The microenvironment and treatment resistance in chronic lymphocytic leukemia
Tromp, J.M.

Link to publication

Citation for published version (APA):
Summary

In this thesis, microenvironmental factors which play a role in proliferation and chemoresistance of CLL cells are addressed. The thesis can be divided into two parts. The first part focuses on the interactions between CLL cells with the microenvironment regarding to survival, drug resistance and proliferation. In the second part, new treatment strategies are investigated which overcome drug resistance in an in vitro model mimicking the lymph node (LN) microenvironment.

Growing evidence suggests that survival signals from the microenvironment (bone marrow and secondary lymphoid organs) promote disease progression due to chemoresistance and increased proliferation of CLL cells. A commonly used model to mimic the LN microenvironment in vitro is stimulation of CLL cells with CD40L which is normally expressed on CD4+ T cells. CD40 stimulation of CLL cells results in an increased anti-apoptotic profile and rescues them from drug-induced apoptosis, but does not induce proliferation of CLL cells. In chapter 2, we analysed whether CpG effected in proliferation and survival of CD40-stimulated mutated versus unmutated CLL cells. CpG motifs are present in unmethylated viral and bacterial DNA, but endogenous ligands released during cellular stress are also thought to activate TLR9 receptors. A dichotomy in NF-κB signaling in mutated versus unmutated CLL cells upon simultaneous TLR9 and CD40 stimulation was found. Unmutated CLL cells showed proliferation, NF-κB activation and drugresistance, whereas mutated CLL cells showed decreased NF-κB activity and reversal of chemoresistance upon TLR9 and CD40 stimulation. In addition, activation of the classical NF-κB pathway resulted in an upregulation of Bfl-1 and Bcl-XL was shown to be a downstream target of the alternative NF-κB pathway in CD40-stimulated CLL cells. Importantly, knock down of Bcl-XL sensitized CD40-stimulated CLL cells to cytotoxic drugs. Importantly, in ex vivo CLL LN samples, activation of the classical and alternative NF-κB pathway, as well as Bcl-XL expression was observed, underlining the relevance of the in vitro observations.

In chapter 3, we investigated whether there are additional T cell related factors, in addition to CD40L, which can also influence survival and proliferation of CLL cells. In this study, we showed that both stimulation of CLL cells with CD40L and co-culturing CLL cells with activated T cells resulted in activation and drug resistance. Microarray data revealed that both in vitro systems resembled the in vivo LN situation according to increased NF-κB activity which is an important determinant of increased survival of CLL cells. Interestingly, proliferation is also an important characteristic of CLL cells in LNs in vivo. CD40 stimulation of CLL cells did not result in proliferation. In contrast, co-culturing CLL cells with activated T cells showed proliferation which was dependent on IL-21. In line with these findings, we found that CD40-stimulated CLL cells in the presence of IL-21 showed massive proliferation.
In chapter 4, we investigated the effects of IL-21 in the CD40L transfectant system. In addition to proliferation, IL-21 abrogated CD40-induced drug resistance to several cytotoxic drugs. RNA and protein analyses revealed a decrease in Bcl-X\textsubscript{L} levels when IL-21 was added to the CD40L transfectant system. These data underscore the findings in chapter 2, that Bcl-X\textsubscript{L} is an important determinant of CD40-induced drug resistance. ABT-737 is a potent small-molecule that binds with high affinity to the anti-apoptotic molecules Bcl-X\textsubscript{L}, Bcl-2 and Bcl-w, but not Mcl-1 or Bfl-1. As expected, CLL cells stimulated with CD40L and IL21 remained resistant to ABT-737. Finally, ex vivo CLL LN samples showed IL-21 levels which were higher compared to PB CLL cells corroborating the in vitro findings.

In the second part of the thesis we focus on new targeted treatment strategies to overcome CD40L-induced drug resistance. In chapter 5 we studied the effects of compound A, a small molecule which is designed to mimic Smac/Diablo. In various cancer cell lines compound A induces cell death via cIAP degradation, NF-κB activation and TNFα mediated cell death by activation of TNFR1. TNFR1 activation results in releasing RIP1 from the TNF receptor and incorporation of RIP1 into a complex with caspase8 and Fas-associated death domain (FADD) leading to apoptosis. We found that unstimulated and CD40-stimulated CLL cells were resistant to compound A treatment. Upon Compound A treatment cIAP1 and cIAP2 were degraded although levels of cIAP2 restored during treatment which was not prevented by the addition of PI3K inhibitors. In addition, combining compound A with other extrinsic cell death inducers such as TRAIL and FAS did not reveal any additive or synergistic apoptotic effects in CLL cells. We expect that resistance to compound A might be due to the fact that upon TNFR1 triggering formation of a death domain does not occur.

In chapter 6, we explored which pro- and anti-apoptotic proteins play a role in ABT-737 resistance in CD40-stimulated CLL cells. In time course experiments we found that an enhanced Noxa/Mcl-1 ratio is associated with increased sensitivity for ABT-737 in CD40-stimulated CLL cells. Furthermore, several anti-leukemia agents such as fludarabine and dasatinib resulted in an increased Noxa/Mcl-1 ratio and showed synergistic apoptotic effects with ABT-737. These data provide a rationale to investigate the combination of fludarabine or dasatinib with ABT-737 in a clinical setting as a novel treatment modality for refractory CLL. Noxa levels are significantly lower in CLL cells from ex vivo LNs when compared to CLL cells from peripheral blood. The role of Noxa in the pathogenesis of CLL was investigated in chapter 7 by knocking out Noxa in commonly used mouse models which have a CLL like phenotype, like TCL1 and APRIL Transgenic (Tg) mice. In both APRIL Tg and Noxaapril mice, an accumulation of B220dim/CD19+ B cells in blood, mesenteric lymph nodes, bone marrow, spleen and peritoneum was found. In 8 months old NoxaKO/TCL1 mice we observed a significant increase in levels of peripheral blood B220dim/CD19+ B cells compared to TCL1 Tg mice. These data indicate that Noxa has additional effects on tumour formation in TCL1 Tg mice.
General discussion

Aim
Despite the development of new treatment strategies, CLL is still an incurable disease due to resistance to cytotoxic drugs. Both impaired apoptosis as well as increased proliferation in the lymph node (LN) microenvironment lead to an accumulation of CLL cells. The aim of this thesis was 1): to address which factors contribute to chemoresistance and proliferation in an in vitro model mimicking the LN microenvironment and 2): to explore novel designed targeted therapies in chemoresistant CLL cells.

Major observations:
1. NF-κB activation is an important determinant of survival of CLL cells. Interaction of CLL cells with activated T cells or with CD40L results in NF-κB activation. Both in vitro systems can be used as a model for the in vivo lymph node microenvironment.
2. Stimulation of CLL with activated T cells results, besides activation and chemoresistance, in proliferation of CLL cells which is dependent on IL-21.
3. Combination of the small molecule ABT-737 and cytotoxic drugs which increase the Noxa/Mcl-1 ratio results in synergistic apoptotic effects in vitro and might be used in a clinical setting as a novel treatment modality for CLL.
4. Ablation of Noxa in TCL1 Tg mice leads to a more aggressive CLL phenotype compared to TCL1 Tg mice which confirms a role for Noxa in the pathobiology of CLL.

Implications and discussion

1. Enhanced survival and proliferation of CLL cells through interactions with the lymph node microenvironment

1.1 In vitro CLL models for in vivo lymph nodes
CLL cells accumulate in vivo but rapidly undergo apoptosis in vitro, implying that in vivo CLL cells are dependent on microenvironmental stimuli for survival, such as cell-cell contact and soluble factors (1-3). Besides displaying an intrinsic defect in apoptosis of CLL cells, in vivo studies with heavy water have demonstrated that a considerable fraction of CLL cells shows proliferation (4).
Several in vitro models have been described which mimic the in vivo LN microenvironment of CLL cells. In lymphoid tissue, CLL cells interact with nurse-like cells (NLCs) which secrete stromal cell-derived factor 1 (SDF1, also known as CXCL12), a proliferation-inducing ligand (APRIL) and B cell activating factor (BAFF) which protect CLL cells from apoptosis in vitro (5, 6). Stimulation of CLL cells with fibroblasts expressing CD40L is another widely used model to mimic the LN microenvironment (7-10). CD40-stimulation of CLL cells results in enhanced survival and resistance to cytotoxic drugs. Importantly, these in vitro models of the LN microenvironment, such as stimulation of CLL cells with APRIL, BAFF
or CD40L do not induce massive proliferation. Thus, other factors must be involved in proliferation. Activated CD4+ T cells express CD40L which can interact with CD40 on CLL cells. In addition, follicular helper T cells produce IL-21 (reviewed in (11)). We demonstrate that, besides enhancing survival and drug resistance, co-culturing activated T cells with CLL cells results in an IL-21-dependent proliferation of CLL cells (chapter 3). Several other studies have also shown that T cells play an important role in enhanced survival and proliferation of CLL cells in vivo. Tinhofer et al have demonstrated that CLL patients with unfavourable prognostic factors (e.g., unmutated IGHV genes) have a significant increase in relative numbers of central and effector memory T cells in the CD4+ pool (12). Furthermore, in an adoptive transfer model of CLL, activated autologous T cells induce proliferation of CLL cells in vivo, indicating that CD4+ T cells have an important role in enhanced survival and proliferation of CLL cells (13). In contrast, in the same mice, it has been shown that increased T cell activation leads to an apparent graft-versus-tumor reaction resulting in apoptosis of CLL cells (13). In addition, IL-21 produced by T cells also abrogates CD40-induced drug resistance due to downregulation of Bcl-XL (chapter 4). In previous studies, pro-apoptotic effects of IL-21 have been described in CLL cells. CLL cells treated with IL-21 produce granzyme B and addition of CpG or anti-B-cell-receptor antibody enhance this granzyme B production (14). Variable expression levels of IL-21Rα have been demonstrated on CLL cells (15). High levels of IL-21Rα correlate with enhanced apoptosis induced by IL-21 through upregulation of Bim (15). Interestingly, stimulation of CLL cells with CpG shows elevated IL-21Rα levels leading to enhanced apoptosis (16). Furthermore, IL-21 has been shown to increase apoptosis levels in CLL cells treated with fludarabine or Rituximab (15). In summary, CD40L-CD40 interaction alone results in enhanced survival due to increased NF-κB signaling and induction of anti-apoptotic proteins. IL-21 has proliferative capacities, but can also partially abrogate CD40-induced chemoresistance. Thus, blocking CD40L-CD40 might be an attractive therapy in CLL. Recently, lucatumumab, a fully humanized anti-CD40 antibody was evaluated in a phase I clinical trial (17). Lucatumumab had acceptable tolerability, but only 1 of 26 enrolled relapsed CLL patients showed a partial response, no complete response was reported and 17 patients had stable disease (17). Future studies should focus on combination-based therapies with lucatumumab and cytotoxic agents.

1.2 Signaling pathways in the lymph node microenvironment

In chapter 2, we show that Bcl-XL plays an important role in drug resistance in vitro and demonstrate an association between alternative NF-κB activity and Bcl-XL levels. Nowadays, there are no specific pharmacological inhibitors available to selectively block the alternative NF-κB pathway. Bcl-XL can be targeted by the small molecule ABT-737 which is a BH3 mimetic and binds to Bcl-XL, Bcl-2 and Bcl-w (18). Interestingly, in vivo phase I studies showed that the oral analog of ABT-737, ABT-263 results in a partial response in 35% of refractory or relapsed CLL patients (19, 20). Another signaling pathway which is
constitutively activated in CLL and involved in enhanced survival is the PI3K pathway (21). In vitro treatment of CLL cells with a PI3K inhibitor, CAL-101, has been demonstrated to inhibit B cell receptor signaling (22). In addition, CAL-101 results in a reduction of interactions that retain CLL cells in the protective LN microenvironment via inhibition of chemotaxis of CLL cells toward CXCL12 and CXCL13 (22). These results are in line with the clinical data showing lymph node shrinkage and lymphocytosis during CAL-101 treatment and reductions in circulating CCL3, CCL4, and CXCL13 levels (reviewed in (23)). The Btk inhibitor PCI-32765 has been shown to exhibit similar clinical responses of sustained reduction of lymphadenopathy and transient lymphocytosis (reviewed in (24)). The clinical response reflects impaired integrinα4β1-mediated adhesion of CLL cells to fibronectin and VCAM-1 (25). As observed in CAL-101 treated CLL cells, PCI-32765 also inhibits CXCL12 and CXCL13 signaling, adhesion and migration of CLL cells (25, 26). Therapeutics that target molecules more upstream of the PI3K and NF-κB pathways are protein tyrosine kinase inhibitors (PTK) such as imatinib and dasatinib, which have been shown to overcome CD40-induced drug resistance in vitro (27). Only dasatinib has inhibitory effects on adhesion and migration of CLL cells (unpublished observation CR Geest). Dasatinib is currently being investigated in a clinical trial in our institute in relapsed and refractory CLL in vivo. In another phase II study of dasatinib a partial response was achieved in 3 of the 15 refractory or relapsed CLL patients (28). Further exploration of activated signaling pathways and their downstream targets which result in enhanced survival of CLL cells is mandatory in order to develop drugs to overcome resistance to cytotoxic agents.

2. New treatment strategies to overcome drug resistance of CLL cells in an in vitro model of the lymph node microenvironment

2.1 Treatment strategies; intrinsic cell death inducers

Previously, we have demonstrated that in vivo lymph nodes of CLL patients reveal higher levels of the anti-apoptotic protein Bcl-X_L when compared to peripheral blood CLL cells (9). In vitro, it has been shown that ABT-737 induces apoptosis in unstimulated CLL cells but CD40-stimulated CLL cells are resistant to ABT-737 (27, 29). Resistance to ABT-737 has been described in several cell lines to be due to enhanced Mcl-1 and Bfl-1 expression (30-33). In agreement, we show that an enhanced Noxa/Mcl-1 balance is important in sensitizing CLL cells to ABT-737. Yet, Vogler et al have described that Bcl-X_L and Bfl-1, and not Mcl-1, play a role in ABT-737 resistance in CD40-stimulated CLL cells (29). A variation in the respective CLL co-cultures to stimulate CD40 is the addition of IL-4 by Vogler et al. We are currently exploring the effect of IL-4 on sensitivity to ABT-737 and other drugs, in relation to expression of Bcl-2 family members. It has been postulated that in CLL cells NF-κB activation is involved in upregulation of Mcl-1 levels (34). However, in CD40-stimulated CLL cells inhibition of NF-κB with BAY-11-7082 does not result in a significant decrease in Mcl-1 levels (CR Geest unpublished observation).
Interestingly, we observe a significant increase in Noxa levels upon inhibition of NF-κB in CD40-stimulated CLL cells (chapter 6). These findings are in contrast with studies in which, increased Noxa levels have been described upon p53 activation in certain systems, which was dependent on NF-κB signalling (35, 36). P53 is a known tumour suppressor gene, which is often inactivated in tumours, and NF-κB is known as a pro-survival pathway which often activated in tumour cells (reviewed in (37)). However, upon certain stimuli NF-κB can also augment cell death via p53 activation resulting in an increase of Noxa levels (35, 36). We hypothesize that in CLL cells the NF-κB pathway inhibits Noxa levels via upregulation of genes which function as transcriptional repressors of Noxa.

Drugs such as fludarabine and dasatinib result in an increase in the Noxa/Mcl-1 ratio and show synergistic apoptotic effects when combined with ABT-737 in CD40-stimulated CLL cells (chapter 6). Current trials are investigating the combination of cytotoxic drugs and ABT-263 in vivo. Another treatment strategy is to combine ABT-263 with Mcl-1 inhibitors, such as roscovitine to induce apoptosis in chemoresistant niches (30). An alternative and more direct strategy is to design Noxa-like BH3 mimetics which specifically bind to Mcl-1 (38). This could be an effective therapy especially for CLL cells residing in the LNs, because in vivo CLL cells from LNs showed lower Noxa levels as compared to CLL cells from peripheral blood (9). In addition, Mcl-1 seems to have an important role in enhanced survival of CLL cells in vitro (39). In vivo, it has been shown that high Mcl-1 levels correlate with unfavourable prognostic factors and are predictive of the patients’ clinical outcome (40, 41). To date, no Noxa-specific BH3 mimic with high Mcl-1 affinity has been identified (42). This may be due to that fact that the hydrophobic grove of Mcl-1 shows more rigidity compared to the more flexible hydrophobic groove of Bcl-XL (43). The development of such Noxa-like BH3 mimetics and combination therapy with ABT-263 could have important implications on the clinical outcome of CLL patients. In chapter 7, we confirm a role for Noxa in the pathobiology of CLL by ablation of Noxa in TCL1 Tg mice. NoxaKO/TCL1 Tg mice show significant higher levels of CD5+ B cells compared to TCL1 Tg mice. In contrast, ablation of Noxa in APRIL Tg mice does not lead to an increase of CD5+ B cells. These differences suggest that the leukemogenic properties of transgenic TCL1 are at least partially suppressed by Noxa.

2.2 Treatment strategies; extrinsic cell death inducers

Besides the development of treatment strategies inducing apoptosis via the intrinsic pathway, there are also advances in inventing strategies which lead to cell death via activation of receptors of the extrinsic apoptotic pathway. A small molecule which has been shown to induce cell death in various cancer cell lines is compound A which mimics the function of Smac/Diablo. Compound A induces cIAP1 and cIAP2 degradation resulting in NF-κB activation and TNFα mediated cell death via binding to TNFR1 (44, 45). CD40-stimulated CLL cells treated with compound A produce TNFα and this leads to TNFR1 activation. However, this does not lead to induction of cell death in
CLL cells (chapter 5). Cancer cell lines which are sensitive for compound A treatment have been shown to form a complex containing a death domain including RIP1, FADD and caspase 8 (46, 47). Whether this complex including RIP1, FADD and caspase 8 is formed in CLL cells is a topic of current investigation. Recently, it has been shown that CLL cells are resistant to necroptosis induced by TNF-α plus the pan-caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD) (48). Necroptosis has been described as programmed necrosis leading to cell death independent of caspase activation (49-51). Necroptosis can be initiated by ligation of death receptors such as TNFR1 and CD95 and leads to formation of a complex including RIP1, RIP3 and caspase 8 (52-54). In CLL cells stimulated with TNFα this complex was not formed due to downregulation of the deubiquitinating enzyme cylindromatosis (CYLD) which is important in deubiquitination of RIP1 (48). When polyubiquitinated chains are removed from RIP1 by CYLD, RIP1 is able to form a complex with RIP3 and caspase 8 which leads to necroptosis (50). These findings have relevance for the observation that CLL cells are resistant to IAP antagonists, since the same RIP1-containing signaling platforms are engaged. Thus, small molecules which mimic CYLD or inhibit LEF1, which is a transcriptional repressor of CYLD, might induce necroptosis in CLL cells upon activation of death receptors, such as TNFR1 and CD95.

Another protein which could play a role in resistance to necroptosis in CLL cells is FLIPL. When the RIP1-RIP3-caspase8 containing complex is formed, necroptosis can be initiated (55). However, in the presence of FLIPL, caspase 8 can form a heterodimer with FLIPL and inhibits necroptosis via cleavage of RIP1 and RIP3 (56). CLL cells express higher levels of FLIP compared to normal B cells (57). In addition, CD40 stimulation of CLL cells results in higher FLIP levels (9) and inhibition of NF-κB significantly decreases FLIP levels (58). Further studies are needed to investigate whether FLIPL plays a role in resistance to necroptosis in CLL cells. Thus, overcoming resistance to necroptosis of CLL cells could be a novel option for treatment strategies.

Despite many advances in treatment strategies there is no curative therapy for CLL. Therefore, more insight is needed into mechanisms which lead to drug resistance of CLL cells in the lymph node microenvironment. In this thesis, we have obtained novel evidence for the importance of the NF-κB pathway and its downstream pro-survival target Bcl-XL in the development of drug resistance in in vitro models of the lymph node microenvironment. Furthermore, we identified that IL-21 is essential to induce proliferation of CLL cells. Besides proliferative capacities, CLL cells have defects in apoptosis and necroptosis regulation. We found that the Noxa/Mcl-1 balance is a key determinant of viability of CLL cells and that combination of agents which enhance the Noxa/Mcl-1 ratio with small molecules targeting the anti-apoptotic protein Bcl-XL is a promising targeted therapy in order to overcome drug resistance in CLL.
Reference List


(3) Lagneaux L, Delforge A, Bron D, De BC, Stryckmans P. Chronic lymphocytic leukemic B cells but not normal B cells are rescued from apoptosis by contact with normal bone marrow stromal cells. Blood 1998 Apr 1;91(7):2387-96.


(38) Billard C. Development of Noxa-like BH3 Mimetics for Apoptosis-Based Therapeutic Strategy in Chronic Lymphocytic Leukemia. Mol Cancer Res 2012 Apr 27.


