Studies on the immune system in CLL

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Summary and discussion
Drug-resistance due to p53 dysfunction

Chronic lymphocytic leukemia (CLL) is frequently regarded as an indolent disease that often does not require therapy. Nevertheless, when progression does occur and treatment is required, a considerable subset of all CLL patients has a poor outcome due to frequent relapses and resistance against most chemotherapeutic agents. Drug-resistance has therefore become the most important issue in the treatment of CLL (54). Especially CLL patients with primary or acquired resistance to fludarabine treatment have a marked decreased overall survival. According to a study by Keating et al., those patients have a median survival of ten months (41). The most important genetic abnormality associated with fludarabine resistance is deletion of the short arm of chromosome 17 (17p-), which contains the p53 locus. Because an intact p53 tumor suppressor pathway is essential for many “classical” chemotherapeutic drugs, CLL patients with 17p- usually respond poorly to chemotherapy (20;82). Unfortunately, fluorescence in situ hybridization (FISH), which is currently used as standard technique to identify chromosomal abnormalities in CLL seems to underestimate the percentage of patients that will become resistant to chemotherapy (6;8). This might be because not only 17p- but also point mutations of p53 (not detectable via FISH) lead to p53 dysfunction and thereby drug resistance (19;90). Therefore, in addition to or even prior to screening for chromosomal abnormalities, it might be useful to assess p53 function in all patients with active disease to identify the patients that should receive p53-independent regimens. Obviously, in order to be applicable in clinical practice, a p53 function test should be easy, reproducible, amenable for standardization and preferably inexpensive. At present, the most frequently used technique to assess p53 function in tumor samples is Western blotting for p53 and p21 (upregulated upon p53 activation (21)) using lysates of irradiated cells. Recently however, several alternatives to this technique have been presented (4;11;18;50). In this thesis, we demonstrate that measuring transcript levels of the p53-responsive genes Puma, p21 and Bax in a reverse transcriptase PCR-based multiplex ligation probe assay (RT-MLPA) may be a reliable technique to test for p53 function in tumor samples (chapter 3). Using RT-MLPA, we also identified p53 dysfunction in samples from CLL patients without chromosomal abnormalities. Our data suggest that upregulation of Puma upon ionizing radiation (IR) treatment is a highly representative parameter for p53 function. In fact, recent observations suggest that Puma is also the most pre-
dominantly upregulated gene in vivo upon chemotherapeutic treatment of locally advanced breast cancer (53). Therefore, RT-MLPA might not only serve to assess p53 function before onset of therapy but might also be used to monitor the effect of drug treatment in vivo in CLL.

Since p53 dysfunction seems to be the most important cause of drug resistance in CLL, there is a definite need for p53-independent therapeutic agents. At this moment, several of these agents are available. Methylprednisolone for example induces p53-independent cell death (14) and indeed seems to be effective in CLL patients with 17p- (80). Also monoclonal antibodies might act p53-independent. This can be deduced from the fact that tumor cells with mutated p53 can be killed in vitro both via antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)(44). Notably, ADCC and CDC are thought to be the key mechanisms via which monoclonal antibodies exert their effect in vivo (27;67). For that reason, it is not surprising that treatment regimens containing alemtuzumab (Mabcampath), a CD52-specific humanized antibody, are effective against CLL with 17p- (47;73). Furthermore, first line treatment with alemtuzumab leads to significantly improved progression-free survival as compared to treatment with chlorambucil (34). Importantly, various clinical studies point out that alemtuzumab also has additive value in the treatment of refractory CLL either as a single agent (55;65), added to fludarabine treatment (23) or used for consolidation (84). Unfortunately, patients treated with alemtuzumab often experience CMV infections due to immune suppression (46;84), probably because alemtuzumab targets all lymphocytes and therefore will affect adaptive immunity.

The mechanism of action of the other monoclonal antibody used in CLL, rituximab (Mabthera), is less clear. Rituximab as a single agent at the usual dosage has only moderate effects against refractory CLL (52). Therefore, in most recent studies it is administered in combination with fludarabine and cyclophosphamide (FCR)(86). The results from a phase II trial point out that FCR treatment results in a longer progression free survival when compared to historical controls (chemotherapy alone)(85). Nevertheless, the response of 17p- CLL to rituximab (in combination with fludarabine) seems to be inferior to that of CLL without 17p- (8). Therefore, this monoclonal antibody may be less effective against chemotherapy-resistant CLL than alemtuzumab.

Modern drug development has lead to the discovery of other drugs that induce apoptosis of tumor cells independent of p53. Some of these drugs, like
 seliciclib (R-roscovitine) or bortezomib have already been tested in clinical trials for CLL (24;61) (seliciclib: phase II trial, results unpublished). Another very interesting group of drugs that might be used for the treatment of p53 dysfunctional CLL are p53 activating small molecules (7;26;36;57;83). It is claimed that these small molecules restore biological activity of inactive and some even of mutated p53, thereby inducing apoptosis in a broad range of tumor types. Results from in vivo studies demonstrate that small molecules CP-31398 and PRIMA-1 used as single agent strongly inhibit tumor growth while not affecting healthy tissues (36;77). Although still to be tested in clinical trials, these drugs seem to have strong potential for the treatment of tumors in which p53 plays a role in the origin or progression of the disease.

The successful application of reduced intensity conditioning regimen in allogeneic stem cell transplantation has recently brought attention to this approach for the treatment of therapy-resistant CLL. Indeed, long-term remissions have been achieved with allogeneic stem cell transplantation in refractory CLL (37;68). The success of this approach is attributed to a graft-versus-leukemia effect mediated via donor-derived cytotoxic T lymphocytes (CTL)(31). Importantly, CTL have been demonstrated to lyse target cells in (an at least partially) p53-independent fashion (79). In line with this observation, promising results have been obtained with allogeneic stem cell transplantation in patients with 11q- or 17p- (9). Therefore, CTL mediated therapies may be an alternative towards therapy for refractory CLL. This is discussed in the following paragraph.

Active immunotherapy for CLL

It has been shown that CLL is accompanied by T cell dysfunction (29;70), which may prevent the formation of efficient CLL-specific T cell responses. Nevertheless, several groups have described successful approaches to overcome T cell tolerance against tumor cells. One approach has been ex vivo transduction of CLL cells with CD40 ligand (CD40L), resulting in increased surface expression of costimulatory molecules on CLL cells. These CD40L-transduced CLL cells could subsequently be used to elicit CLL specific T cell responses in vitro (40). Moreover, CLL-specific T cell clones emerged in patients upon administration of autologous CD40L-transduced CLL cells (87). The latter was accompanied by reduction of tumor mass in these patients,
suggesting that infusion of CD40L-transduced CLL cells even broke immuno­tolerance against non-transduced tumor cells. Alternative approaches towards active immunotherapy for CLL, such as \textit{ex vivo} generation of allogeneic or autologous CLL-specific CTL using either CLL cells (3;35) or DC pulsed with CLL lysates (43), have demonstrated promising results \textit{in vitro} but have not been successfully applied \textit{in vivo} as yet. A possible explanation for the latter might be that all methods described above require either \textit{ex vivo} manipulation of tumor cells or the use of allogeneic T cells. As an alternative, we therefore studied the possibility of redirecting virus-specific cytotoxic T cells (CTL) towards autologous CLL cells. We hypothesized that cytomegalovirus (CMV)-specific CTL would be proper candidates for this task, because they are unaffected by the general T cell dysfunction in CLL and, importantly, are not heavily dependent on co-stimulation to become activated. Indeed, previous studies from our group confirmed the power of CMV-specific CTL since CLL cells loaded with the CMV immunodominant peptide pp65 were lysed by autologous CMV-specific CTL, even without \textit{in vitro} pre-stimulation (39).

Because the technique described above still requires \textit{ex vivo} manipulation of tumor cells, we modified this technique by replacing the CMVpp65 peptide by targeted complexes (TC) consisting of HLA-I molecules containing CMVpp65 peptide coupled to a CD20 antibody fragment (chapter 4). We demonstrate that these TC coated CLL cells are lysed by autologous CMV-specific CTL as efficiently as CMVpp65 loaded CLL cells, but that cytokine production by TC stimulated CTL is less than cytokine production triggered by CMVpp65 (chapter 5). We demonstrate that the latter is probably due to inefficient immunological synapse formation between CTL and TC-coated CLL cells. Nevertheless, the reduced cytokine production by TC triggered CMV-specific CTL might be an advantage for the future clinical application of the TC because the reduced production of cytokines by CMV-specific CTL will possibly reduce the risk of the induction of a cytokine storm (74) while the targeted tumor cells still are effectively lysed.

The results from a clinical trial with the CD20 antibody fragment suggest that TC should have excellent tissue penetration \textit{in vivo} (25). In this study, even tumor cells in immune-privileged sites were efficiently targeted upon intravenous infusion of the CD20 antibody fragment. At present, animal experiments are being performed to see whether this also holds true for the complete CMV peptide-containing TC. Subsequently, additional experiments should also address whether CTL against CMV can be successfully activated to lyse all tu-

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mor cells in vivo. If the concept of redirecting virus-specific T cell responses against tumor cells without ex vivo manipulation indeed proves to be valid, it might be applicable for the treatment of other malignancies as well (13;63;64) because of the flexibility that the dual-component targeting construct offers. In chapter 6, we investigated the possibility to enhance tumor cell death induced by CMV-specific CTL. It is known that many tumor types (including CLL) express high levels of X-linked inhibitor of apoptosis protein (XIAP)(76). This may have consequences for active immunotherapy in CLL, since cell death induced by Granzyme B (an important mediator of CTL-induced cell death) can be antagonized by XIAP (28;38). Therefore, we tested whether CTL mediated lysis could be optimized by pre-treating tumor cells with small molecule inhibitors of XIAP. We observed that small molecule XIAP inhibitor 1396-11 indeed synergized with Granzyme B induced DNA fragmentation (which is caspase-mediated) in Jurkat cells but surprisingly did not enhance CTL-induced cell death of CLL cells. In contrast to Jurkat cells, CLL cells express high levels of proteinase inhibitor 9 (PI-9), an inhibitor of Granzyme B. This suggests that Granzyme B may not play an important role in CLL cell death induced by CMV-specific CTL. Alternatively, it also confirms that CTL-induced cell death is at least partially caspase-independent (17;51). It would therefore be interesting to further explore the mechanism behind CTL-mediated cell death in CLL cells, thereby possibly identifying new targets for drugs that enhance active immunotherapy in CLL.

Regulatory T cells: target for anticancer therapy?

Cancer patients (including CLL patients) frequently have increased numbers of regulatory T cells (Treg) in the peripheral blood (5;88). The data presented in chapter 2 of this thesis suggest that in CLL, tumor cells might facilitate the formation of Treg in a lymph node environment via CD70 ligation. This is in line with recent observations in non-Hodgkin’s lymphoma, where the tumor cells also seem to play a crucial role in the formation of Treg via CD70 ligation (89). It is tempting to speculate that increased formation of regulatory T cells through CD70 ligation by tumor cells might be a general mechanism behind immunotolerance in B cell malignancies. Although at present it is unclear whether these Treg indeed play a role in the disease, it has been described that eradicating Treg in vivo leads to enhanced tumor cell clearance (30;33;69;71).
Moreover, it has been suggested that Treg play an essential role in the initial phase of tumor formation and that eradication of Treg at this stage of the disease even prevents tumorigenesis (22). Strikingly, there are reports stating that active immunotherapy results in increased numbers of tumor-specific Treg which diminish the effect of tumor vaccination (12;92). If Treg indeed play such a crucial role in tumorgenesis and may also hamper active immunotherapy, it would be very interesting to see what effect eradicating Treg in cancer patients will have. Because Treg, in contrast to naïve and memory T cells, have high surface expression of CD25 (beta subunit of the IL-2 receptor), this molecule would be a putative target for anti-Treg therapy. In this perspective, one of the agents that seems very interesting is denileukin diftitox or ONTAK (91). This drug is composed of recombinant human IL-2 coupled to diphtheria toxin. It has been approved by the FDA for the treatment of cutaneous T cell lymphoma (62), a tumor that has high expression of CD25. However, in experimental therapeutic settings for melanoma and renal cell carcinoma this agent also reduced frequencies of Treg, which was accompanied formation of tumor-specific T cell responses (16;49). Thus, the antitumor effect of denileukin diftitox in the treatment of cutaneous T cell lymphoma may partially be explained by its effect on regulatory T cells. A comparable phenomenon may also account for the potent antitumor effect of fludarabine treatment in CLL. A recent study by Beyer et al. showed that fludarabine treatment does not only eradicate tumor cells but also results in reduced frequencies and activity of regulatory T cells (5). Fludarabine probably has this effect on Treg because Treg are highly sensitive to apoptosis induction (75). Studies from our group demonstrate that Treg (in general) have a highly apoptosis-prone phenotype characterized by low expression of anti-apoptotic Bcl-2 and high expression of pro-apoptotic Noxa and high surface expression of Fas (CD95) (chapter 2). Surprisingly, we found an altered apoptotic gene expression profile in Treg of CLL patients which highly resembles the apoptotic gene expression profile of CLL cells (48). Therefore, some new drugs that have been designed to overcome apoptosis resistance in CLL might also be very effective against Treg in CLL, especially the ones targeting cells with high expression of Noxa (like seliciclib (32) and bortezomib (58)) and Bcl-2 (like oblimersen, a Bcl-2 antisense oligonucleotide (66)).
Targeting Noxa and Bcl-2: killing two birds with one stone

It is generally assumed that CLL cells do not accumulate *in vivo* by increased proliferation but due to disturbed apoptosis. This is most likely due to increased expression of anti-apoptotic Bcl-2 (42). Strikingly, our data suggest that Treg from CLL patients also have increased expression of Bcl-2 compared to Treg of healthy donors (chapter 2). In contrast to Bcl-2 expression in CLL cells, the increased Bcl-2 expression in Treg from CLL patients is probably not caused by deletions involving Bcl-2-regulating microRNA (10), but more likely via other pathways, such as cytokine stimulation (15;78) or T cell receptor triggering (1). Because of its increased expression in both CLL cells and Treg of CLL patients, Bcl-2 seems an attractive target for the development of novel therapeutic strategies for CLL. Various agents that target Bcl-2 have been developed (66;72;81), of which oblimersen (an antisense oligonucleotide targeting Bcl-2) seems the most promising. This drug is now used in phase II and III trials, either as single agent or in combination with other drugs. Although less effective as a single agent due to severe side effects at therapeutic levels (60), at lower dosages it seems to have additive effect to treatment with classical cytotoxic drugs (59). Nevertheless, considering the apoptotic gene expression profile of CLL (48), oblimersen would be even more effective in combination with drugs that preferably target cells with high expression of pro-apoptotic Noxa. Conveniently, Treg from CLL patients (as well as Treg from healthy individuals) also express high levels of Noxa. Therefore, it is not surprising that both CLL cells and Treg are very sensitive to apoptosis induction via R-roscovitine (2)(chapter 2), a cyclin-dependent kinase inhibitor that acts via the Noxa/Mcl-1 axis (32). Unfortunately, R-roscovitine is less potent against cells that have increased expression of Bcl-2 (32). In this perspective, it would be very interesting to explore the effect of combined treatment with R-roscovitine and oblimersen in CLL. In addition, combining these therapeutic agents may also be very effective against therapy-resistant CLL because R-roscovitine induces apoptosis in a p53-independent fashion (2) while oblimersen also enhances (radiation-induced) cell death in p53-deficient cells (56).
Concluding remarks

From the data discussed above, it is clear that extensive knowledge of tumor biology and drug resistance mechanisms is essential for the development of treatment strategies for CLL patients that are resistant to “classical” chemotherapy. However, care should be taken when extrapolating *in vitro* and even *in vivo* effects (in animals) to the clinic (45;74). Nevertheless, with our current knowledge of tumor biology, it should be possible to design therapeutic strategies that target tumor cells in a highly specific way with minimal side effects.

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