Concepts of Chronic Lymphocytic Leukemia Pathogenesis: DNA Damage Response and Tumor Microenvironment

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Introduction

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disorder that accounts for approximately 30% of adult leukemias and 25% of non-Hodgkin lymphomas [1]. It is the most common form of leukemia in the western world with an incidence rate of 4.5–100,000 [1]. CLL is a disease of the elderly with less than 10% of the patients being 40 years or younger and a median age at diagnosis of 72 years [2]. In the clinical setting, CLL represents a very heterogeneous disease, with various forms of manifestation ranging from lymphadenopathy to isolated leukemic effusion with or without B symptoms. Furthermore, treatment response and disease course vary dramatically between patients. Some patients live for decades and do not require any therapeutic intervention, while others suffer from rapidly progressive and refractory disease [3]. This extreme heterogeneity results in a therapeutic dilemma, as it is still not entirely clear who will benefit from early and aggressive intervention and who should not be treated. Although the introduction of novel targeted agents (e.g. the BTK inhibitor ibrutinib, the PI3Kδ inhibitor idelalisib or the BCL2 inhibitor ABT-199) into our therapeutic armamentarium has opened new therapeutic horizons, there is currently no curative therapy besides allogeneic stem cell transplantation, for which most patients do not qualify due to age or lack of fitness [4–6]. Inefficiency of the current gold standard chemoimmunotherapy (fludarabine, cyclophosphamide and rituximab, FCR) to cure CLL patients is caused by 2 central aspects. On the one hand, common cytogenetic alterations are associated with inherent resistance against genotoxic therapies. For instance, losses on the short arm of chromosome 17 (del(17p)), affecting the prominent tumor suppressor TP53, are commonly observed in CLL [7]. In addition, losses on the long arm of chromosome 11 (del(11q)), in large part affecting the proximal DNA damage response (DDR) kinase ATM, which is critical for p53 activa-
tion in response to genotoxic stress, are frequently present in CLL [7–9]. Moreover, aberrant pathways in CLL include core components of the DDR (p53, ATM), DNA damage-induced apoptosis (BCL2, MCL1, certain microRNAs) and B-cell receptor (BCR) signaling [7, 10–12]. In addition, recent data obtained by whole-genome sequencing of CLL patients suggest that the ATM, TP53, CHEK2, NOTCH1, XPO1, SF3B1, IKZF3, MAP2K1 and MYD88 genes are recurrently mutated in CLL cells [13, 14] (fig. 1). However, the functional consequences and prognostic value of these mutations still need to be elucidated in detail. Indeed, a novel comprehensive prognostic index for CLL patients, obtained by taking 1,948 patients from 3 independent prospective clinical trials into consideration, identified 8 independent predictors of overall survival: sex, age, ECOG status, del(17p), del(11q), IGHV mutation status, serum β2-microglobulin, and serum thymidine kinase [15]. Besides genetics, the second driver of CLL is its tumor microenvironment, which even has an impact on the DDR itself. Novel therapeutic strategies, therefore, try to circumvent defective DDR signaling and to suppress the pro-survival stimuli received from the tumor microenvironment.

**CLL Cells Receive Critical Survival Signals from Their Microenvironment**

A hallmark feature of CLL is its dependence on extracellular stimuli, such as those received through (auto)antigens signaling via (stereotyped) BCRs, signals received through CD40/CD40L engagement, insulin-like growth factor receptors (IGFRs), VEGF receptors, chemokine receptors and Toll-like receptor (TLR) signaling [16–20]. Signals received through these various receptor molecules trigger numerous cellular responses, including cell cycle progression and the activation of critical intracellular survival pathways, which counteract the apoptotic machinery in CLL cells [21]. In fact, CLL is commonly considered as a paradigm for a malignancy of failed apoptosis [22], as CLL cells circulating in the blood are largely non-proliferating and arrested in the G0/G1 phase of the cell cycle. Cell division must occur, presumably in proliferation centers in specialized niches, accounting for the inevitable rise in white blood cell counts in some patients and evidenced by the shortening of telomeres in CLL cells [23]. However, lack of apoptosis is considered a major component of the dysregulation of normal B-cell homeostasis in all subsets of this malignancy. This apoptosis resistance of CLL cells is the result of both cell-autonomous and non-cell-autonomous mechanisms. Indeed, cell-autonomous overexpression of X-linked inhibitor of apoptosis protein (XIAP) and acyl-protein thioesterases (A PTs) 1 and 2 causes resistance of CLL cells towards CD95- and TRAIL-mediated killing [24, 25]. Furthermore, core components of the DDR that are required to relay the presence of genotoxic lesions to the apoptotic machinery, such as ATM and p53, are frequently mutationally inactivated in CLL [7]. On the other hand, CLL cells receive a variety of extracellular pro-survival signals, which ultimately blunt the apoptotic signaling network within CLL cells [16–20]. Important in this regard is the observation that survival of CLL cells requires sustained ligand-dependent or ligand-independent (‘tonic’) BCR signaling via auto- or superantigens [26]. BCR stimulation results in intracellular recruitment and activation of the kinases Syk, ZAP-70, Lyn and BTK at tyrosine-based activation motifs of the BCRs [26]. As a result, the canonical MEK/ERK MAPK, the PI3K/AKT and the NFkB pathways become activated to antagonize (DNA damage-induced) apoptosis and to stimulate cell proliferation. For instance, AKT is critical for sustained expression and p38 for suppression of the anti-apoptotic molecule MCL1 in CLL cells [27, 28]. Furthermore, NFkB-dependent transactivation of the anti-apoptotic genes Bcl2, Bcl-xl and Bfl1/A1, as well as different inhibitor of apoptosis proteins (IAPs) has been shown to occur downstream of BCR and TLR signaling in CLL cells [29–31]. Given these observations, it might not be surprising that expression of the above-mentioned
BCR-related proteins, as well as the mutational status of the BCR itself have prognostic impact in CLL, further underscoring the pathophysiological importance of extracellular stimuli for CLL cell survival [10, 27, 32–41]. Notably, the lymph node microenvironment for CLL patients promotes BCR signaling associated with NFκB activation [42].

**Hypoxia, an Underestimated Factor**

A major determinant, which is at the heart of cell-autonomous and non-cell-autonomous mechanisms and links the tumor microenvironment with the DDR and drug-response, is the physiological oxygen tension in the tumor microenvironment. While experiments that investigate the tumor microenvironment in vitro are mostly performed under 21% oxygen tension, the ‘real’ oxygen concentration in the tumor microenvironment is usually far below 5%, in most areas of the lymphatic tissues even around 1% [43]. Despite its evident importance in solid tumors, the impact of hypoxia on CLL is only poorly understood [44]. Indeed, it was shown that hypoxia directly impacts on DDR signaling by protecting CLL cells from fludarabine- and bendamustine-triggered apoptosis, which is a reasonable explanation for why CLL cells are not efficiently targeted in the tumor microenvironment and display a niche for relapse [28]. In contrast, the efficacy towards the BH3-mimetic ABT-737 and the clinical compound venetoclax (ABT-199) was increased [28]. This was due to uncoupled activation of central signaling pathways: while phosphorylation of AKT, ERK1/2 and NFκB was reduced, p38 became hyp- or phosphorylated under hypoxic conditions [28]. Phospho-p38 (pp38) down-regulated Mcl-1 and thereby increased sensitivity to BH3 mimetics, hence pp38 inhibition could reduce susceptibility [28]. The importance and specificity of the pp38-Mcl-1 axis for killing by BH3 mimetics was further emphasized by shifted pp38-Mcl-1 levels in B-cell lines with acquired ABT-737 resistance [28]. In contrast, pp38-Mcl-1 levels were not affected in the same cell lines with acquired fludarabine resistance [28]. This uncoupling from the signaling pattern of the other investigated phosphokinases indicates that pp38 might play a unique role in the signaling in hypoxia [28]. This observation makes CLL cells under hypoxic condition ideal candidates to be targeted by the BH3-mimetic venetoclax (ABT-199) [28]. Hypoxic conditions (1% oxygen tension) thus need to be considered for further drug tests.

**Misregulated NFκB Signaling Contributes to CLL Pathogenesis**

Beyond BCR signaling, a plethora of additional immune-related signaling cascades have been shown to be involved in CLL leukemogenesis and maintenance, including CD40/CD40L-mediated signals, and chemokine and TLR signaling [16]. A striking commonality of all these signaling modules is the activation of the NFκB pathway, which in turn has been shown to mediate CLL cell survival and is further emerging as a prognostic marker in CLL [16, 29]. An important consequence of NFκB activation in CLL cells is the expression of anti-apoptotic genes, such as Bcl-xl and Mcl1, whose gene products have both been shown to mediate resistance against p53-driven apoptosis following DNA-damaging chemotherapy or treatment with BH3 mimetics, which target anti-apoptotic BCL-2 family proteins [16, 45–48]. In fact, the NFκB pathway has been proposed to act as a functional antagonist of p53 signaling, which is the major driver of apoptosis following DNA damage [49]. The tumor suppressor p53 limits the consequences of (genotoxic) stress through the induction of apoptosis or senescence [50], while the oncogene NFκB promotes cell division and initiates innate and adaptive immune responses following extrinsic stress, such as cytokine activation or infection [51]. Thus, these 2 transcription factors have adopted diametrically different strategies to cope with cellular stress and cannot function in the same cell at the same time. On activation of 1 of these transcription factors, the other is inactivated through several regulatory signaling nodes in the p53 and NFκB pathways. For instance, AKT, which is known to be a downstream component of BCR signaling, phosphorylates and inactivates Bad, a potent pro-apoptotic protein, which is transcriptionally activated by p53 [52]. AKT also phosphorylates MDM-2 to increase its ubiquitin ligase activity, resulting in p53 ubiquitination and subsequent degradation [53]. AKT further phosphorylates and activates IKK. Once activated, IKK phosphorylates IκB, resulting in β-TrCP-dependent degradation of IκB, ultimately leading to NFκB activation [49]. In parallel IKK also directly phosphorylates p53 on Ser-362 and Ser-366. These phosho-epitopes are subsequently recognized and bound by β-TrCP, leading to p53 poly-ubiquitination and degradation [49]. In contrast, the tumor suppressor ARF acts as an inhibitor of MDM-2, leading to increased cellular p53 levels, while simultaneously activating the ATR/Chk1 checkpoint kinase complex, which in turn phosphorylates and inhibits NFκB [49].

Recently, the involvement of an additional class of immuno-receptors – the TLRs – in the pathobiology of CLL has moved into the focus of scientific investigation. TLRs are expressed on the majority of immune cells characterized to date. CLL cells, in particular, express a distinct pattern of TLRs, namely TLR1, TLR2, TLR6, TLR7, TLR9 and TLR10, that is similar to the repertoire present in activated B cells [19]. TLRs recognize and engage a set of different molecular patterns present on a diversity of invading microbes. They bridge the innate and adaptive immune responses through the delivery of costimulatory signals in B cells after antigen recognition, ultimately contributing to maturation, proliferation and antibody secretion [54]. On a molecular level, TLR stimulation has been shown to drive NFκB activation [31]. There is accumulating evidence suggesting that TLRs are not only expressed but also active in CLL cells, as their expression correlates with the ability to respond to specific TLR agonists [19]. When activated by ligand binding, TLRs recruit adaptor molecules to their cytoplasmic domains [54]. Most TLRs express cytoplasmic TIR domains that are responsible for binding of the adaptor protein MyD88. Curiously, mutation (p.L265P) in the MYD88 gene was recently identified as a recurrent mutation in 9 of 310 CLL patients by whole genome se-
quencing [55, 56]. MyD88 immunoprecipitates from CLL cells carrying the p.L265P mutation revealed large amounts of co-precipitating IRAK1, in contrast to cells lacking this mutation [55]. Furthermore, the NfκB p65 subunit showed increased phosphorylation in MYD88-mutated compared to unmutated CLL cells, and there was an increased DNA-binding activity of NfκB in MYD88-mutated CLL cells [55]. These data support the hypothesis that the MYD88 p.L265P mutation constitutes an activating mutation of this novel proto-oncogene. Stimulation of TLRs in MYD88-mutated CLL cells resulted in an increased expression of IL-6, as well as CCL2, 3 and 4, when compared to the secretion of these cytokines by MYD88-unmutated CLls after TLR stimulation [55]. The increased secretion of these cytokines has been implicated in the recruitment of macrophages and T lymphocytes by CLL cells, creating a favorable niche for their survival [57]. Moreover, activation of TLRs in CLL cells promotes the proliferation of tumor cells and protects them from spontaneous apoptosis [19]. Patients with MYD88-mutated CLL were diagnosed at a younger age than those with wild-type MYD88 and the disease presented with a more advanced clinical stage, although no differences were observed in progression or survival rates [55]. Furthermore, recent CLL re-sequencing data suggest that the MYD88 p.L265P mutation occurs early during CLL development and might constitute an initiating event in CLL leukemogenesis [56].

**Misregulated DDR Signaling Contributes to CLL Pathogenesis**

A second hallmark feature of CLL cells, besides their dependence on extracellular stimuli, is a high frequency of genomic aberrations, which can be observed in the majority of CLL patients [7, 58–60]. From a clinical perspective, the failure of all conventional chemotherapies to induce long-lasting remissions might suggest that the induction of apoptosis, which is initiated through the DDR, is dysfunctional in CLL. Mutational inactivation of different components of the DDR is an established hallmark of cancer [61, 62]. The proximal DDR consists of 2 major kinase branches, the ATM/Chk2 and the ATR/Chk1 pathways [63]. The upstream kinase ATR is activated largely in response to single-strand breaks and bulky DNA lesions, such as those induced by platinum-based drugs. Once activated, ATR phosphorylates and activates its effector kinase Chk1. In addition, the upstream kinase ATM, signaling through its effector Chk2, is activated primarily in response to DNA double-strand breaks (DSBs), such as those induced by alkylating agents, topoisomerase inhibitors or ionizing radiation [63]. Both kinase pathways converge on p53, which becomes stabilized upon phosphorylation and locates to the nucleus where it acts as a transcription factor transactivating numerous target genes, such as the pro-apoptotic BH3-only genes PUMA and NOXA [64]. Interestingly, losses or mutations of TP53 and ATM are common events in CLL and are associated with poor response to conventional chemo-immunotherapy and adverse prognosis [7, 65].

In agreement with an increased genomic instability of CLL cells, numerous recurrent genomic aberrations have been identified. The clinically most relevant aberrations affect the prominent tumor suppressor TP53, which can be inactivated through at least 2 distinct mechanisms in CLL. First, large deletions on the short arm of chromosome 17 (del(17p)) are found in approximately 7% of treatment-naive patients [7]. These del(17p) almost invariably include band 17p13, which harbors the TP53 gene. In CLL patients, del(17p) has been shown to be associated with marked resistance against genotoxic chemotherapies, which cannot be overcome by the addition of anti-CD20 antibodies in the context of state-of-the-art chemo-immunotherapy [9]. Besides chromosomal losses, recurrent protein-damaging TP53 point mutations have been detected in 4–37% of patients with CLL, and are associated with very poor prognosis [11]. Among cases with confirmed del(17p), the majority show mutations in the remaining TP53 allele (> 80%) [16]. In cases without del(17p), TP53 mutations are much rarer, but have a similarly detrimental effect on chemotherapy response and overall survival [16]. TP53 mutations are also associated with higher genomic complexity in CLL, indicating that a crippled DDR promotes a ‘mutator phenotype’ in CLL [16]. Lastly, inactivating TP53 mutations are substantially enriched in patients who have been exposed to genotoxic chemotherapy, suggesting that an inactivation of the pro-apoptotic ATM-Chk2-p53 signaling cascade is selected for in CLL [13, 14, 55, 56].

Besides del(17p), deletions of the long arm of chromosome 11 (del(11q)) are detected in approximately 25% of previously untreated CLL patients with advanced disease stages and approximately 10% of patients with early stage disease [14, 55, 66]. Furthermore, del(11q) CLLs are associated with bulky lymphadenopathy and ZAP-70 expression [67]. These deletions frequently encompass band 11q23 where the ATM gene is located. Approximately 40% of del(11q) CLL patients display inactivating mutations of the second ATM allele and these cases show a poor chemotherapy response, reminiscent of that described for TP53-mutant CLls [68]. In addition, patients carrying a del(11q) clone typically show rapid disease progression, and reduced overall survival [16]. Reminiscent of CLls carrying a TP53 alteration, disabling ATM mutations are enriched in chemotherapy-exposed patients, further suggesting that an inactivation of the pro-apoptotic DDR is selected for in CLL [14, 55].

Deletions on the long arm of chromosome 13, specifically involving band 13q14 (del(13q14)) is the single most frequently observed cytogenetic aberration in CLL, occurring in approximately 55% of all cases. An isolated del(13q14) is typically characterized by a benign course of the disease. The miRNAs, miR-15a and 16–1, were recently identified to be located in the critical region of del(13q14) [69]. The pathophysiological role of these miRNAs is further underscored by the phenotype of genetically engineered mice carrying a targeted deletion of the mir-15a/16–1 locus in combination with a deletion of the non-coding RNA gene DLEU2. These animals develop a monoclonal B-cell lymphocytosis-like disorder, CLL and lymphoma, suggesting that the miRNAs 15a and 16–1 indeed play a role in CLL leukemogenesis [70].
Lastly, trisomy 12 is observed in 10–20% of CLL patients. However, the genes involved in the pathogenesis of CLLs carrying a trisomy 12 are largely unknown, and the prognostic relevance of trisomy 12 remains a matter of debate [16].

A number of recently reported comprehensive DNA sequencing projects in CLL have revealed a number of recurrent somatic gene mutations that occur in parallel to the above-mentioned structural genomic aberrations. These include, but are not limited to, the genes NOTCH1, MYD88, TP53, ATM, CHEK2, SF3B1, FBXW7, POT1, CHD2, RPS15, IKZF3, and MAP2K1 [13, 14, 55, 56]. Of note, TP53, ATM, POT1 and CHD2 encode for proteins critically involved in DNA damage signaling and DNA repair [71–74]. Intriguingly, both del(17p) and del(11q), as well as inactivating somatic mutations in TP53 and ATM, are critical in patients with secondary resistance to DNA-damaging chemotherapy [14, 55]. This observation underscores the critical importance of the ATM-Chk2-p53 signaling axis in mediating apoptosis in response to DNA damage in CLL.

Upon excessive DNA damage, such as that induced by chemoradiation therapy, the DDR network promotes the induction of apoptosis through the transactivation and post-translational stabilization of pro-apoptotic Bcl2 family members. An impaired apoptotic response upon cellular stress or DNA damage is a further established hallmark of cancer and a central feature of CLL [61, 75]. Mitochondria represent the central regulatory node of the apoptotic machinery by releasing pro-apoptotic factors, such as cytochrome c [76]. This process is tightly controlled by the Bcl2 protein family [77], including BH3-only pro-apoptotic Bcl2 proteins such as PUMA and NOXA. Altered expression and function of several Bcl2 protein family members (e.g. BCL2, MCL1, and NOXA) are well documented in CLL [10]. Accordingly, repression of anti-apoptotic BCL2 family members through the use of BCL2 inhibitors, such as ABT199, BCL2 antisense nucleotides or BH3 mimetics have shown some therapeutic efficacy in CLL [6, 78–83].

Concluding Remarks

Given the above-mentioned alterations in cellular signaling circuits in CLL cells, a picture emerges in which the central pro-apoptotic ATM-Chk2-p53 axis, which ultimately results in the transactivation of pro-apoptotic p53 target genes, such as PUMA, NOXA, BAX and BAK, is affected on multiple levels in CLL cells. First, losses or disabling mutations in TP53 and ATM are frequent events in chemotherapy-naive patients and are further enriched in chemotherapy-resistant patients. Second, pathways that are known to directly antagonize pro-apoptotic p53 signaling, such as AKT-, Notch- or MAP kinase signaling are hyperactivated either through mutational activation (stereotyped IGHV, NOTCH) or tonic signaling through niche-dependent transmembrane receptors (BCR, CD40, IGFR and chemokine receptors). Lastly, TLR-dependent NFkB signaling, which acts as a functional antagonist of p53 is frequently activated in CLL. Thus, the cellular DDR and DDR-driven apoptosis are blunted through multiple molecular antagonists in CLL, likely explaining the resistance to apoptosis that is characteristic for this type of leukemia. Blunting of the pro-apoptotic ATM-Chk2-p53 axis likely promotes a ‘mutator phenotype’, which not only promotes the acquisition of additional CLL-driving mutations, but also renders CLL chemotherapy refractory in almost all cases.

Thus, some of the essential biological features of CLL cells can be tied into the concerted malfunction of essential cellular DNA damage-sensing and cell death systems [11, 27, 38, 52, 68, 84, 85]. The diversity of potential alterations of these regulatory mechanisms could contribute to genome instability and explain – at least in part – the impressive heterogeneity of the clinical course of this leukemia [59, 60, 86, 87]. Moreover, each of these physiological systems may represent a suitable target for novel therapeutics in CLL.

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