Microenvironment and anti-CD20 based therapies in CLL

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Chapter 7

Concluding remarks
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One focus of the studies described in this thesis is the bidirectional CLL/T cell interaction and its consequences for the (patho)biology of CLL. In chapter 2 we describe the role of T cells in both microenvironment induced activation, proliferation and drug resistance of CLL cells. In chapter 3 we show that CLL cells induce Treg in a CD70-dependent manner, thereby elucidating one of the underlying mechanisms of increased Treg formation in CLL. Furthermore, we find remarkable similarities in activation, drug resistance and gene expression profiling between in vitro CD40-stimulated CLL cells and CLL cells cocultured with activated T cells (chapter 2). This latter finding supports the physiological role of CD40 stimulation for drug resistance in CLL and the important role of T cells in the pathophysiology of the disease. In this chapter we discuss the relevance of our findings and their implications for future (immuno)therapeutic approaches.

A second aspect of this thesis concerns the mechanism of direct cell death induced by type I and type II anti-CD20 mAbs in CLL cells. In chapter 4 we describe the Ca^{2+} and reactive oxygen species (ROS) dependent cell death induced by the type I anti-CD20 mAb rituximab and in chapter 5 we describe the lysosomal cell death induction by GA101, a novel type II anti-CD20 mAb. Furthermore, we show CD40-induced sensitization to both types of anti-CD20 mAbs. We show that CD40 stimulation sensitizes to rituximab-induced cell death by increasing basal ROS production, and that CD40-induced sensitization to GA101 is based on an increase in lysosomal volume and activity. The different mechanisms of cell death induction and CD40-induced sensitization to type I- and type II anti-CD20 mAbs in CLL cells are summarized in Figure 1. The effect of CD40 stimulation on anti-CD20 mediated cell death in vivo was studied in chapter 6. We conclude that CD40 stimulation in vivo does not play a role in resistance to anti-CD20 mAbs, and also that CD40-stimulation induces strong B cell proliferation, which argues for caution in using CD40 stimulation for the treatment of B cell malignancies.

These observations have 2 important implications

1. The mutual effects of CLL/T cell interaction provide insight into the role of T cells in CLL in vivo. This could possibly lead to T cell targeted therapies in CLL.
2. The effect of anti-CD20 mAb based therapies in CLL might be improved by modulation and sensitization of CLL cells to anti-CD20 induced, non-apoptotic and p53 independent cell death pathways.

1. The mutual CLL/T cell interaction has different consequences. First, CLL cells induce Treg and other studies have shown inhibition of T cells in the presence of CLL cells. Eradicating Tregs in CLL could result in increased T cell function and restored activity of tumor-specific
T cells that eliminate the malignant CLL cells. Interesting new drugs that overcome drug resistance in CLL may also be effective against Tregs in CLL, especially those targeting cells with high expression of Noxa, like seleciclib. The site where CLL cells induce Tregs in a CD70-dependent manner in vivo is probably in proliferation centers in lymph nodes. Here, CLL cells are activated by CD40L+ T cells and upregulate CD70. In this respect, anti-CD70 mAbs could kill two birds with one stone: CD70+ drug resistant CLL cells from the LN niches are eliminated and CLL-induced Treg formation is prevented.

Second, signals derived from activated T cells are instrumental in inducing and maintaining specific features of CLL cells. Activated T cells can induce drug resistance and proliferation of CLL cells. These effects are only partially dependent on CD40L, which suggests a role for yet unknown factors. We show the IL-21 dependent proliferation induction in CLL cells by activated T cells. Recently, a model for CLL cell biology was suggested by Calissano et

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**Figure 1. Mechanisms of type I (Rituximab, top) and type II (GA101, bottom) anti-CD20 mAb induced cell death and underlying mechanisms of CD40-induced sensitization to both types of anti-CD20 mAbs in CLL.** Rituximab induces a Ca\(^{2+}\) flux (inhibited by EGTA) resulting in an increase in cytoplasmic Ca\(^{2+}\) concentration \([\text{Ca}^{2+}]_c\) which leads to a rise in mitochondrial Ca\(^{2+}\) concentration \([\text{Ca}^{2+}]_m\). Increased \([\text{Ca}^{2+}]_m\) leads to the formation of ROS and ROS dependent cell death (inhibited by NAC). CD40 stimulation sensitizes CLL cells to rituximab-induced cell death by increasing basal ROS production. GA101 induces homotypic aggregation (HA) which can be blocked by cytochalasin D (CytoD). CD40 stimulation increases the number of lysosomes, which are susceptible to swelling and burst via GA101 engagement. Both GA101 induced HA and increase in lysosomal number are required to result in Lysosomal Membrane Permeabilisation (LMP) leading to cell death. An increase in lysosomal volume and LMP can be inhibited by concanamycin A (concA). Release of cathepsins into the cytosol after LMP leads to caspase activation. Caspase activation, but not cell death is inhibited by Z-VAD and Q-VD.
al. where the leukemic clone contains a spectrum of cells. It consists of a proliferative fraction, enriched in recently divided robust cells that are lymphoid tissue emigrants (CXCR4(dim) CD5(bright)) and a resting fraction enriched in older, less vital cells (CXCR4(bright)CD5(dim)) that need to immigrate to lymphoid tissue or die. This model fits our T cell model where CLL cells are stimulated in the lymph node by CD40L+ T cells that produce IL-21 and leave the lymphoid tissue as proliferating CLL cells. Interfering in this process by blocking IL-21R on CLL cells seems an attractive therapeutic approach.

From these studies the question arises whether the CD40L system is a supraphysiological model in contrast to the activated T cell model. Several in vitro models have been used to mimic the natural microenvironment. CLL cells co-cultured with nurselike cells (NLCs), bone marrow stromal cells (MSCs) or follicular dendritic cells are protected against apoptosis. In CLL tissues pseudofollicular structures are found consisting of CLL cells, antigen (Ag)-presenting cells and numerous CD4+CD40L+ T cells. In vitro stimulation of CLL cells with CD40L results in an increased anti-apoptotic profile and rescues them from drug-induced apoptosis. All these in vitro models might be oversimplified. The important question on how to construct a model which most faithfully reflects the complex influence of membrane-bound and soluble factors on CLL cells in vivo remains to be answered. We conclude in chapter 2 that both the CD40L system and the activated T cell system at least partially reflect the in vivo LN microenvironment, however only activated T cells induce proliferation of CLL cells, an important characteristic of CLL cells in vivo. This underscores the importance of T cells in vivo.

Should activated T cells then be eliminated in CLL patients? On one hand it seems that by inducing proliferation and drug resistance of CLL cells, activated T cells might negatively affect the course of the disease. On the other hand, in mouse models increased autologous T cell activation can lead to an apparent graft-versus-tumor reaction. Also injection of autologous CLL cells transduced with CD40L leads to T cell activation and CLL cell elimination. Furthermore, cross-priming of apoptotic CLL cell proteins after for instance anti-CD20 therapy could result in cytotoxic T cell responses against the tumor. Therefore, simple elimination of activated T cells in the treatment of CLL will not result in a favourable outcome per se. The occurrence of opportunistic infections and CMV reactivation as a result of T cell depletion following anti-CD52 therapy in CLL are serious adverse events. Blocking CD40L and IL-21 signaling in CLL seems a more attractive targeted immunotherapeutic approach.

2. The mechanisms of direct cell killing by anti-CD20 mAbs are not well understood. The ability of anti-CD20 mAb to induce anti-tumour effects through cell signalling remains controversial. Only few studies show that intracellular signals are generated following rituximab treatment of CLL cells. Further controversy exists as to the actual cell death mechanism induced by CD20 ligation. Many have shown that CD20 crosslinking induces classical apoptosis, however growing evidence (including this thesis) exists...
that non-classical apoptotic cell death is induced by anti-CD20 mAbs\textsuperscript{25-28}. By inducing non-classical apoptosis, anti-CD20 mAbs can circumvent the anti-apoptotic machinery in CLL cells. After CD40 stimulation CLL cells increase their anti-apoptotic profile and become drug resistant\textsuperscript{13-15,29}. On the other hand, the studies in this thesis show CD40-induced sensitization to anti-CD20 mAbs. And combination treatment of CD40-stimulated CLL cells with anti-CD20 mAbs and cytostatic drugs shows significant additive effects resulting in 80-100% cell death. Altogether these results are promising and provide a rationale for combining cytostatic drugs and anti-CD20 mAbs in the treatment of CLL. The results of a large randomized phase III trial combining fludarabine, cyclophosphamide and rituximab (FCR) which improved both progression free survival (PFS) and overall survival (OS) in p53 functional, previously untreated CLL patients\textsuperscript{30} are the first in vivo confirmation of this finding.

In light of the different mechanisms underlying CD40-induced sensitization of CLL cells to rituximab and GA101 respectively (Figure 1), several interesting candidates for combination treatments exist. We show CD40-induced sensitization to rituximab by increasing basal ROS production in CLL cells. Various studies show that cancer cells have altered redox balance. Malignant cells are under oxidative stress: high amounts of ROS are produced and cells have relatively low antioxidant capacity (reviewed in\textsuperscript{31}), resulting in a vulnerable balance. Also CLL cells are under enhanced oxidative stress, which is associated with oxidative damage to nuclear and mitochondrial DNA\textsuperscript{32-34}. Therefore, ROS inducing compounds are attractive in the treatment of CLL. Increased sensitivity of CD40-stimulated CLL cells to the ROS inducing agent cisplatin confirms the hypothesis that ROS inducing agents are potent cell death inducers in CD40-stimulated CLL cells. Combining rituximab and cisplatin results in even stronger cell death induction (Chapter 4). Whether this combination is also potent in vivo has to be determined. A recent interim analysis of a phase 2 study with 20 patients treated with rituximab, cisplatin, cytarabine and dexamethason (R-DHAP) shows effective remission-induction for fludarabine-refractory or early relapsed patients\textsuperscript{35}. Also Bendamustine acts independently from p53 and induces ROS dependent cell death in CLL\textsuperscript{36}. Combination treatment of rituximab and bendamustine could therefore be interesting to test in CD40-stimulated CLL cells. The German CLL Study Group is comparing bendamustine plus rituximab (BR) versus FCR as first-line therapy in patients with CLL who require therapy. A phase 1 study showed that BR is effective and safe in patients with relapsed CLL and has notable activity in fludarabine-refractory disease\textsuperscript{37}. The ultimate goal in the treatment of CLL patients is to eliminate the malignant clone with a treatment with lowest toxicity. The effect of other ROS inducing agents less toxic than cisplatin or bendamustin could be interesting to explore. It has been shown that 2-methoxyestradiol (2-ME) substantially reduces CLL cell survival by inhibiting the superoxide dismutase enzyme (SOD) leading to an accumulation of superoxide (O$_2^-$) and cell death\textsuperscript{34}. CLL cells with higher basal O$_2$ contents are more sensitive to 2-ME in vitro than those with lower O$_2$ contents\textsuperscript{34}. Combining 2-ME with rituximab could therefore enhance anti-leukemic activity and overcome drug resistance in CD40-stimulated
CLL cells. Other ROS inducing agents that have shown cytotoxicity in CLL cells as single agents are ascorbic acid and arsenic trioxide. Of interest and also most attractive in light of lowest possible toxicity, are mAbs that induce ROS and subsequent non-apoptotic cell death in CLL, such as Hu1D10 (apolizumab), a humanized HLA-DR beta-chain-specific antibody.

The induction of lysosomal cell death could also be promising in the treatment of CLL. Lysosomal membrane permeabilization (LMP) initiates a lysosomal cell death mechanism. LMP results in a release of lysosomal hydrolases (cathepsins, calpains, nucleases) into the cytosol, that causes digestion of vital proteins and the activation of additional hydrolases including caspases (reviewed in ). The cathepsins released into the cytosol upon LMP can therefore initiate the intrinsic apoptosis pathway, but lysosomal leakage can also trigger a caspase-independent nonapoptotic cell death pathway, indicating that lysosomal hydrolases are capable of acting both as initiators and as effectors of programmed cell death.

LMP is induced by a plethora of distinct stimuli (reviewed in ) including lysosomotropic agents (hydroxychloroquine, sphingosine, antibiotics such as ciprofloxacin) and microtubule toxins (vincristine, vinorelbine). Lysosomal alterations and perturbed regulation of LMP is common in cancer cells. The increased expression and altered trafficking of lysosomal enzymes is involved in tissue invasion, angiogenesis and sensitization to the lysosomal death pathway (reviewed in ). The CD40-induced sensitization to lysosomal cell death induction by GA101 suggest that LMP induction might lead to novel therapeutic avenues in CLL. It would be very interesting to test for instance the effect of combination treatment of GA101 and hydroxychloroquine (HCQ) on CD40-stimulated CLL cells. Especially since HCQ has been used for the treatment of malaria since decades and safety profiles are well known. Cancer cells can be resistant to LMP because of high Hsp70. In malignant cells Hsp70 translocates to the lysosomal compartment where it promotes cell survival by inhibiting LMP. Whether Hsp70 expression levels in CLL cells correlate with sensitivity to GA101 needs to be determined.

Whether combination treatment of CD40 stimulation and anti-CD20 mAbs in vivo is effective and safe needs to be determined. First of all, the contrasting effects of CD40 stimulation on drug resistance on one hand and sensitization to anti-CD20 mAbs on the other seem contradictory. This observation is likely to put any clinician in doubt whether CD40 stimulation in vivo is sound. In vitro treatments of anti-CD20 mAbs and cytostatic drugs however show significant additive effects resulting in 80-100% cell death (chapter 4 and 5). Furthermore, in chapter 6 we show massive B cell depletion in hCD2OTG mice with both rituximab and GA101, also after CD40 stimulation. The results suggest at best that CD40 stimulation in vivo does not induce resistance to anti-CD20 mediated cell death. On the other hand, a large increase in B cell numbers is observed after CD40 stimulation in vivo. Furthermore, the CD40-induced upregulation of CD70 on CLL cells and hence CD70-induced Treg formation could be a pitfall in CD40 treatment in vivo. However, CD40-induced cross priming of antigen from necrotic or apoptotic tumor after anti-CD20 therapy will eventually lead to anti-tumor cytotoxic T
cell formation. This latter process could be catalyzed by CD70 costimulation delivered by CD70+ CLL cells. Altogether, CD40 treatment in vivo should be approached with caution. In conclusion, our increasing insight into the effects of the microenvironment on both the biology of the CLL cells and their sensitivity to cytotoxic drugs and mAbs offers novel opportunities for future improvement of the treatment results in CLL.
Reference List


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