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B lymphoid malignancies: insights from mouse models

Coordinator: Prof. Andrea Biondi
Tutor: Prof. Paolo Ghia

Dr. Giorgia Simonetti
Matr. No. 725273

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INTRODUCTION

1.1 B cell development

B lymphopoiesis takes place in specialized microenvironments, including the fetal liver and spleen during embryonic life and the bone marrow after birth and throughout life[1]. B cell development in the bone marrow is a multistep process involving different B cell precursors which finally differentiate into mature, antigen-reactive B lymphocytes. All developmental stages, from the progenitors to the mature B lymphocytes, share the expression of the specific surface marker CD19, that appears before the organization of the precursor-B cell receptor (pre-BCR) and decreases only on plasma cells, both in humans and mice[2]. In addition, within the bone marrow, B cell precursors express CD10 which is then lost on mature B cells[3]. The definition of the intermediate stages of B cell differentiation in both mouse and human bone marrow is based on the differential expression of some surface and intracellular proteins as well as the analyses of the rearrangement of the immunoglobulin (IG) heavy (H) and light (L) chain genes. In particular, the rearrangement of the IGH genes is one of the first events occurring along the B cell differentiation pathway, that is dependent for its progression on cell-cell interactions, hematopoietic growth factors and stimulation through the pre-B and B cell antigen receptor[4].

Progenitor-B (Pro-B) cells originated from the hematopoietic stem cells (HSC) are considered to be the first cells already committed to the B cell lineage, where the IGH genes recombination process is actually initiated. The pro-B cell stage is characterized by effective production of the H chain that associates with a surrogate light chain (SLC), giving rise to a pre-BCR expressing pre-B cell. Subsequent rearrangement of the IGL chain locus leads to the surface expression of a complete IgM molecule that marks the immature B cell stage. Cells that did not succeed in BCR formation and immature cells that react strongly to autoantigens are eliminated through apoptosis. On the contrary, B cells expressing a functional and non-self-reactive IgM finally differentiate into mature B lymphocytes. The last developmental stage consists in the acquisition of a surface IgD molecule, with the same specificity of IgM, resulting in double positive $IgM^{+}IgD^{+}$ cells, also called *naïve* B cells as they have not encountered an antigen yet.

Although cells at the pro-B and pre-B stages are found predominantly in embryonic liver and bone marrow, they have also been observed at low frequencies in the fetal lung and omentum[5]. The hypothesis that during embryonic development B lymphopoiesis may occur outside of the major hemopoietic tissues is sustained by evidences that B-1 cells in the mouse arise from progenitors located in fetal omentum[6] (paragraph 1.2.1). In addition, it is well established that in the mouse the transition from immature to mature B lymphocytes takes place in the spleen[7], while in human it is yet to be determined.

Mature *naïve* B cells circulating in the peripheral blood may be exposed to antigens, that drive their activation and their homing to primary B cell follicles in the spleen and other secondary lymphoid organs such as lymph nodes, where they establish germinal centres (GCs) (Figure 1). The *naïve* IgM^+IgD^+ B cells that constitute the primary B cell follicle are located to the outside of the follicle, where they form a mantle zone around the GC.

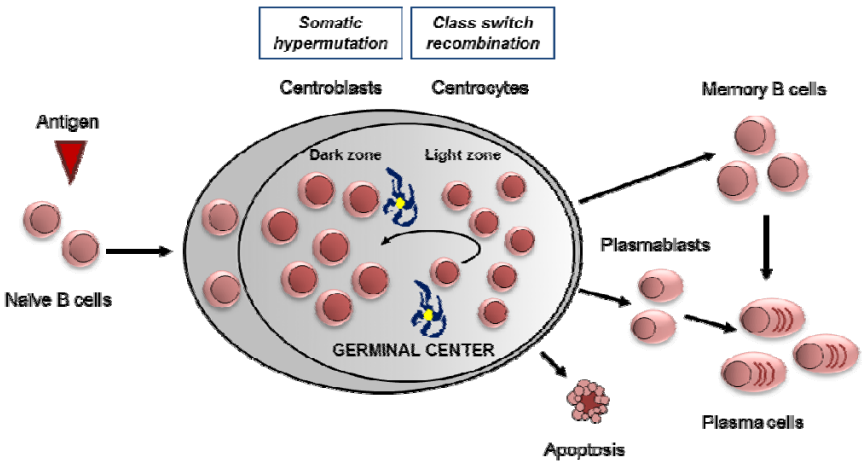

FIGURE 1. **B cell differentiation in the GC.**

The GC consists into a dark zone and a light zone. The dark zone is populated by proliferating centroblasts that have acquired all the features of GC B lymphocytes: they express peculiar surface markers including CD10 and CD71; they show reduced levels of CD5, CD23 and CD44 that typically mark follicular mantle B cells; they express Fas/CD95, but low B cell

CLL/lymphoma 2 (BCL-2) protein; they undergo spontaneous apoptosis during culture; about 30% of these cells express the proliferation associated nuclear antigen Ki-67[8,9]. Centroblasts undergo the process of somatic hypermutation (SHM), leading to the randomised introduction of mutations into the IG genes thereby modifying the antigen binding site of the molecule. Most mutations are disadvantageous for the cells and drive them to apoptosis. Only GC B cells synthesizing high-affinity antibodies are positively selected in the light zone, where they form a population of resting centrocytes that is in close contact with follicular dendritic cells (FDCs). A fraction of centrocytes undergoes the class swith recombination (CSR) process that changes the constant (C) region of the IGH chain, from IgM to IgG, IgA or less commonly IgE. The processes of SHM,CSR and clonal expansion of B lymphocytes are induced by the cooperation with activated T lymphocytes that express CD40-ligand (CD40L) and secrete interleukin (IL)-4 and IL-10 after antigen specific recognition[10]. Finally, GC B cells may repeat another round (or repeated rounds) of proliferation, hypermutation and selection or differentiate into plasma cells through the plasmablastic stage or into memory B cells and leave the GC. Memory B cells can eventually differentiate into plasma cells upon secondary antigen exposure.

1.2 B cell populations in the mouse

1.2.1 From one to three B cell lineages

Different B cell populations can be distinguished in mice based on anatomical localization, surface phenotype and ontogeny[11], namely B-1 and B-2 cells. B-1 cells are $B220^{low}IgM^{high}IgD^{low}CD23^{-}$ lymphocytes, divided into B-1a ($CD5^{+}$) and B-1b ($CD5^{-}$). The B-2 subset includes marginal zone (MZ) and follicular (FO) B cells. Two theories have been developed to explain B-1 and B-2 cell origin. The "activation model" focuses on the role played by the BCR signalling intensity, resulting from the integration of receptor cross-linking, BCR accessory signals, non-BCR signals and cytokine/chemokine receptor stimuli for driving B cell commitment[12]. Otherwise the "lineage model" supports the existence of two separate lineages deriving from distinct hematopoietic progenitors.

B-1 lymphocytes develop early in ontogeny: embryonic day 9 yolk sac and intra-embryonic homogenic endothelium generate progenitors for the B-1 lineage[13]. B-1 cell progenitors reside in fetal omentum and liver starting from day 13 and 14 of fetal life and differentiate into B-1a and B-1b cells. B-1a cells appear around day 16 of fetal life and they predominate in the total B cell pool at the time of birth both in the spleen and in the peritoneal cavity. Their frequency then decreases in adult mice, but often increases again in aging mice[14] (paragraph 1.5.1). B-

1b cells become detectable at the same time of the B-1a subset or shortly after and represent only a small proportion of the B-1 population. In the adult B-1a and B-1b cells predominate in peritoneal and pleural cavities, they variably make up about 5% of splenic B lymphocytes and they are extremely rare, if present at all, in lymph nodes and Peyer's patches.

B-1 cells are characterized by a limited diversity in the IG repertoire, which results from minimal expression of Terminal deoxynucleotide transferase (TdT) in early B cell progenitors[15]. TdT activity leads to insertion of non-coded nucleotides (N) during IG gene rearrangement. Cells that express very low levels of the protein, show rare N-region sequences at the IGH variable(V)-diversity(D) and IGHD-joining(J) junctions and are prone to assume a B-1 phenotype. B-1 cell differentiation from precursors terminates somewhere between 3 and 6 weeks of age, when the presence of a mature B-1 population is sufficient to trigger a feedback mechanism and prevent *de novo* B-1 cell maturation. The B-1 subset persists throughout life thanks to self-replenishment (division of fully mature B-1 cells), that may be sustained by constitutively activated signal transducer and activator of transcription 3 (STAT3). This nuclear factor regulates Cyclin D2 expression that contributes to lymphocytes proliferation[16]. B-1 cell self-renewal capacity has been proved by reconstitution experiments showing that mature peritoneal B-1 cells completely and permanently reconstitute themselves but not the B-2 cell population in irradiated mice[17]. Besides, splenic B-1 cells can also propagate themselves, when transplanted

into immunodeficient recipient mice, while B-2 cells cannot[18]. Adoptive transfer experiments revealed that B-1 cell homing to the peritoneal cavity is driven by Chemokine C-X-C motif ligand 13 (CXCL13), a chemokine produced by cells in the omentum and by peritoneal macrophages[19], and is characterized by upregulation of the marker CD11b on the surface of B-1 cells[20].

Both B-1a and B-1b cells maintain their numbers by self-replenishment though they have also the ability to develop from progenitors in adult bone marrow, as demonstrated for B-1b cells first[21] and for B-1a cells more recently[22]. Indeed by inducing Recombination-activating gene 1 (RAG1) expression *in vivo* in *Rag1*-targeted mice, that lack mature B lymphocytes, *Duber et al.* observed that the adult bone marrow can generate both B-1a and B-1b cell subsets, together with FO and MZ B cells. Unlike fetal liver-derived B-1a cells, bone marrow-derived B-1a cells express IG with abundant N-region, reminiscent of B-2 cells[23].

However, the bulk of B-1a cells seems to be mainy generated during the fetal life, as suggested by the identification of phenotypically and temporally distinct bone marrow progenitors[24]. *Montecino-Rodriguez et al.* showed that $B220^{+}CD19^{-}$ pre-pro-B and $B220^{+}CD19^{+}$ pro-B cells from adult bone marrow mainly repopulate B-2 lymphocytes in bone marrow and spleen of SCID mice. $B220^{+}CD19^{+}$ cells also repopulated the peritoneal cavity with B-1 cells. Otherwise $B220^{low\text{-}neg}CD19^{+}$ B cells reconstituted peritoneal B-1 cells, with a bias toward $CD11b^{+}CD5^{+}$ B-1a cells, when derived from fetal

bone marrow, and toward $CD11b^{+}CD5^{-}$ B-1b cells, when originated from adult bone marrow. The analysis of CD138 expression in the early-stage of B cell development confirmed the differential representation of adult and neonatal B lineage progenitors[25]. Indeed $CD138^{high}$ pre-B cells are enriched in the adult B-2 progenitor subset, while $CD138^{-}$ cells constitute about 90% of the neonatal pool that gives rise to B-1a lymphocytes and $CD138^{int}$ progenitors compose 50% of the adult subset that predominantly originate B-1b cells.

This findings lead to the conclusion that three separated B cell lineages exist in mice, but do not exclude that the BCR signalling strength may be involved in the maturation of the different progenitor cells. Multiple lines of evidence indicate that a stronger BCR signalling is required to generate B-1 cells compared to B-2 cells. Accordingly, mice carrying a mutation in the Bruton agammaglobulinemia tyrosine kinase (*Btk)* gene or lacking Protein kinase C beta (PKCβ) or delta (PKCγ) or other positive regulators of the BCR signalling, like B cell linker protein (BLNK) and VAV1 have a decreased number of B-1 cells[26] and BCR-deficient mice, in which B cell survival is sustained by the EBV protein LMP2A, fail to develop B-1 cells[27]. On the contrary, mice deficient for negative regulators such as protein tyrosine phosphatase (SHP-1), LYN and Sialic acid-binding immunoglobulin-like lectin G (SIGLEC-G), show an expansion of B-1 cells due to the inability to dampen the cell response to BCR stimulation[28,26,29].

1.2.2 Marginal zone and follicular B cell development

B-2 cells, also called “conventional” B cells, develop later in ontogeny from progenitors located in fetal liver and in the adult bone marrow and spleen that may both generate B-2 cells when transferred into irradiated or immunodeficient mice[30,18]. B-2 cells become detectable during the post-natal period and are preserved throughout life by *de novo* differentiation of HSC in the bone marrow. B cell lymphopoiesis in the bone marrow is strictly related to stromal cells.

After becoming independent of stromal factors, sIgM-expressing B cells enter the blood stream and are passively transported to the spleen. In addition to high levels of sIgM, B cells that have recently emerged from the bone marrow express very low levels of CD21 and have yet to acquire IgD and CD23 markers. This phenotype identifies cells at the transitional type 1 (T1) stage that are drawn into follicles following CXCL13 chemokine gradient. In the secondary lymphoid organs T1 cells mature into transitional type 2 follicular precursors (T2-FP) cells ($IgM^{high}IgD^{high}CD21^{int}CD23^{+}$). T2-FP cells persist in primary follicles of secondary lymphoid organs probably because they are neither eliminated by self-tolerance mechanisms nor possess the BCR specificity to complete the differentiation pathway. Splenic follicles also hold transitional type 2 marginal zone precursors (T2-MZP) cells, defined as cycling precursors of MZ B cells, differing from T2-FP cells for higher levels of CD21 and CD23 expression[31]. Adequate BCR signals together

with proper stromal microenvironment and the ability to respond to locally produced growth factors are required for the development of T1 into T2 cells and further into mature B-2 lymphocytes[32].

The late phases of differentiation take place in the spleen: B cells gather in the primary follicles and externally in the MZ, giving rise to the FO ($IgM^{low}IgD^{high}CD21^{+}CD23^{+}$) and the MZ ($IgM^{high}IgD^{low}CD21^{high}CD23^{-}$) B cell pools respectively (Figure 2). BCR specificity, NOTCH2 and Nuclear factor κB (NF-κB) signalling determine for FO or MZ B cell fate specification. In particular, it was observed that relatively strong BCR signals commit peripheral B cells preferentially to a FO B cell fate, whereas weak BCR signals associated with low affinity for self-structures appear to be permissive for MZ B cell development[31].

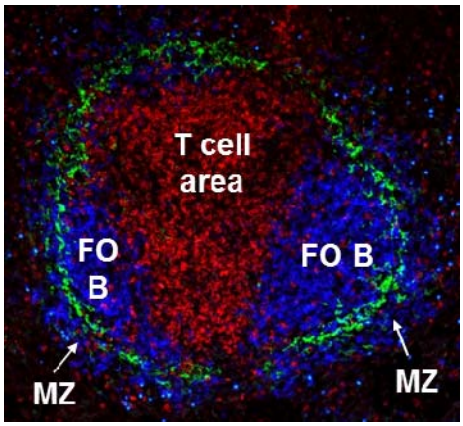

FIGURE 2. **Localization of FO and MZ B cells in the splenic primary follicles.**

1.2.3 Inside the specificities of the B cell lineages

During the early phases of infection and before specific antibodies production, “natural” (or polyreactive) antibodies limit the dissemination of pathogens. In mice, B-1a cells are the main source of “natural” antibodies, characterized by low affinity and broad specificities, due to the expression of germline IGHV genes that is a prerequisite for proteins with great flexibility and capacity to fit different antigenic configurations. They mediate a type of T cell-independent response and recognize lipids, polysaccharides and proteins of microrganisms coat antigens and autoantigens such as cellular membrane proteins, IG molecules, DNA or phosphatidylcholine[33]. In addition B-1a lymphocytes are the only defence against encapsulated bacteria and aid in the clearance of senescent cells and degraded proteins or lipids[32,12]. They secrete IgM especially in the spleen and together with B-1b cells contribute to IgA-producing plasma cells in lamina propria of the gut. B-1b cells mainly take part to the adaptive arm of the immunological response. They secrete antigen-specific antibodies upon priming and give rise to long lasting T-cell independent IgM memory against pathogens such as *Streptococcus pneumoniae* and *Borrelia hermsii*[34,35].

B-2 cells are the major player of T-dependent immune responses. They interact with T lymphocytes and produce high affinity and fine specificities antibodies to control and clear virus, fungal and bacterial infections. FO B cells are freely

recirculating lymphocytes involved in adaptive immunity: they respond to protein antigens and undergo IGH class switching and affinity maturation thanks to T cell-help. MZ B cells are sessile mature B cells located in the spleen at the border of the white pulp (Figure 2) and respond without priming to blood-borne pathogens[36]. Along with B-1a lymphocytes, they have been described as cells endowed with "natural memory" thereby providing a bridge between innate and adaptive immune responses. B-1a and MZ B cells may carry out a similar function with defined competences: B-1a lymphocytes may be the mobile arm of the B cell protection system against infection while MZ B cells may be the local defence against pathogens that reach the spleen. MZ B cells participate both in T-dependent as well as in T-independent immune responses, where high levels of CD21 presumably facilitate the efficiently capturing of complement coated polysaccharides [37].

1.3 B lymphoid malignancies

1.3.1The role of genetic events in lymphomagenesis

The beneficial function of the germinal center reaction in the establishment of the immune reponse is counterbalanced by the fact that the vast majority of B cell malignancies arise from GC or post-GC B cells (Table 1). Indeed many types of non-Hodgkin lymphoma show highly mutated IG genes, as an

indication that the cell of origin has undergone SHM. Though evidence suggests that SHM may also take place outside the GC, this specific structure is the preferential place for the process. In addition, some of these tumors as follicular lymphoma (FL) display distinct IG rearrangements in the malignant clone that is a hallmark of ongoing SHM[38].

TABLE 1. **Mature B cell malignancies**

Malignancy	Major genetic abnormalities	SHM	Putative cell of origin
Mantle cell lymphoma	CCND1-IGH	Yes and no	Pre- and post-GC B cell
Chronic lymphocytic leukemia	Deletion 13q14	Yes and no	Antigen-experienced B cell
Burkitt lymphoma	MYC-IGH, MYC-IGL	Yes	GC B cell
Follicular lymphoma	BCL-2-IGH	Yes	GC B cell
Marginal zone lymphoma	Deletion 7q22-36	Yes	GC or post-GC B cell
Diffuse large B cell lymphoma	BCL-6 translocations, BCL-2-IGH, MYC-IGH, MYC-IGL	Yes	GC or post-GC B cell
Hodgkin lymphoma	REL amplifications, IκBα, IκBε, CD95 mutations	Yes	GC or post-GC B cell

The processes of SHM and CSR, together with early VDJ recombination, represent potentially dangerous steps in the development of B lymphocytes since they generate DNA breaks[39,40]. This may partly explain why malignant transformation occurs more frequently in B than in T cells that do not undergo SHM or CSR. Following the DNA breaks, reciprocal chromosomal translocations involving one of the IG

loci and a proto-oncogene induce the deregulated expression of the oncogene in different B cell lymphoma types. An example is the translocation t(14;18) that juxtaposes the *BCL-2* gene to the IGH locus and forces the expression of the anti-apoptotic molecule in centroblasts leading to the disruption of the physiologic apoptotic program of the cells. The BCL-2-IGH translocation occurs at the VDJ recombination stage[41] similarly to the t(8;14) involving the IG locus and *MYC* and occurring in endemic-type Burkitt lymphoma[42]. In this case, ectopic expression of MYC, that is normally absent in GC cells, alters B cell growth and genomic stability.

Other genes may be affected during SHM as in the case of B cell lymphoma 6 (*BCL-6*), a master regulator of the GC response. BCL-6 is constitutively expressed in 50% diffuse large B cell lymphoma (DLBCL) and 10% FL cases as a result of a translocation to the IG loci during SHM or of the introduction of mutations by aberrant SHM. These mutations disrupt the regulatory regions of the proto-oncogene and prevent its downregulation that is required to activate the plasma cell differentiation program[43].

In DLBCL, other genetic lesions including amplifications and deletions disrupt cell homeostasis by affecting cell cycle and cell death regulation and terminal differentiation. Accordingly, *PRDM1*, the gene encoding the plasma cell differentiation factor B lymphocyte-induced maturation protein 1 (BLIMP1), can be inactivated by two-hit mechanism or by epigenetic silencing in activated B cell-like subtype (ABC) DLBCL[44,45]. This aberration

is mutually exclusive with BCL-6 deregulation since they both prevent the terminal differentiation of the cell.

In general, in B cell lymphomas, the upregulation of BCL-2 and NF-κB represent the most commonly deregulated antiapoptotic pathways. Multiple components of the NF-κB pathway are targeted by mutations in B cell malignancies, like ABC DLBCL while Hodgkin lymphoma cells show constitutive activation of the pathway due to the Ebstein-Barr virus (EBV) protein LMP-1 that mimics CD40 stimulation[46]. Constitutive activation of the NF-κB pathway favors not only survival but also proliferation of the malignant clone.

1.3.2 The role of the microenvironment in lymphomagenesis

In addition to the genetic lesions, stimulation through the BCR has an important role during lymphomagenesis. Both tonic and antigen-dependent BCR signals are involved in the pathogenesis of B cell lymphomas. Evidence is provided by *in vivo* studies on the Eμ-MYC transgenic (tg) mouse model[47]. These mice develop lymphoma of B cell precursors. However when crossed with tg mice expressing a BCR specific for hen egg lysozyme (HEL), they preferentially succumb of mature B cell malignancies that display a more aggressive phenotype in presence of the HEL antigen[48]. Antigenic stimulation has a pivotal function in chronic lymphocytic leukemia (CLL) cells as supported by the presence of sterotyped receptors in one third of the cases (paragraph 1.4.4). Chronic BCR-dependent

stimulation seems also to favor the retention of a proliferation and survival program in ABC DLBCL and to be involved in FL pathogenesis as suggested by the presence of tumor cells carrying autoreactive BCR[49] or showing ongoing SHM. In addition, the translocations occurring in the IG loci selectively affect the non-productive rearranged loci in most cases, underlying that a functional BCR is necessary for the malignant cells.

Neoplastic cells also express chemokine receptors like Chemokine CXC motif receptor 4 (CXCR4), Chemokine CXC motif receptor 5 (CXCR5) and Chemokine C-C motif receptor 7 (CCR7) that favor their homing to the lymphoid organs and their further dissemination[50]. The lymphoid tissue is organized by a network of stromal cells that attract cancer cells, “reinforce” them and then stimulate their release. Stromal cells promote survival and proliferation of lymphoma cells by producing interleukins (e.g. IL-6, IL-7, IL-4, IL-8) and expressing adhesion and stimulatory molecules (e.g. Jagged-1, Macrophage inflammatory protein 1 (MIP-1), insulin-like growth factor 1 (IGF-1)). In the lymphoid organs malignant B cells are in contact with other immune cells that contribute to the formation of GCs, including T cells and FDCs. In particular FL cells depend on costimulatory signals delivered by follicular T helper cells for their growth[51]. Malignant cells may take advantage of the presence of regulatory T cells (Treg) that in some cases can suppress tumor-associated reactive lymphocytes, facilitating tumor immune escape[52]. Accordingly, the reduction and

phenotypic alteration of DCs peculiar of NHL contribute to the loss of tumor control. Immune escape may also be favoured by genetic lesions in the malignant cells, such as mutations in the *β2-microglobulin* gene (encoding a protein involved in cell surface expression of MHC class I dimers) that impair recognition of neoplastic cells by cytotoxic T cells[53,54]. Finally, the role of the microenvironment is preminent in Hodgkin lymphoma since the tumor is made by a small fraction of malignant lymphoid cells surrounded by a large infiltrate of T cells, eosinophils, macrophages, B cells, plasma cells[55].

1.4 Chronic Lymphocytic Leukemia

1.4.1 Definition and morphology

CLL is the most frequent adult leukemia in the Western world and accounts for approximately 11% of all hematologic malignancies with an incidence of about 2-6 new cases every 100.000 individuals annually.

CLL originates from the relentless proliferation and accumulation of kinetically resting mature $CD5^+$ B lymphocytes in the peripheral blood, bone marrow, lymph nodes and spleen. The immunophenotype of leukemic cells is characterized by the expression of CD19, low levels of CD20 and high levels of CD5, together with the marker CD23. In addition CLL cells show low levels of surface IG[56], mostly IgM and/or IgD, and restriction of

the κ or λ light chain, indicating that they underwent a monoclonal expansion[57].

Peripheral blood of CLL patients contains a large number of small rounded B lymphocytes that are blocked in the G_0/early G_1 phase of the cell cycle[58]. The expansion of circulating lymphocytes is sustained by the presence of proliferation centres (PCs) in the spleen, lymph nodes and especially in the bone marrow. The PCs, also known as pseudofollicles, are thought to be the CLL proliferative compartment. They appear like nodular areas without mantles, filled with prolymphocytes and para-immunoblasts expressing CD5, Ki-67 and high levels of CD20 and CD23[59], together with the anti-apoptotic protein BCL-2 and Survivin, a member of the family of apoptosis inhibitors[60]. The last two markers may justify the predisposition of PC-generated cells to accumulate. Proliferating lymphocytes are surrounded by newly formed vessels[61] and bystander cells, especially $CD3^+CD4^+$ T lymphocytes. Most of them express CD40L on their surface[62], suggesting that they are in an activated state and they are able to deliver survival stimuli to the neoplastic cells (paragraph 1.4.5).

1.4.2 Genomic aberrations and genetic variation

CLL cells carry several recurrent genomic alterations, detectable by Fluorescent In Situ Hybridization (FISH) in up to 80% of patients. In contrast to other lymphoproliferative disorders, traslocations are very rare, while the most common

abnormalities are deletions or amplifications of specific chromosomal regions. Most aberrations become manifest during the disease course, therefore they are considered to influence the disease progression rather than to be early transforming events. In particular the presence of multiple chromosomal abnormalities in CLL has been associated with poor prognosis[63]. However, cyogenetic lesions have been identified, with variable prevalence, in CLL-like Monoclonal B cell Lymphocytosis (MBL)[64-66], an expansion of $CD5^+$ B lymphocytes in the peripheral blood of otherwise healthy individuals at a concentration of $<5x10^9$/L, that is thought to preceed virtually all CLL[67].

The most frequent aberrations include deletion in the long arm of chromosome 13 (13q14), trisomy of chromosome 12, deletion in chromosome 11 between bands q22 and q23 (11q22-q23) and deletion in bands 17p13.

- 13q14 Deletion is present in more than 50% of CLL patients. A *minimal deleted region (MDR)* has been identified that includes the *deleted in leukemia (DLEU) 2* gene, encoding a noncoding RNA, the first exon of the *DLEU1* sterile gene and the *microrRNA (miR)-15a/16*-1 cluster, located intronic to *DELU2*. Evidence suggests that this locus contains tumor suppressor genes for CLL. The microRNAs has been reported to negatively regulate the expression of BCL-2[68,69] and the *DLEU2/miR-15a/16-1* cluster has been shown to control B cell proliferation by the downregulation of genes promoting cell cycle entry[70].

A larger deletion is frequently observed in CLL, namely *common deleted region (CDR)* that comprises the *DLEU1, DLEU7 and RNASEH2B* genes in addition to the *DLEU2/MiR-15a/16-1* cluster. DLEU7 expression was shown to be downregulated in CLL and associated with promoter methylation[71].

- Trisomy 12 was found by FISH analysis in 10-25% of CLL cases and is responsible for the upregulation (not followed by increased protein level) of a number of genes, though so far it is not clear which correlates with the leukemic phenotype: Cyclin-dependent kinase 2 (CDK2), Cyclin-dependent kinase 4 (CDK4), Signal transducer and activator of transcription 6 (STAT6), Apoptotic protease activating factor 1 (APAF-1) and Mouse double minute 2 (MDM-2), that play a role in oncogenesis, cell cycle control and apoptosis[72].
- 11q22-q23 Deletion is detected in 10-20% of the patients and is associated with reduced expression of Ataxia telangectasia mutated (ATM), a tumour suppressor gene involved in the activation of the p53-induced cell death. The deletion results in tumour cells resistance to this apoptotic pathway[73].
- 17p13 Deletion is present in 7-12% of the cases and implies the loss of p53 tumour suppressor gene and the consequent acquisition of a remarkable survival advantage for leukemic cells that can accumulate further genetic defects. At clinical level, this aberration

correlates with reduced survival expectances, due also to resistance to the most common treatments[74].

In addition most cases are characterized by BCL-2 overexpression[75,76] and virtually all cases show expression of T cell leukaemia/lymphoma 1 (TCL1)[77] that is frequently upregulated[78] (see paragraph 1.5.5).

Recently, whole-genome sequencing and exome sequencing analysis have identified new recurrent mutations in CLL, that target the *NOTCH1*, Myeloid differentiation primary response gene 88 (*MYD88*), Splicing factor 3B, subunit 1 (*SF3B1*) and Baaculoviral IAP repeat contining 3 (*BIRC3*) genes and are distributed in a mutually exclusive fashion with *TP53* abnormalities[79]. *NOTCH1* mutations lead to the synthesis of a truncated protein lacking the PEST sequence in 8-12% of CLL cases[80,81]. The frequency of *NOTCH1* mutations increases during disease progression toward Richter syndrome and in chemorefractory CLL[81,82]. In addition *NOTCH1* mutations preferentially associate with trisomy 12[82-84] and with aggressive disease course[81-83]. Mutations in the *MYD88* gene (2.9% of CLL cases) are thought to induce the activation of this novel proto-oncogene and associate with younger age of diagnosis[80]. The gene encoding SF3B1, a subunit of the spiceosomal U2 small nuclear riboprotein (snRNP), is somatically mutated in 9.7% of CLL cases[85] and in 17% of fludarabine-refractory cases[86]. The mutations associate with faster disease progression and poor overall survival[85]. Similarly, deletions and/or inactivating mutations of the *BIRC3* gene are an independent prognostic

factor of poor outcome and are preferentially detected in fludarabine-refractory CLL[79]. According to the role of negative regulator of alternative NF-κB signaling pathway exerted by BIRC3, CLL cases carrying a truncated BIRC3 protein show constitutive activation of NF-κB2.

These abnormalities help the classification of CLL patients and have a prognostic value, but they can be acquired during the course of the disease (e.g *TP53* abnormalities) thus not associating with the genetic predisposition characteristic of CLL. It is well known that CLL shows a strong familial basis[87] and first degree relatives have a ~sevenfold increased risk of developing the disease. This observation prompted the investigation of new risk variants. A genome wide association study identified ten susceptibility loci for CLL[88,89] that have been confirmed by further studies performed on independent patient cohorts[90,91]. These single nucleotide polymorphisms (SNPs) map in regulatory or intronic regions or near to genes like Interferon regulatory factor 4 (*IRF4*), GRAM domain containing 1B (*GRAMD1B*), SP140, Protein kinase D2 (*PRKD2*), BCL-2-like 11 (*BCL2L11*), Interferon regulatory factor 8 (*IRF8*), Neural precursor cell expressed, developmentally downregulated 4 (*NEDD4*) and Methyl-CpG-binding domain protein 1 (*MBD1*) that have been demonstrated to play a role in lymphocytes. Among the identified SNPs, the strongest statistical evidence for an association was found for the allelic variants rs872071 and rs9378805 mapping within the 3' UTR and 10kb centromeric to the 3' UTR of the *IRF4* gene, respectively[88]. The

IRF4 gene is an interesting candidate, since it plays an important function in the terminal differentiation of antigen experienced B lymphocytes. The rs872071 variant was associated with reduced IRF4 expression and aggressive disease course and the second one with younger age at diagnosis. Fine scale mapping of the locus revealed the presence of additional risk variants[92] and some of the previously mentioned SNPs have been associated with MBL risk, suggesting that genetic variation may influence CLL pathogenesis through predisposition to MBL[93].

1.4.3 Prognostic factors

In contrast with the homogeneous phenotype of CLL cells, the clinical course of the disease is very heterogeneous. In some cases, it develops as an indolent disease characterized by stable or slowly increasing lymphocytosis, no or delayed need for therapy and survival up to decades after the initial diagnosis. However, approximately 20% of patients experience an aggressive disease, with progressive lymphocytosis, lymphoadenopathy, splenomegaly, anemia, thrombocytopenia and autoimmune phenomena. These patients need early and frequent treatments and die within few years from diagnosis. The majority of patients have an intermediate clinical course and survival expectancy. This picture clearly indicates that proper management of CLL patients requires prognostic factors

able to predict the disease prognosis already at the time of the initial diagnosis.

The traditional clinical prognostic factors define the extension of the disease and are useful to monitor its progression, but not to predict its evolution. They are: the clinical stage according to Rai[94] or Binet[95], the lymphocyte doubling time and the bone marrow infiltration pattern.

Several biological factors have a prognostic value in CLL and give information about the extension of the leukemic clone: presence of prolymphocytes in the peripheral blood[96], β2-microglobulin levels[97], thymidine kinase and soluble CD23 serum levels[98,99] and p53 expression[100].

In the last decade, new biological markers have been introduced that can predict patients' risk already at the moment of the initial diagnosis and all carry an independent prognostic value: somatic mutations of IGHV genes, surface expression of CD38, Intracellular ζ-associated protein 70 (ZAP70) expression, chromosomal abnormalities and Hematopoietic cell-specific Lyn substrate (HS1) phosphorylation status.

Somatic mutations of IGHV genes

CLL patients can be divided in two groups based on their IGHV gene mutational status: ≥98% identity of the IGHV sequence with the corresponding germline (unmutated-CLL, U-CLL) is associated with a more aggressive disease and a shorter survival time (8-9 years), as compared to mutated-CLL (M-CLL) cases, having a median life expectancy of 24 years[101,102].

Surface expression of CD38

CD38 is a type II membrane glycoprotein and functions as an ectoenzyme and a receptor. In particular, it was demonstrated to activate a genetic program relevant for proliferation and migration in CLL cells[103] and to associate with the BCR complex resulting in a synergistic response in both normal and leukemic B cells[104,105]. Accordingly, CD38 expression defines an activated subset within CLL clones[106] and CD38 positivity is associated with faster disease progression and shortened life expectancy[107].

Intracellular ZAP70 expression

ZAP70 is a tyrosine kinase expressed in developing and mature B cell subsets in mice[108,109] and in normal and malignant human B lymphocytes at distinct stages of differentiation[110]. ZAP70 displays redundances with Spleen tyrosine kinase (SYK) in early B cell development[109] and contributes to signal transduction downstream of the BCR in mature B cells. In CLL cells, antigen stimulation drives ZAP70 recruitment to the cell surface where it functions as an adaptor protein to activate the pathway. It has been reported that CLL cells expressing ZAP70 (e.g. in >20% of the leukemic cells) are more competent than ZAP70 negative ones (having <20% of positive cells) to receive proliferation and/or survival stimuli[111]. This could account for the relatively aggressive clinical behaviour of patients showing ZAP70 positivity[112,113].

Chromosomal abnormalities

Genomic aberrations can help to define risk groups. As expected from the roles played by ATM and p53 in the cell response to DNA damage, cell cycle progression and cell death, the presence of *ATM* (11q deletions) or *TP53* (17p deletion) mutations correlates with poor prognosis and poor response to therapy. Trisomy of chromosome 12 does not significantly affect survival as compared to normal karyotype while patients with deletions 13q have the best survival expectancy[63]. However, though some of these abnormalities may be detected at the initial diagnosis, others (e.g. deletions at 17p and 11q) may be acquired during the disease course and alter patients' risk.

HS1 phosphorylation status

HS1 is a hematopoietic-specific intracellular protein[114]. Immunofluorescence analysis and confocal microscopy have revealed different subcellular localizations of HS1 in normal and malignant B cells. It forms fiber-like structures mainly in the cytoplasm of resting B lymphocytes, MEC1 CLL cell line and CLL cells[115], but it moves into the nucleus after anti-IgM induced tyrosine-phosphorylation[116] and it can be found in lipid rafts of B lymphocytes[117] where it activates Actin-related protein 2/3 (Arp2/3) complex-mediated actin nucleation and branching[118,119]. In normal and leukemic B lymphocytes HS1 also participates to a protein complex including also HPK1-interacting protein (HIP-55), Cortactin and ZAP70 that interacts

with intermediate filament protein Vimentin and polymerized F-actin to modulate the actin cytoskeleton organization[115].

According to HS1 role downstream of BCR stimulation, HS1-deficient B cells show an altered response to sIgM cross-linking[120]. In particular *Hs1*$^{-/-}$ splenic B lymphocytes have a significant impairment in anti-IgM induced proliferation and *Hs1*$^{-/-}$ peritoneal B cells fail to dye by apoptosis upon stimulation through the BCR, in contrast to what observed in normal B cells[121].

HS1 phosphorylation status has been shown to correlate with CLL prognosis together with CD38 expression and IGHV mutational status in a large cohort of patients[122]. The proteomic analysis of circulating leukemic B cells revealed that cells from patients affected by the indolent form of the disease express two differentially-phosphorylated isoforms of HS1, whereas the hyperphosphorylated form prevails in cells from patients with poor prognosis, meaning that most HS1 is constitutively phosphorylated.

Since HS1 was demonstated to have a prognostic value in CLL and to be involved in BCR signalling and cytoskeleton organization of B cells, that have a central role in CLL pathogenesis, we analyzed the effect of HS1 deficiency in CLL both *in vitro* (by downregulating HS1 in MEC1 cell line) and *in vivo* (by crossing HS1$^{-/-}$ with Eμ-TCL1 tg mice (paragraph 1.5.5 and chapter 3)).

1.4.4 From immunoglobulin features analysis to CLL origin hypothesis

The BCR is a multiproteic complex composed by a sIg (IgM in immature B lymphocytes, IgM and IgD in mature B lymphocytes and every possible isotype in memory B cells) with antigen binding specificities paired with Igα/Igβ heterodimers (CD79a/CD79b) involved in signal transduction. The IG molecule consists of two identical IGH and IGL (κ or λ) chains that comprise a V region for antigen binding and a constant C one for effector functions. The IGH and IGL chain arise from the rearrangement of the IGH and IGK or IGL loci, respectively. The IGH gene results from the association of the germline segments IGHV, IGHD, IGHJ and C, whereas the L chains lack the D region. These rearrangements originate a large number of combinations that contributes to the antibody repertoire.

During lymphocyte development in the bone marrow, additional diversity is provided by random nucleotide insertion/deletion at the junction sites and by three possible reading frames for the D gene. Altogether these events account for more than 10^{12} combinations of the antibody "germline" (or unmutated) repertoire. Antigen encounter together with stimulation by helper T cells induces SHM of the IG genes, including point mutations and, less frequently, insertions and deletions in the IG loci that concentrate preferentially in the complementary determining regions (CDRs), hypervariable regions in the IGHV genes, involved in the antigen binding site

formation. Among them, the HCDR3, spanning along the IGHV-D-J junction is the most variable region[123].

The human IGHV genes are grouped into 7 gene families (IGHV1-IGHV7) based on at least 80% sequence homology and CLL cells show a biased use of certain IGHV genes compared to the normal peripheral blood $CD5^+$ repertoire, such as overexpression of the IGHV1 family and underrepresentation of the IGHV3 family. In particular the most frequent IGHV rearrangements include IGHV1-69, IGHV3-07 and IGHV4-34[124]. The IGHV1-69 gene prevails in the U-CLL group[125], while in the mutated group restricted usage of the IGHV3-07 and the IGHV4-34 genes has been observed[124].

Furthermore, regardless the IGHV mutational status, ~30% of CLL cases carry “stereotyped receptors”[126]. This definition refers to recurrent IGs molecules that share multiple CDR3 structural features such as length, amino acidic composition and unique residues at recombination junctions. Cluster analyses have revealed that CLL cases with “stereotyped CDR3” sequences also share phenotypic characteristics and marked similarities in terms of clinical outcome[127]. The striking homology noticed among distinct and geographically distant cases is unlikely to be a random phenomenon, rather it suggests the involvement of discrete antigens or classes of structurally related epitopes that select and activate the leukemic clones. Indeed CLL IGs can recognize antigenic targets on apoptotic cells, self-antigens interacting with microbes and bacterial components[128-131].

The presence of “stereotyped receptors” and the non-stochastic pairing of H and L chains in both mutated and unmutated cases suggest that CLL most likely originates from a clonal expansion of mature immunocompetent B cells. Two additional evidences support this hypothesis. First, CLL cells have a surface phenotype similar to B cells activated by antigen encounter (overexpression of the markers CD23, CD25, CD69, CD71 and downregulation of CD22, CD79b and IgD)[132]. Second, their gene expression profile resembles that of memory B cells, irrespective of the IGH mutational status[133], confirming that CLL is a disease antigen-experienced B cells. Though they share phenotypic similarities with memory B cells, leukemic cells do not entirely accomplish the “memory B cell” definition[134], and the question regarding the cell of origin of CLL is still open. It has been proposed that CLL may derive from B cells located in the MZ that are often exposed to antigens and may have previously undergone a GC reaction leading to the acquisition of SHM. However this hypothesis would imply that expression of CD5 and CD23 on CLL cells results from an unusual activation status of the leukemic cells rather than from their developmental stage or identity. On the other hand the surface expression of the CD5 molecule, together with the production of “natural antibodies” that mainly characterizes U-CLL cases, suggest that the disease may originate from human B lymphocytes with the feature of murine B-1 cells, that so far have not been isolated. To add furher complexity to the picture, it has been recently demonstrated that self-renewing HSCs

purified from CLL patients have the propensity to generate clonal B cell expansions and may thus be skewed toward a clonal CLL-like phenotype during maturation[135].

1.4.5 The BCR at the center of antigenic and microenvironmental stimuli in CLL B cells

The BCR is expressed on the surface of all mature B cells and coordinates a cascade of signalling events regulating B cell selection, proliferation, differentiation and antibody production. BCR engagement normally occurs on translocation into lipid rafts and subsequent phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) within the cytoplasmic tails of CD79a and CD79b. ITAM phosphorylation is mediated by Src family kinases (FYN, Tyrosine kinase, B lymphocyte specific (BLK), Hematopoietic cell kinase (HCK), FGR, LYN) that also activate (BTK), CD19 and Phosphoinositide 3-kinase (PI3K). As a result, the BCR oligomerize in microlusters and ITAMs phosphorylated residues recruit the protein tyrosine kinase SYK that is phosphorylated and activated by Src-family kinases. The signal is then transduced through enzymatic activation of kinase proteins and phosphorylation of the relevant substrates, placed into two parallel cascades (Figure 3).

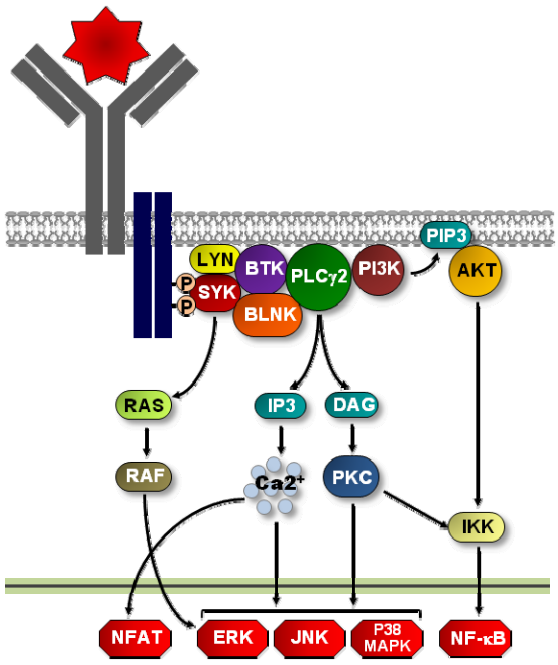

FIGURE 3. **BCR signalling pathways**.

The first pathway is dominated by LYN and SYK that contact and phosphorylate Phospholipase Cγ2 (PLCγ2) through BLNK interface and induce activation of RAS. RAS in turn activates RAF and the signalling cascade results in the activation of MAP-ERK kinase 1 (MEK1) and 2 (MEK2), while PLCγ2 leads to the production of Inositol-1,4,5-triphosphate (IP3) and Diacylglycerol (DAG) which are required for the subsequent release of intracellular calcium ($Ca2^{+}$) and activation of PKC, respectively. In the other pathway, PI3K generates the second messenger Phosphatidylinositol-3,4,5-triphosphate (PIP3) which recruits Protein kinase B (AKT) and other BCR signalling molecules to the cellular membrane, a step required for their

activaction. Altogether these pathways carry the signal from the surface membrane to the nucleus, where Mitogen-activated protein kinases (MAPKs), such as Extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK), transcription factors including NF-κB and Nuclear factor of activated T cells (NF-AT) and anti-apoptotic molecules as Myeloid cell leukemia-1 (MCL-1) regulate cell activation, proliferation, survival and differentiation[136].

CLL B cells differ from normal B lymphocytes because of the altered activation status of various signalling molecules. In particular they have high levels of LYN tyrosine kinase activity[137] and SYK phosphorylation, together with active nuclear NF-κB and NF-AT in the absence of stimuli[138,139]. These findings prompted the development of therapeutic strategies based on inhibition of BCR signalling[140]. Small molecule inhibitors of SYK, BTK and p110δ isoform of PI3K (PI3Kδ) have recently entered the clinical arena and show promising results in CLL treatment.

In addition, subsets of CLL patients show different responses to antigen stimulation. It has been demonstrated that a fraction of patients displays constitutive phosphorylation of ERK1/2, that associates with constitutive expression of phospho-MEK1/2 and activation of NF-AT, but is not linked to AKT and SYK phosphorylation status and to LYN and NF-κB activity[141]. Moreover, in phospho-ERK^+ cases, B cells do not respond to anti-IgM antibody stimulation, while BCR cross-linking induces a strong increase of phospho-ERK in the negative cases. The

presence of constitutive ERK1/2 phosphorylation correlates with a low stage disease, but it does not seem to correlate significantly with any known prognostic factor.

That notwithstanding, a different responsiveness to BCR engagement has been reported between mutated and unmutated cases. *In vitro* stimulation with anti-IgM antibodies is able to trigger BCR translocation to lipid rafts[137] and signal transduction, and to favour survival of leukemic cells in the bad prognosis cases, but not in the others[142]. This result could be explained *in vivo* by a "constitutive" activation of the BCR signalling pathway promoted by persistent exposure to T-independent antigens in U-CLL cases[143], in agreement with the absence of mutations in the IGHV genes. Conversely, in the case of M-CLL, the behaviour of leukemic lymphocytes reflects the malignant clone quiescence and resembles the anergic status of normal B lymphocytes, that is characterized by a failure of BCR translocation to lipid rafts in response to cross-linking[144]. The oucome of antigenic stimulation is also affected by the expression of ZAP70 and CD38 that are both BCR signalling-associated molecules and prognostic factors in CLL (paragraph 1.4.3).

The central role of the BCR in CLL is underlined by the fact that it integrates antigenic and microenvironmental signals (Figure 4) and the microenvironment is thought to shape CLL cells by promoting B cell transforming events and by sustaining the proliferation and survival of leukemic cells. Accordingly, the gene signature of CLL cells isolated from patients' lymph nodes

shows BCR and NF-κB signalling activation[145]. Increased BCR response and NF-κB activation also characterize peritoneal $CD5^+$ B cells of SIGLEC-G-deficient mice[29,146]. SIGLEC-G is a negative regulator of BCR-mediated Ca^{2+} response and *Siglecg*$^{-/-}$ mice has been reported to develop an expansion of polyclonal B-1a cells early in life. We therefore monitored the evolution of the $CD5^+$ B cell population over time and described the malignant transformation occurring in aged *Siglecg*$^{-/-}$ mice (chapter 5).

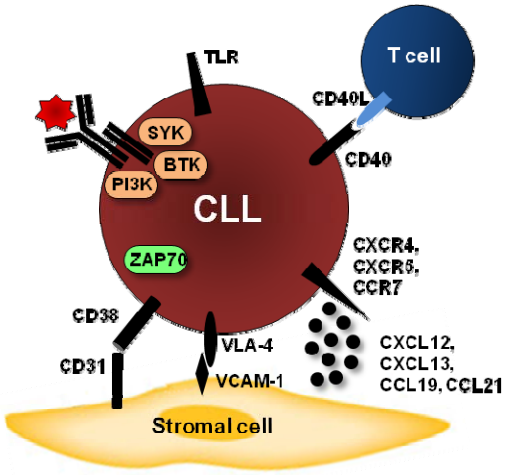

FIGURE 4. **Major interactions in the CLL microenvironment.**

As mentioned earlier, in the PCs of lymph nodes and bone marrow, CLL cells are in contact with T cells and are exposed to stimuli delivered through CD40. CD40 engagement is considered one of the costimulatory signals required for activation of normal B cells after BCR cross-linking. Full activation is likely achieved by simultaneous stimulation of the Toll-like receptor (TLR) pathway[147]. TLRs recognize a set of

different molecular patterns found in microbial components and support B cell proliferation, maturation and antibody production[148]. The expression pattern of TLRs in CLL cells resembles that of human tonsils and memory B cells[149], confirming once more that CLL originates from antigen-experienced B cells. In addition, both normal and leukemic B cells express low levels of Single immunoglobulin domain-containing IL1R-related protein (TIR8/SIGIRR), a transmembrane receptor belonging to the interleukin 1 receptor (IL-1R)/TLR family that functions as a negative regulator of TLR signaling[150,151]. Since a fraction of CLL IGs reacts with microbial components[143], it is likely that TLR pathway could be activated together with the BCR, as previously described during autoimmune reactions[152,153]. To elucidate the role of TLRs in CLL *in vivo*, we crossed TIR8-deficient and Eμ-TCL1 tg mice and analyzed cell responsiveness and CLL development in the context of a sustained TLR activation (chapter 4).

1.4.6 Cytoskeleton abnormalities and migration properties of CLL B cells

Circulating CLL cells have a fragile cell membrane, that often breaks during blood smear preparations, giving rise to the so-called “smudge cells”[154]. This event is not only a morphological hallmark of CLL, but it is also suggestive of defects involving cytoskeletal components. By studying the organization of Actin-containing microfilaments and Vimentin-containing intermediate

filaments it has been demonstrated that leukemic cells are able to organize adhesion structures and to adhere *in vitro*[155]. However CLL cells have impaired cell motility, decreased capping by multivalent ligands, increased shedding of membrane proteins and enhanced susceptibility to microtubule-disrupting drugs[156]. The reduction in Vimentin expression in leukemic cells as compared to normal B lymphocytes is considered to play a causative role in their defective motility[157]. Additional molecules that have been recently found to be implicated in F-actin dynamics (e.g. Wiskott-Aldrich syndrome protein (WASP) and VAV family, HS1 and Cortactin)[119,115] may affect the cytoskeletal properties of CLL cells. Actin polymerization and cytoskeleton re-organization also occur during the formation of immunogical synapses. It has been shown that this process is defective in CLL due to alterations in both the CLL T and B cell compartments[158]. The defects include suppressed F-actin polymerization and impaired recruitment of key molecules, such as Lymphocyte function-associated antigen 1 (LFA-1) and Lymphocyte-specific protein tyrosine kinase (LCK), Cell division cycle 42 (CDC42), WASP, Dynamin-2 and Filamin A to the T cell synapse site.

Besides these cytoskeleton abnormalities, chemokine responsiveness has an important function in CLL pathogenesis, since it regulates lymphocyte recirculation between the proliferative compartments and the periphery. Circulating CLL B cells show upregulation of CCR7, that responds to Chemokine C-C motif ligand 19 (CCL19) and Chemokine C-C motif ligand

21 (CCL21), expressed on high endothelial venules at the entry barrier of lymph nodes. Increased expression of CCR7 may therefore contribute to leukemic cells homing to the lymph nodes[159]. In addition CLL B cells have high surface levels of CXCR4[160] and CXCR5[161]. CXCR4, by interacting with stromal cell-derived factor-1 (SDF-1), protects malignant cells from apoptosis[162] and drives their spontaneous migration beneath bone marrow stromal cells[163], appearing as an important *in vivo*-confirmed mechanism for bone marrow infiltration[164]. Otherwise CXCR5 allows for neoplastic lymphocytes trafficking and migration to CXCL13-secreting cells in lymphoid tissues and favours their accumulation within lymphnodal follicles[161]. Altogether overexpression of CCR7, CXCR4 and CXCR5 in malignant B cells may explain the widespread lymphoid organ dissemination observed in CLL[165].

Evidence suggests that expression of chemokine receptors and/or adhesion molecules and chemokine responsivess may correlate with prognostic factors, such as IGHV mutational status, ZAP70 and CD38 expression[166]. $ZAP70^+$ CLL cells have increased migration capabilities toward SDF-1[167], CCL21 and CCL19 and exhibit sustained ERK1/2 activation upon SDF-1 stimulation as compared to $ZAP70^-$ cells[166]. In agreement, ectopic expression of ZAP70 in B cells induce enhanced BCR signaling following IgM cross-linking end increased ERK1/2-mediated expression of CCR7[168]. CD38 expression associates with increased migration to SDF-1 and increased SDF-1 signaling, and *in vivo* blocking of CD38 impairs CLL cell homing

to lymphoid organs in a xenograft mouse model[169]. Further, ZAP70 and CD38 double positivity define CLL cells with high migration capacity[167] that may easily access the PCs and receive survival and proliferative stimuli responsible for the aggressive course of the disease.

1.5 Spontaneous and genetically-engineered mouse models of CLL

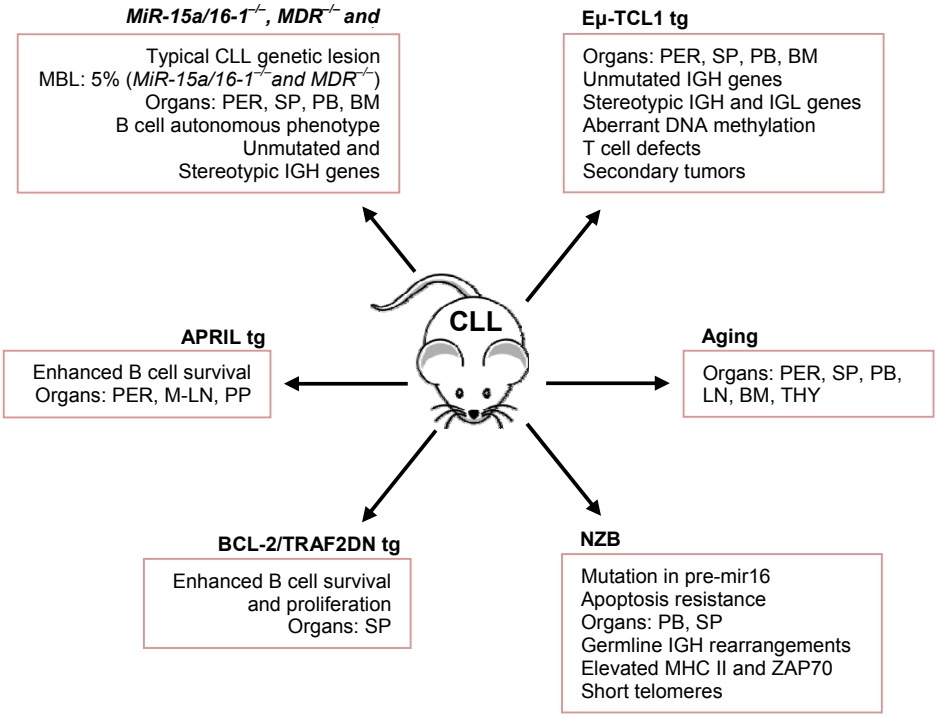

FIGURE 5. **Features of spontaneous and genetically-engineered mouse models of CLL.** PER: peritoneal cavity; SP: spleen; PB: peripheral blood; BM: bone marrow; M-LN: mesenteric lymph nodes; PP: Peyer's patches; THY: thymus.

1.5.1 Aging mice

It has been demonstrated that mice over the age of 18 months develop clonal populations of phenotypically homogeneous B-1a cells resembling B cell clones of human CLL[170]. In addition they produce dysregulated humoral responses characterized by limited heterogeneity with respect to isotype (IgM versus IgG), antigen-binding affinity, idiotype and IGHV gene usage that may be responsible for the impaired antibody responses to foreign antigens and the consistent shift to self-specificities[171]. During aging, $CD5^+$ B cells become predominant over $CD5^-$ cells, they accumulate within the peritoneal cavity, where they naturally reside, and they start to disseminate and expand in other lymphoid organs, such as the spleen[170]. More than 85% C57BL/6 mice older than 18 months of age have splenic B cell clonal populations, that appear like a single dominant peak at the analysis of size of CDR3 mRNA of IGH chain[172] and are detectable by flow cytometry and Southern blot analysis[170]. Some of these clones can be also identified in the bone marrow, lymph nodes and thymus, but not in the peripheral blood of the same animals, meaning that B cell clonal expansions are selected in the peripheral immune system and they can disseminate and expand in different lymphoid compartments[172]. Most stable B cell clonal populations may be dominated by cells at two stages of differentiation: plasma cells that form small clonal expansions in the spleen and mature B lymphocytes originating large clonal populations that are

detectable in the spleen, bone marrow and thymus of old mice[173]. In synthesis, aging mice show typical features of CLL patients: poor responses to foreign antigens, enhanced production of autoantibodies, increased frequency of serum monoclonal IGs and predominance of the $CD5^+$ B cell subset (Figure 5).

1.5.2 NZB mouse model

The New Zealand Black (NZB) strain is a naturally occurring model of late-onset CLL. It is characterized by early B cell hyperproliferation and autoimmunity restricted to IgM anti-erytrocyte and anti-DNA autoantibodies, together with progressive accumulation of clonal $IgM^+B220^{low}CD5^+$ small B lymphocytes in the peripheral blood and in the spleen[174]. This expansion is partly due to the decreased expression of miR-15a and miR-16, two microRNAs that are frequently deleted or downregulated in CLL[175]. In NZB mice the precursor of miR-16 is targeted by a point mutation in the 3' flanking region resulting in apoptosis resistance of B-1 cells that can be reverted *in vitro* by delivery of exogenous miR-16[176]. In addition, systemic *in vivo* lentiviral delivery of miR15a/16 is able to reduce the disease burden[177]. NZB mice share with CLL patients other additional features, as the presence of germline IGH gene rearrangements and elevated MHC class II and ZAP70 expression (Figure 5). Finally increased telomerase activity and short telomeres, that correlate with shorter survival of CLL

patients[178], have been also associated with disease progression in NZB mice[179].

1.5.3 BCL-2/TRAF2DN double tg mouse model

It has long been known that BCL-2 and tumour necrosis factor (TNF) receptor-associated factor 2 (TRAF2) play a role in leukemic B lymphocytes survival and proliferation. In particular BCL-2 is upregulated in CLL[75,76] and its overexpression in mouse lymphoid cells results in polyclonal expansion and prolonged survival of B cells *in vitro*[180]. TRAF2 is an adaptor protein involved in the TNF-mediated activation of NF-κB and JNK and mice expressing a dominant negative form of the gene (TRAF2DN) develop lymphoadenopathy, splenomegaly and increased B cell count that never lead to a clear haematological disease[181]. In order to understand wheather the two factors may cooperate in the malignant transformation of B cells, *Zapata et al.* crossed BCL-2 and TRAF2DN tg mice and in double tg mice they observed spleen enlargement and clonal expansion of chemotherapy-resistant $B220^{+}CD5^{+}$ malignant cells resembling human CLL lymphocytes (Figure 5).

1.5.4 APRIL tg mouse model

A proliferation-inducing ligand (APRIL) is a member of the TNF family, it is expressed quite exclusively in hematopoietic cells[182] and, after secretion, interacts with various receptors and

contributes to NF-κB pathway stimulation. The protein has been reported to be overproduced in different B cell malignancies, including CLL[182], where it enhances B lymphocyte survival, as confirmed by *in vitro* and *in vivo* studies[183]. Tg mice overexpressing APRIL in T cells show increased serum levels of the protein that favors the expansion of peritoneal $IgM^+B220^+CD5^+$ cells. The malignant lymphocytes also infiltrate the mesenteric lymph nodes and Pleyers patches around 9-12 months of age (Figure 5). However APRIL tg mice develop a milder disease compared to BCL-2/TRAF2DN and Eμ-TCL1 tg models (see paragraph 1.5.5) and do not die prematurely, indicating that, though they are prone to lymphoid malignancies, additional components need to be deregulated for a full transformation[184].

1.5.5 TCL1 tg mouse model

TCL1 expression and function in normal and malignant lymphoid cells

The identification of the the T cell leukemia 1 (*TCL1*) gene dates at the cloning of the breakpoint of chromosome 14 that is responsible for abnormal rearrangements associated with mature T cell leukemias and lymphomas[185]. TCL1 is normally expressed in restricted subsets of cells within the lymphoid lineage, preferentially in the early stages of differentiation. In T cells it is detectable only in $CD3^-CD4^-CD8^-$ thymic progenitors[77], while in the B cell compartment is expressed at

low levels in pro-B cells, it is upregulated in pre-B cells, sIgM-expressing *naïve* B cells, mantle cells, it is downreguated in GC cells and absent in terminally differentiated plasma cells.

TCL1 is a co-activator of the serine/threonine kinase AKT, a downstream effector of the PI3K oncogenic pathway. PI3K activation induces AKT recruitment to the plasma membrane, a step required for the phosphorylation of AKT that in turns upregulates the expression of antiapoptotic and pro-survival factors including BCL-2-antagonist of cell death [54], Nuclear receptor subfamily 4 group A member 1 (NUR77), Programmed cell death 4 (PDCD4), T cell acute lymphocytic leukemia 1 (TAL1), IκB kinase-α (IKKα), Glycogen synthase kinase-3 (GSK3) and Cyclic AMP-responsive element binding protein (CREB). Co-expression of AKT and TCL1 in lymphoid cells leads to increased activity and nuclear translocation of AKT[186,187]. It has recently been reported that TCL1 has additional AKT-independent roles at least in CLL B cells[188]. In particular it activates NF-κB activity by physically interacting with the co-activator p300/CREB binding protein and it inhibits transactivation of the AP-1 complex at the level of the single components (c-JUN, JUN-B and c-FOS), preventing AP1-mediated apoptosis in CLL cells.

Deregulation of TCL1 activity is involved in both B and T cell tumours. TCL1 is constitutively overexpressed in T lymphocyte malignancies, namely T-prolymphocytic leukemias (T-PLL) and acute and chronic T cell leukemia arising in ataxia-telangiectasia patients, by insertion in the T cell receptor (TCR)

regulatory region, leading to TCL1 activation and to late-onset clonal expansion of $CD3^+CD4^+CD8^-$ T cells. The oncogenic role of TCL1 in T cell leukemia pathogenesis has been confirmed by expressing the human (h)*TCL1* gene under the control of the T cell-specific gene promoter $p56^{lck}$ in a tg mouse model[189]. Fifteen- to twenty-month old $p56^{lck}$-hTCL1 mice have elevated white blood cell (WBC) count and enlargement of the spleen, lymph nodes and thymus, that are highly infiltrated by clonal $CD3^+CD4^-CD8^+$ cells (a subset of mature T lymphocytes differing from the human neoplasia).

High levels of TCL1 expression have been found also in a variety of B cell tumors, such as AIDS-related NHL, lymphoblastic lymphoma, CLL, mantle cell lymphoma (MCL), FL, DLBCL and primary cutaneous B cell lymphoma. The pEμ-B29-hTCL1 tg[190] and the Eμ-TCL1 tg[68] mouse models helped to elucidate TCL1 role in B cell malignancies by recapitulating respecitively mature B cell lymphoma and CLL pathogenesis. In the first mouse model the expression of the *hTCL1* gene is regulated by the B29 minimal promoter and IGH intronic enhancer, leading to similar levels of TCL1 protein in T and B cells. At 7-13 months of age pEμ-B29-TCL1 tg mice show expansions of $IgM^+B220^{low}CD5^{low}$ B cells in the spleen, with oligoclonal or monoclonal IG rearrangements. The presence of mutations in the IGH genes and the histopathological analysis revelead similarities with different GC or post-GC B cell lymphoma types, including DLBCL with histiocyte-associated and T cell-rich features, Burkitt-like lymphomas and FL. The

onset of B cell tumors in these mice precludes the appearance of T cell malignancies that probably require longer latencies as observed in the $p56^{lck}$-hTCL1 mouse model[189]. However, B cell lymphoma latency remains long, meaning that high levels of hTCL1 expression offer a proliferative advantage to the cells, but other genetic or epigenetic changes, presumably involving GC-related functions and pathway may be required for the malignant transformation[191].

The *TCL1* gene in CLL and Eμ-TCL1 tg mouse model

Unlike the genomic alteration present in T cell malignancies, the TCL1 gene is not activated as a classical oncogene in CLL, a disease characterized by modulated TCL1 expression[78]. Strong TCL1 expression is associated with high levels of sIgM and intracellular ZAP70 and unmutated IGHV genes. In addition it correlates with the clinical outcome of penthostatin-, ciclophosphamide- and rituximab-based chemotherapy, becoming a potential prognostic factor for treatment efficacy[192].

Clear evidences of TCL1 involvement in CLL have emerged from the Eμ-TCL1 tg mouse model[68] (Figure 5). In this model the *hTCL1* gene is located under the control of the IGHV promoter and IGH enhancer (Eμ), together with the 3' end and untranslated region of the human *β-globin* gene, resulting in a constitutive expression of hTCL1 throught the B cell lineage. Eμ-TCL1 tg mice develop a progressive expansion of $CD19^+CD5^+Ig\kappa^+IgM^+$ B cells[68,193], that is detectable at 2 months in the peritoneal cavity, at 3-5 months in the spleen, at 6 months in the peripheral blood and at 8 months in the bone

marrow. This population is mainly composed of not-dividing B lymphocytes, arrested in the G_0/G_1 phase of the cell cycle, as circulating human CLL cells. Between 13 and 18 months of age, Eμ-TCL1 tg mice develop an overt leukemia resembling the aggressive form of human CLL, characterized by elevated WBC counts and massive infiltrations of monoclonal $CD5^+$ B cells in the spleen, lymph nodes and liver with a 100% penetrance. Variable macroscopical and/or microscopical alterations have been also observed in the lungs, kidneys, heart, salivary glands, esophagus, thymus, trachea and thoracic cavity[194].

The analysis of the BCR repertoire of Eμ-TCL1 tg confirmed the clonal origin of the $CD5^+$ B cell expansion and revealed skewed usage of IGHV, IGHD, IGHJ and IGKV family genes compared to normal $CD5^+$ B cells, together with the presence of unmutated IGH gene rearrangements (less than 2% of difference from germline) and stereotypic IGH and IGL gene rearrangements[195]. All these molecular aspects reproduce the features of human U-CLL and suggest that in mouse leukemic cells may originate from peritoneal B-1a cells with restricted BCR structure, selected by autoantigens or microbial antigens and forced to clonal expansion.

The Eμ-TCL1 tg mouse model also recapitulates other features of the human disease, including epigenetic and T cell function alterations and the development of secondary malignancies. Aberrant DNA methylation is detectable in B cells of Eμ-TCL1 tg mice even before the disease onset and the level of methylation increases along with leukemia progression[196]. As

regards the T cell compartment, the mouse model carries similar T cell defects to that seen in CLL patients[197]. In particular leukemic mice show decreased *in vivo* antigen-specific T cell activation, suppressed T cell mitogenic proliferation, and impaired induction of idiotype specific $CD8^+$ T cells capable of killing CLL cells. Though Eμ-TCL1 tg mice progressively accumulate an increased number of T cells as previously observed in CLL patients[198], tumor cells can activate *in vivo* immunomodulatory mechanisms that result in defective T cell function and successful escape from the host immune system.

The profound B and T cell defects affecting Eμ-TCL1 tg mice favour infectious morbidity[199] and the development of secondary malignancies, that are a frequent complication in human CLL and the most common cause of death. Even if patients often develop secondary lymphoid malignancies, several cases of solid tumours are reported, especially of skin cancer. Pathologic analysis of leukemic mice have revealed some cases of intestinal histiocytic sarcoma, spindle cell sarcoma of the gallbladder, malignant pilomatricoma of the skin, all unrelated to CLL and to the abnormal TCL1 expression[194].

Taken together, these observations make Eμ-TCL1 tg mouse a suitable model for the aggressive form of human CLL, which helps to investigate further mechanisms and to test new therapeutic strategies[200-205].

Nevertheless, this model shows some limitations related to the long time required to develop leukemia and to the

introduction of a transgene that is not associated with a genetic lesion in human CLL.

1.5.6 MiR-15a/16-1$^{-/-}$, MDR$^{-/-}$ and CDR$^{-/-}$ mice

MiR-15a/16-1$^{-/-}$, MDR$^{-/-}$ and *CDR$^{-/-}$* mice represent the most faithful model of CLL pathogenesis, since the disease arises from a genetic lesion that is frequently detected in CLL[63] (paragraph 1.4.2) and MBL[206] and occasionally in other mature B cell lymphoma types[207]. Similarly to what observed in the Eμ-TCL1 tg model, *MDR$^{-/-}$* mice first develop an expansion of CD5^{+} B cells in the peritoneal cavity, that progresses in an overt CLL-like tumor involving the spleen, the peripheral blood and the bone marrow at 15-18 months of age[70] (Figure 5). This phenotype characterizes 27% of *MDR$^{-/-}$* and 21% of *MiR-15a/16-1$^{-/-}$* mice and the CD5^{+} clonal cells display "stereotypic" IGH gene usage. Five percent of the mice develop a peripheral blood lymphocytosis resembling human MBL and the remaining fraction show hystologic and phenotypical features of CD5^{-} NHLs. This mouse model is the first *in vivo* evidence of the tumor suppressor role of the 13q14 locus and the more aggressive phenotype observed in *MDR$^{-/-}$* compared to *MiR-15a/16-1$^{-/-}$* mice indicates that besides the microRNAs, other genetic elements in the *MDR* locus contribute to the anti-tumorigenic function. In addition the analysis of mice carrying a B cell specific deletion of the locus, demonstrates that lymphoproliferations in *MDR$^{-/-}$* and *MiR-15a/16-1$^{-/-}$* mice are B

cell autonomous. B cell-specific deletion of the entire *CDR* locus (*CDR*$^{-/-}$ mice) leads to a similar phenotype, with accumulation of clonal CD5^{+} B cells in the peripheral blood, spleen, bone marrow and with liver involvement. *CDR*$^{-/-}$ mice show a penetrance of lymphoproliferation and age of onset similar to *MDR*$^{-/-}$ mice[208]. However the disease progresses faster, bypassing a detectable MBL stage and resembles CLL in almost all cases, suggesting that multiple genes in the *13q14* locus may have a tumor suppressor function in CLL in addition to the *DLEU2*/*MiR-15a/16-1* cluster.

1.6 Xenograft models of human CLL

Several attempts have been made in the past years in order to obtain the engraftment of CLL cell lines and primary cells with multiple difficulties due primarly to the lack of mice sufficiently immunodeficient.

A highly immunocompromised mouse model is required for a successful engraftment. Indeed engraftment of MO1043[209] and of JVM-3[210] CLL cell line was achieved in *nude* mice (that are characterized by T cell deficiency and partial development of B cells) only after irradiation. Better results were obtained in *nude* female compared to male recipients, thanks to hormonal inhibition of the cytolitic function of natural killer (NK) cells, suggesting that this cell type may play an important immunosurveillance role in the early phases of the disease. An

increased efficiency of primary and CLL cell lines engraftment was achieved in SCID[209] mice that completely lack T and B cells and in NOD/SCID[211,212] mice that have additional defects in the innate immune system. Finally, through the introduction of the IL-2RG (γ-chain) deficiency, *Rag2*$^{-/-}$*Il2rg*$^{-/--}$ (*Rag2*$^{-/-}\gamma_c^{-/-}$) mice and NOD/Sci-scid-*Il2rg*$^{-/-}$ (NSG) mice, that lack T, B and NK cells appear to be the most promising background for successfull human cell engraftment[213,214].

However the low and variable level of primary CLL cell recovery and/or the poor extraperitoneal dissemination of the leukemia, especially to the bone marrow and in the peripheral blood, still represent limiting factors[209,211,212,215,216]. The variability observed in the engraftment capacity associates with the properties of the leukemic cells. It was demonstrated that the engraftment of primary CLL cells correlates with the disease stage and with prognostic markers such as the IGHV gene mutational status and ZAP70 expression, while CD38 positivity accounts for an increased homing of the tumor cells to the bone marrow in a short-term migration assay[217].

Recently two studies have given new insights on the role of T cells in the pathogenesis of CLL. By co-transfering CLL PBMCs with normal antigen presenting cells (APCs) (preferably CD14$^+$) from unrelated subjects, CLL cell survival and proliferation was obtained *in vivo* in NSG mice[218]. From this model the two most important lymphohematopoietic elements involved in CLL engraftment have emerged: autologous T lymphocytes and allogeneic APCs (CD14$^+$ or CD19$^+$ cells). APCs-primed T cells

in turn are able to sustain the engraftment and proliferation of the leukemic cells. Accordingly *Aydin et al.* showed that the proliferation index and CD38 expression level of CLL cells recovered from xenograft mice correlates with T cell engraftment[217].

The low level of reproducibility limits the applicability of these primary CLL cells xenograft models to preclinical studies. These findings prompted us to develop a new xenograft model by injecting the MEC1 cell line either subcutaneously or intravenously in *Rag2*$^{-/-}\gamma_c^{-/-}$ mice (chapter 2).

1.7 Scope of the thesis

Aim of this thesis was understand the molecular mechanisms leading to mature B lymphoid malignancies by using different mouse models, in order to identify novel potential therapeutic targets and obtain useful models for preclinical studies.

In chapter 2 we show the establishment of a new xenograft model obtained by injecting the CLL cell line MEC1 in immunodeficient *Rag2*$^{-/-}\gamma_c^{-/-}$ mice. This model resembles the aggressive form of human CLL and is conceivably useful to test the efficacy of new therapeutic agents.

In chapter 3 we show that HS1 is involved in the trafficking and homing of leukemic B cells and that its deficiency is responsible for an earlier onset of the disease and a reduced survival in the Eμ-TCL1 mouse model of CLL.

The same animal model was used to investigate the role of TLR pathway in CLL. In chapter 4 we report that the absence of TIR8 accelerates the appearance of the disease and favors the progression into an aggressive form characterized by the accumulation of “prolymphocytoid” cells.

Finally in chapter 5 we demonstrate that mice lacking the negative regulator SIGLEC-G are susceptible to B cell lymphoma development with age indicating that the downregulation of SIGLEC10 may be involved in the malignant transformation of human B lymphocytes.

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2. A novel *Rag2*$^{-/-}\gamma_c^{-/-}$ -xenograft model of human CLL

Maria Teresa Sabrina Bertilaccio,[1] Cristina Scielzo,[1,2] Giorgia Simonetti,[3,4] Maurilio Ponzoni,[5,6] Benedetta Apollonio,[1,7] Claudia Fazi,[3] Lydia Scarfò,[3,8] Martina Rocchi,[6] Marta Muzio,[1] Federico Caligaris-Cappio,[1,5,7] and Paolo Ghia[3,5,7]

[1]Laboratory of Lymphoid Malignancies, Division of Molecular Oncology, Istituto Scientifico San Raffaele, Milan; [2]Università degli Studi di Milano, Milan; [3]Laboratory of B cell Neoplasia, Division of Molecular Oncology, Istituto Scientifico San Raffaele, Milan; [4]Università degli Studi di Milano-Bicocca, Milan; [5]Unit of Lymphoid Malignancies, Department of Oncology, Istituto Scientifico San Raffaele, Milan; [6]Pathology Unit, Istituto Scientifico San Raffaele, Milan; [7]Università Vita-Salute San Raffaele, Milan; and [8]Haematology Unit, Azienda Ospedaliera Sant'Anna, Ferrara, Italy

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ABSTRACT

Easily reproducible animal models that allow for study of the biology of chronic lymphocytic leukemia (CLL) and to test new therapeutic agents have been very difficult to establish. We have developed a novel transplantable xenograft murine model of CLL by engrafting the CLL cell line MEC1 into *Rag2*$^{-/-}\gamma_c^{-/-}$ mice. These mice lack B, T, and natural killer (NK) cells, and, in contrast to nude mice that retain NK cells, appear to be optimal recipient for MEC1 cells, which were successfully transplanted through either subcutaneous or intravenous routes. The result is a novel in vivo model that has systemic involvement, develops very rapidly, allows the measurement of tumor burden, and has 100% engraftment efficiency. This model closely resembles aggressive human CLL and could be very useful for evaluating both the biologic basis of CLL growth and dissemination as well as the efficacy of new therapeutic agents.

3. HS1 has a central role in the trafficking and homing of leukemic B cells

Cristina Scielzo[1,2]*, Maria T.S. Bertilaccio[1]*, Giorgia Simonetti[3,4], Antonis Dagklis[3], Elisa ten Hacken[3], Claudia Fazi[3], Marta Muzio[1], Valeria Caiolfa[6,7], Umberto Restuccia[2,8], Angela Bachi[8], Martina Rocchi[9], Maurilio Ponzoni[9,10], Paolo Ghia[3,5,10] and Federico Caligaris-Cappio[1,5,10]

[1]Unit of Lymphoid Malignancies, Division of Molecular Oncology, San Raffaele Scientific Institute, Milano, Italy; [2]Università degli Studi di Milano, Milano, Italy; [3]Unit of B Cell Neoplasia, Division of Molecular Oncology, San Raffaele Scientific Institute, Milano, Italy; [4]Università degli Studi Milano-Bicocca, Milano, Italy; [5]Università Vita-Salute San Raffaele, Milano, Italy; [6]Unit of Dynamic Fluorescence Spectroscopy, Division of Molecular Oncology, San Raffaele Scientific Institute, Milano, Italy; [7]Italian Institute of Technology Network Research, Unit of Molecular Neuroscience,San Raffaele Scientific Institute, Milano, Italy; [8]Biomolecular Mass Spectrometry Unit, Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milano, Italy; [9]Pathology Unit, Division of Molecular Oncology, San Raffaele Scientific Institute,Milano, Italy; [10]Clinical Unit of Lymphoid Malignancies, Department of Oncology, San Raffaele Scientific Institute, Milano, Italy.

** these authors equally contributed to the work*

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ABSTRACT

The function of the intracellular protein Haematopoietic cell specific Lyn substrate-1 (HS1) in B lymphocytes is poorly defined. To investigate its role in migration, trafficking and homing of leukemic B lymphocytes we have utilized B cells from $HS1^{-/-}$ mice, the HS1-silenced human Chronic Lymphocytic Leukemia (CLL) MEC1 cell line and primary leukemic B cells from CLL patients. We have used both *in vitro* and *in vivo* models and found that the lack of expression of HS1 causes several important functional effects. *In vitro*, we observed an impaired cytoskeletal remodeling which resulted in diminished cell migration, abnormal cell adhesion and increased homotypic aggregation. *In vivo*, immunodeficient $Rag2^{-/-}\gamma_c^{-/-}$ mice injected with HS1-silenced CLL B cells showed a decreased organ infiltration with the notable exception of the bone marrow (BM). The leukemic-prone Eμ-TCL1 transgenic mice crossed with HS1-deficient mice were compared with Eμ-TCL1 mice and revealed an earlier disease onset and a reduced survival. These findings demonstrate that HS1 is a central regulator of cytoskeleton remodeling that controls lymphocyte trafficking and homing and significantly influences the tissue invasion and infiltration in CLL.

4. Lack of TIR8/SIGIRR triggers progression of chronic lymphocytic leukemia in mouse models

Maria Teresa Sabrina Bertilaccio[1], Giorgia Simonetti[1], Antonis Dagklis[1], Martina Rocchi[2], Tania Veliz-Rodriguez[1], Benedetta Apollonio[1], Alberto Mantovani[3], Maurilio Ponzoni[2,4], Paolo Ghia[1,4,5], Cecilia Garlanda[3], Federico Caligaris-Cappio[1,4,5], Marta Muzio[1]

[1] Division of Molecular Oncology, San Raffaele Scientific Institute, Milano, Italy; [2] Pathology Unit, Department of Oncology, San Raffaele Scientific Institute, Milano, Italy; [3] Fondazione Humanitas per la Ricerca, Rozzano, Italy; [4] Clinical Unit of Lymphoid Malignancies, Department of Oncology, San Raffaele Scientific Institute, Milano, Italy; [5] Vita-Salute San Raffaele University, Milano, Italy

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ABSTRACT

Inflammation is involved in the initiation and progression of several chronic lymphoid malignancies of B-cell type. Toll-like receptors (TLRs) are transmembrane inflammatory receptors that upon recognition of pathogen-associated molecular patterns trigger an innate immune response and bridge the innate and adaptive immune response by acting as costimulatory signals for B cells. Fine tuning of TLR and IL-1R like (ILR) activity is regulated by TIR8 (SIGIRR), a transmembrane receptor of the TLR/ILR family which inhibits other family members. To test the hypothesis that TLR and/or ILR may play a role in the natural history of chronic B-cell tumors, we crossed Eμ-TCL1 transgenic mice, a well established model of chronic lymphocytic leukemia (CLL), with mice lacking the inhibitory receptor TIR8 that allow an unabated TLR-mediated stimulation. We here report that in the absence of TIR8 the appearance of monoclonal B-cell expansions is accelerated and mouse life span is shortened. The morphology and phenotype of the mouse leukemic expansions reproduce the progression of human CLL into an aggressive and frequently terminal phase characterized by the appearance of prolymphocytes. This study reveals an important pathogenetic implication of TLR in CLL development and progression.

5. SIGLEC-G deficiency increases susceptibility to B cell lymphoma development

Giorgia Simonetti[1,2*], Maria Teresa Sabrina Bertilaccio[1*], Tania Veliz Rodriguez[1], Antonis Dagklis[1], Martina Rocchi[3], Thomas H Winkler[4], Lars Nitschke[5], Maurilio Ponzoni[3,6], Federico Caligaris-Cappio[1,6,7] and Paolo Ghia[1,6,7]

[1] Division of Molecular Oncology, San Raffaele Scientific Institute, Milano, Italy; [2] Università degli Studi di Milano-Bicocca, Milano, Italy; [3] Pathology Unit, San Raffaele Scientific Institute, Milano, Italy; [4] Department of Biology, Nikolaus Fiebiger Center for Molecular Medicine, Friedrich Alexander University Erlangen-Nuremberg, Erlangen, Germany; [5] Department of Biology, Friedrich Alexander University Erlangen-Nuremberg, Erlangen, Germany; [6] Clinical Unit of Lymphoid Malignancies, Department of Oncology, San Raffaele Scientific Institute, Milano, Italy; [7] Vita-Salute San Raffaele University, Milano, Italy.

** these authors equally contributed to the work*

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6. CONCLUSIONS AND FUTURE PERSPECTIVES

Despite several recent advancements in the treatment of B lymphoproliferative disorders, still a considerable number of lymphoma cases either cannot be cured (as for indolent cases) or become incurable when relapsing (as for aggressive lymphomas). This issue reflects the need for better and more effective therapies that should target in a tailored manner pathogenetic mechanisms specifically acting in each form of the disease. The process of mechanism-based targeting implies several steps (Figure 1): first, identify genes that are involved in lymphomagenesis; second study the function of the protein through *in vitro* and *in vivo* approaches leading to the selection of the most promising targets; third design new specific drugs and investigate their effectiveness and overall toxicity in preclinical testing and fourth identify biomarkers predicting for response in early-phase clinical trials. Mouse models have a central role in drug development as demonstrated by their usage at multiple stages of the process. In particular they are useful to elucidate the mechanism of action of the protein through genetic disruption of the molecule of interest *in vivo* or through adoptive transfer of human cell or cell lines previously manipulated to deregulate the expression of the gene of interest. In addition both genetic-engineered and xenograft mouse models are valuable tools for the first-line testing of new therapeutic strategies.

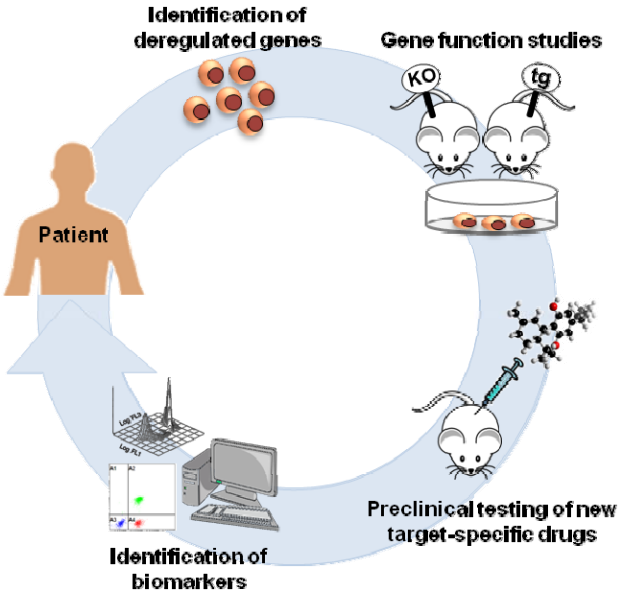

FIGURE 1. **Preclinical steps of targeted drug design including uses of mouse models.**

In this thesis, we have developed a new xenograft model suitable for testing new therapies and in addition we have investigated the role of different molecular pathways (involving HS1, TLRs and SIGLEC-G) in the pathogenesis of B lymphoid malignancies using genetic-engineered mouse models.

Eμ-TCL1 tg mice represent to date the most helpful animal model for studying CLL[1,2]. Though the murine disease does not completely resemble the human situation due to lack of specific molecular abnormalities[3], differences in the surface markers of the leukemic cells (e.g. sIG and CD23) and site of origin of the malignant expansion (peritoneal cavity vs. bone marrow and/or

lymph nodes), this mouse model displays several advantageous features including the homogeneous phenotype of the disease and the high penetrance (100%). Therefore it has been extensively used to study the role of multiple cellular components in the onset and progression of the disease through genetic ablation (Frizzled 6[4], PKCβ[5], Receptor tyrosine kinase-like orphan receptor 1 (ROR1)[6], RAS homolog gene family, member H (RHOH)[7]), or ectopic expression (BAFF[8]) of the gene of interest in the Eμ-TCL1 tg background. This animal model also shows similar clinical and therapeutic response properties to human CLL[9] and has been used for pre-clinical purposes. In particular it has been demonstrated that the SYK inhibitor R788 prolongs the survival of leukemic mice by inhibiting antigen-dependent BCR signals in Eμ-TCL1 tg mice[10]. However, as expected for a mouse model of a chronic indolent disease, the murine leukemia has a long latency and may not be optimal to study specific anti-human therapeutic strategies.

To overcome this limitation, we have developed a novel xenograft model of CLL by engrafting the CLL cell line MEC1 into *Rag2*$^{-/-}\gamma_c^{-/-}$ mice through either subcutaneous or intravenous routes (chapter 2). The disease arising in the recipient mice shows systemic involvement, resembles the aggressive human CLL and has 100% engraftment efficiency. In addition responsiveness to systemic treatment with fludarabine alone or in combination with cyclophosphamide indicates that this model is a reliable preclinical tool for testing new therapeutic agents. Accordingly, it has been used for

studying the *in vivo* efficacy of a novel immunotherapy strategy based on anti-CD23 chimeric antigen receptor (CD23.CAR), a molecule containing the extracellular binding moiety of CD23 coupled to the CD28-costimulatory endodomain and the ζ chain of the TCR/CD3 complex[11]. Since CLL cells express high levels of CD23, human T cells transduced with the CD23.CAR are able to selectively recognize and kill them *in vitro* and to control tumor growth in our xenograft model leading to delayed disease progression.

This xenograft model is also a valuable tool to dissect how innate and adaptive immune cell populations contribute to antitumor response and how microenvironmental influences shape leukemic growth. It has been shown that MEC1 (chapter 2) and MEC2[12] cell lines do not engraft in nude mice, an athymic model lacking T cells. Conversely MEC1 cells can be transplanted into *Rag2*$^{-/-}\gamma_c^{-/-}$ mice lacking B, T and NK cells. These results suggest that NK cells play an important role in the immunosurveillance of the leukemic cells. Indeed NK cells isolated from CLL patients, especially in the advanced stage of the disease, show defective cytotoxic activity[13,14]. Therefore investigating the pathways involved in the interplay between NK and leukemic cells and the functional alterations induced by malignant cells would be important to design improved immunotherapeutic strategies[15]. In addition adoptive transfer experiments would help to clarify the role of distinct T cell subsets in the disease history and to better understand the

interaction between neoplastic cells and microenvironmental components.

To this purpose, one has to keep in mind the central role of the microenvironment as underlined by the presence of specific proliferative compartments, the PCs, in the spleen, lymph nodes and bone marrow that sustain the bunch of resting leukemic cells in the periphery. Understanding the mechanisms that drive trafficking and homing of CLL cells from and to the tissues remains one major challenge. Given that the cytoskeleton controls cellular shape modelling e.g. in the context of cellular movements and migration, and that HS1, a prognostic factor for CLL interacts with multiple cytoskeletal components[16], we aimed at elucidating whether HS1 absence interfered with the motility of leukemic cells (chapter 3). We have demonstrated that HS1 is important for the proper organization of the cellular cytoskeleton and for spontaneous migration, adhesion and homotypic aggregation of the cells. We took advantage of the previously established xenograft model to study the migration properties of HS1-deficient MEC1 cells and we observed that, in the absence of HS1, MEC1 cells preferentially infiltrate the bone marrow of the transplanted mice. Similarly, *in vivo* migration assays showed that the absence of HS1 leads to an early onset of the disease and a more severe involvement of the bone marrow in Eμ-TCL1 tg animals. This phenotype may be explained by dysregulated response of HS1-deficient cells to chemoattractant stimuli, inability to exit the bone marrow after entering it, increased

survival or proliferation in the bone marrow microenvironment. Interestingly, primary CLL cells carrying hyperphosphorylated HS1 accumulate in the bone marrow of recipient mice, prompting us to investigate the link between HS1 absence/hyperphosphorylation and the bone marrow localization of leukemic cells, in the future.

Results from these and similar studies might lead to the design of specific therapeutic agents favouring the mobilization of CLL cells from the PCs into the blood stream where they are more easily targeted by current pro-apoptotic chemotherapies, that are, conversely, rather inefficacious against leukemic cells embedded in the tissues. A similar though initially unexpected effect has been recently achieved by the use of an inhibitor of the BCR downstream molecule PI3Kδ, namely CAL-101[17]. Beside blocking the BCR signalling pathway and prompting apoptosis of CLL cells, CAL-101 causes rapid lymph node shrinkage and transient lymphocytosis in the clinical practice by reducing the interactions that retain CLL cells in the tissue microenvironments[18]. In particular it inhibits CLL cell chemotaxis toward CXCL12 and CXCL13 and migration beneath stromal cells.

Therefore understanding HS1 dynamics may help to design novel therapeutic strategies able to decrease leukemic cell homing to the bone marrow. To this purpose we plan to first characterizate HS1 phosphorylation status in proliferating cells located in the lymph nodes and bone marrow. Since HS1-deficient cells resemble CLL cells carrying hyperphosphorylated

HS1 (that characterizes the bad prognosis subset of patients) and since lack of HS1 causes a more aggressive phenotype *in vivo*, we think that high level of HS1 phosphorylation most likely induces inactivation or functional alterations of the protein. Therefore it will be relevant to identify the sites of tyrosine phosphorylation of HS1 in CLL cells in order to study their specific role in the recruitment of molecular interactors in the context of BCR signalling pathway and cell migration.

Along the same reasoning, we are now aware that leukemic cells in CLL are still responsive to external stimuli originating from the microenvironment including stimulation through the BCR. This is a central event in CLL pathogenesis[19] and the finding of HCDR3 stereotypes in more than 20% of unrelated and geographically distant CLL cases corroborates the hypothesis that antigenic exposure is crucial[20]. Evidence also suggests that costimulatory signals are needed for CLL development and progression such as those delivered through TLRs. We have demonstrated that the lack of the negative regulator of TLR/IL1R signalling, TIR8, accelerates disease progression in Eμ-TCL-1 tg mice and favours transformation of the disease into a prolymphocytoid-like phenotype (chapter 4).

B cell prolymphocytic leukemia is a rare aggressive disease that in some cases originates from transformation of CLL[21]. It is characterized by the accumulation of large B cells (prolymphocytes) that may lose the expression of CD5. Our results indicate that chronic inflammation, as the one occurring after unabated TLR signalling, may favour CLL progression to

this aggressive form. Indeed histopathological evaluation of lymphoid tissues of TIR8-deficient Eμ-TCL1 tg mice revealed clear areas of "prolymphocytoid" transformation. We plan to characterize the phenotype and molecular features of this population in comparison with small CLL-like lymphocytes to identify the main mechanisms involved in the transformation. This analysis may also give us new insights in the function of CD5 in B cell malignancies.

To further elucidate the role of TLRs in CLL, we plan to study different components of the pathway. We have started from MYD88, a TIR-domain-containing adaptors for the TLR/IL-1R superfamily that is engaged by several receptor complexes to positively regulate activation of downstream transcription factors[22]. MYD88 is required for the activation of NF-κB, JNK and p38, moreover a recurrent mutation in the MYD88 gene has been recently identified in 2.9% of CLL patients[23,24]. We have hypothesized that blocking the signal transduction pathway may delay the disease onset and/or progression in the Eμ-TCL1 tg model. However preliminary results seem to rule out a specific function of the molecule in the history of CLL. This may have at least two explanations. First, since MYD88 has multiple functions in different cell types, the analysis of a chronic B cell phenotype may be compromised by other immunological defects due to the complete deficiency of MYD88[25]. Second, TLR4 and TLR3, the only receptors that can transduce the signal by the alternative use of TIR-domain-containing adaptor protein inducing IFNβ (TRIF), independent

of MYD88, may be the key players in the disease. Future studies and alternative strategies will clarify this issue.

Finally, given the pivotal role of tonic BCR stimulation in CLL pathogenesis, we have analyzed mice deficient for the negative regulator of BCR-mediated calcium response SIGLEC-G. *Siglecg*$^{-/-}$ mice show an early expansion of CD5^{+} B cells in the spleen and peritoneal cavity, that we hypothesized might anticipate leukemia onset, as suggested by CLL mouse models[1,3,26]. Surprisingly, the lack of this negative regulator leads to development of CD5^{-} B cell lymphomas that are compatible with diagnosis of DLBCL, FL and medium-to-large B cell monomorphic lymphoma at histopathological evaluation. Similarly, *in vitro* studies revealed a dramatic decrease in the expression level of the human counterpart, SIGLEC10, in B cell lymphoma and leukemia cell lines compared to normal circulating and tonsillar B cells. We are now planning to analyze primary tissues from lymphoma patients and compare to normal lymphoid tissue in order to confirm our result.

To better understand the biological consequences of the downregulation we will clone SIGLEC10 and reintroduce it in human B cell lymphoma cell lines (e.g. DHL4), followed by xenograft transplantation, as previously reported[27]. This approach will allow us to elucidate whether SIGLEC10 downregulation affects tumour development, cell proliferation, activation and/or dissemination based on the hypothesis that SIGLEC10 expression will delay tumour engrafment and expansion. The engineered cell lines will also offer a good tool

to dissect the molecular components of SIGLEC10 pathway in order to design novel therapeutic strategies. A potential role of SIGLEC10 in B cell lymphoma is supported by previous studies showing that SHP-1, a signal transduction molecule recruited by SIGLEC10, is silenced by aberrant CpG methylation in several types of B cell lymphoma including FL and DLBCL and especially in cases with high-grade disease[28]. We plan to confirm the interaction between SIGLEC-G and SHP-1 by immunoprecipitation and to identify new partners.

In particular we will focus on SIGLEC-G/10 ligands. Though the sialoside specificity of Siglecs has been deeply investigated[29], little is known about specific biologically-relevant antigens. The sialoglycoprotein CD24 has been shown to bind SIGLEC10 in order to downregulate immune response to proteins released by damaged cells[30] and to negatively regulate B cell development and activation[31]. CD24 is expressed in early B cell progenitors[32] and resting mature B cells but is downregulated from the GC stage to plasma cell differentiation. We are planning to test the biological effect of exogenous CD24 on the growth and survival of normal and malignant B cells. Based on these results, new small molecules may be designed aimed at forcing activation of SIGLEC10 or of downstream signaling molecules and thus restoring the antitumorigenic function of the pathway.

Overall our study and perspectives highlight the importance of dissecting the molecular mechanism of cancer and specifically of lymphomagenesis, in order to select mechanism-

based targeting strategies. In this process mouse models have a double beneficial function: genetically-engineered mouse models which can mimic the progression of specific types of B lymphoid malignancies can contribute to the prioritization of the therapeutic targets while xenograft model can add new insights on human disease and facilitate preclinical testing.

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