναφορικά με το ποιοι TLR εκφράζονται από τα Β λευχαιμικά κύτταρα στη ΧΛΛ. Μέχρι τώρα, το προφίλ έκφρασής τους φαίνεται να μοιάζει σε εκείνο των Β λεμφοκυττάρων που έχουν έρθει σε επαφή με αντιγόνο. Αρκετές ερευνητικές ομάδες αναφέρουν σταθερή έκφραση των TLR7 και TLR9 στα ΧΛΛ κύτταρα [140-141,144] και οι αντίστοιχοι αγωνιστές δοκιμάζονται ήδη σε κλινικές μελέτες με σκοπό να αυξήσουν την ανοσογονικότητα των λευχαιμικών κυττάρων ενισχύοντας την έκφραση συνδιεγερτικών μορίων και/ή τον ρυθμό πολλαπλασιασμού και συνεπώς την ευαισθησία τους σε κυτταροτοξικές θεραπείες [155,158]. Παρόλα αυτά, η επίδραση της διέγερσης των TLR στα ΧΛΛ κύτταρα παραμένει σχετικά αδιευκρίνιστη. Υπάρχουν εξάλλου μελέτες οι οποίες υποστηρίζουν ότι η διέγερση των TLR, και ειδικά του TLR9, μπορεί να οδηγεί σε διαφορετικά αποτελέσματα ανάλογα με την ύπαρξη ή όχι σωματικών μεταλλάξεων στα IGHV γονίδια [144].

Η παρούσα μελέτη αποσκοπεί στο να διερευνήσει τη λειτουργία των TLR και NLR υποδοχέων σε υποομάδες ΧΛΛ, όπως αυτές ορίζονται από τη χρήση επιλεγμένων IGHV γονιδίων, την ύπαρξη ή όχι σωματικών μεταλλάξεων στα IGHV γονίδια ή τη στερεοτυπία του Β κυτταρικού υποδοχέα. Υπό αυτό το πρίσμα, αναλύσαμε μια σειρά από 67 ασθενείς με ΧΛΛ, επιλεγμένους με προτίμηση για περιπτώσεις με στερεότυπους Β κυτταρικούς υποδοχείς οι οποίες υπάγονται στα υποσύνολα #4 και #1, καθότι τα υποσύνολα αυτά μπορούν να θεωρηθούν πρότυπα «μεταλλαγμένων» περιπτώσεων/καλής πρόγνωσης και «αμετάλλακτων»

περιπτώσεων/κακής πρόγνωσης, αντίστοιχα. Αρνητικά επιλεγμένα, κεκαθαρμένα λευχαιμικά Β λεμφοκύτταρα από κάθε περίπτωση διεγέρθηκαν με ειδικούς προσδέτες για όλους τους TLR που έχουν βρεθεί ως τώρα να εκφράζονται -έστω και σε ελάχιστα επίπεδα- σε ΧΛΛ κύτταρα, καθώς και για τον NOD2 της οικογένειας NLR˙ εν συνεχεία, ελέγχθηκε με κυτταρομετρία ροής η αύξηση της έκφρασης των CD25 και CD86, που αποτελούν δείκτες ενεργοποίησης.
Διαπιστώθηκε ότι ο TLR9 ήταν λειτουργικός σε όλες πρακτικά τις περιπτώσεις (αύξηση της έκφρασης του CD25 και/ή CD86 στο 98.5% των περιπτώσεων μετά από διέγερση με CpG). Επιπλέον, ο TLR1/2, ο TLR2/6 και ο NOD2 βρέθηκαν λειτουργικοί στις περισσότερες περιπτώσεις (86.6%, 89.6% και 86.6%, αντίστοιχα) και μάλιστα διαπιστώθηκε ισχυρή συσχέτιση της λειτουργικότητάς τους. Πάνω από δύο τρίτα των περιπτώσεων εμφάνιζε λειτουργικότητα και των τριών αυτών υποδοχέων ταυτόχρονα. Δεδομένου ότι οι TLR1/2 και TLR2/6 αναγνωρίζουν τριακετυλιωμένες και διακετυλιωμένες βακτηριακές λιποπρωτεΐνες, αντίστοιχα, ο δε NOD2 αναγνωρίζει MDP το οποίο ανευρίσκεται τόσο σε Gram+ όσο και σε Gram- βακτήρια, θα μπορούσε κανείς να υποθέσει ότι αυτοί οι τρεις υποδοχείς σηματοδοτούν μαζί κάτω από την επίδραση κοινού βακτηριακού αντιγόνου σε συγκεκριμένες περιπτώσεις ΧΛΛ. Ο TLR7 βρέθηκε λειτουργικός σε λιγότερες περιπτώσεις (61.2%), και μόνο όταν διεγέρθηκε με το συνθετικό του αγωνιστή imiquimod. Ο φυσικός προσδέτης του (loxoribine) δεν είχε πρακτικά καμία επίδραση στην έκφραση των CD25 και CD86. Τέλος, ο TLR4 και ο TLR8 δε βρέθηκαν λειτουργικοί σε καμία περίπτωση.
Στη συνέχεια διερευνήσαμε την ύπαρξη πιθανών συσχετίσεων μεταξύ των προφίλ TLR/NLR λειτουργικότητας και μοριακών χαρακτηριστικών του Β κυτταρικού υποδοχέα, δηλαδή χρήση επιλεγμένων IGHV γονιδίων, ύπαρξη ή όχι σωματικών μεταλλάξεων στα IGHV γονίδια και παρουσία στερεοτυπίας. Η πρώτη σύγκριση αφορούσε «μεταλλαγμένες» και «αμετάλλακτες» περιπτώσεις ΧΛΛ. Διέγερση του TLR9 φάνηκε να έχει ισχυρή επίδραση στην αύξηση της έκφρασης του CD86 στις «μεταλλαγμένες» περιπτώσεις ΧΛΛ, ενώ δεν είχε παρόμοιο αποτέλεσμα στις «αμετάλλακτες» περιπτώσεις. Αντιθέτως, διέγερση του TLR7 με imiquimod είχε ως αποτέλεσμα την έντονη αύξηση της έκφρασης του CD25 στις «αμετάλλακτες», αλλά όχι στις «μεταλλαγμένες» περιπτώσεις ΧΛΛ.

Κατόπιν εστιάσαμε στα υποσύνολα #4 και #1. Συγκρίνοντας περιπτώσεις του υποσυνόλου #4 με όλες τις άλλες περιπτώσεις που έφεραν μεταλλαγμένα IGHV γονίδια, καθώς και με τις περιπτώσεις που έφεραν το IGHV4-34 γονίδιο σε μη-στερεότυπες αναδιατάξεις, διαπιστώσαμε ότι η διέγερση των TLR1/2, TLR2/6 και NOD2 έχει πολύ ισχυρότερη επίδραση όσον αφορά την αύξηση του CD25 ή/και του CD86 στο υποσύνολο #4. Επιπλέον, 6/7 περιπτώσεις από το υποσύνολο αυτό ανταποκρίνονταν λειτουργικά και στους τρεις συνδέτες - Pam3CSK4, MALP-2 και MDP. Θα μπορούσε λοιπόν κανείς να υποθέσει ότι αυτό το μοτίβο TLR/NLR λειτουργικότητας στο υποσύνολο #4 αντιστοιχεί σε κάποιον κοινό αντιγονικό ερεθισμό. Όταν συγκρίναμε το υποσύνολο #4 με το υποσύνολο #1, διαπιστώσαμε ότι η διέγερση των TLR9, TLR1/2, TLR 2/6 και NOD2 δεν είχε καμιά επίδραση όσον αφορά την έκφραση του CD86 στο υποσύνολο #1. Τουναντίον, η διέγερση του TLR7 αύξησε σημαντικά την έκφραση του CD25, σε έντονη αντίθεση με το υποσύνολο #4. Βάσει αυτών θα μπορούσε κανείς να υποθέσει ότι πίσω από αυτά τα ξεχωριστά προφίλ λειτουργικότητας των TLR/NLR στα υποσύνολα #4 και #1 κρύβονται διαφορετικοί παθογόνοι μικροοργανισμοί.

Όλα τα παραπάνω μπορούν να θεωρηθούν ως επιπρόσθετη απόδειξη του πόσο ετερογενής είναι η ΧΛΛ, τόσο σε μοριακό όσο και σε λειτουργικό επίπεδο. Θα μπορούσε άραγε η σηματοδότηση μέσω των TLR/NLR να λειτουργεί ως μη-ειδική διέγερση για τα ΧΛΛ κύτταρα, και αν ναι, πόσο μη-ειδική είναι; Διαφορετικά προφίλ TLR/NLR λειτουργικότητας μεταξύ υποσυνόλων πιθανώς αντιστοιχούν σε διαφορετικά αντιγονικά στοιχεία και ενδέχεται να χρήζουν διαφορετικής θεραπευτικής προσέγγισης όσον αφορά TLR αγωνιστές ή ανταγωνιστές. Άλλα καταληκτικά σημεία της TLR/NLR σηματοδότησης, όπως απόπτωση και πολλαπλασιασμός, χρειάζεται επίσης να διερευνηθούν προκειμένου να κατανοήσουμε καλύτερα πώς λειτουργούν αυτά τα μονοπάτια στη ΧΛΛ και πώς μπορούν να αξιοποιηθούν από θεραπευτική σκοπιά.