Studies on the immune system in CLL
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CLL: a paradigm of tumor-associated immune dysfunction

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Introduction

Abstract

Disorders of the immune system occur in a large range of malignancies. Whereas some of the abnormalities may affect different components of host defense against pathogens, others may contribute to the survival or even expansion of tumor cells. In B cell chronic lymphocytic leukemia (CLL) various immune disorders may be observed in the course of the disease. In CLL, an increased amount of regulatory T cells, skewing of the immune system towards Th2 and down modulation of costimulatory molecules combined with enhanced surface expression of CD200 by tumor cells may all serve as a protective mechanism against tumor-directed immune responses. Other coinciding immune disorders (like autoimmunity and hypogammaglobulinaemia) also have an effect on host immunity but offer no obvious advantage for the tumor cells. Strikingly, amidst all these impairments, immunity against chronic (herpes) viruses like cytomegalovirus (CMV) and Epstein Barr Virus (EBV) is conserved in CLL patients. Studying these unique T cell populations in CLL may provide new insights into the requirements for the maintenance of immunity against chronic (herpes) viruses. Moreover, these virus-specific T cells may also hand us a powerful tool for active immunotherapy for CLL if they can be redirected against tumor cells.
Chapter 1

Introduction

Cancer is one of the most important causes of death in the western world (WHO 2002). For many types of cancer, curative treatment is still lacking. Fortunately, recent research has led to the development of new therapeutic tools that are claimed to have a stronger and more tumor-specific effect. Whereas some of these new agents attack the weak spots of the cancer cells, others aim at directing the attention of the immune system towards tumor cells(27;49;69). Under normal circumstances however, the immune system should be able to eliminate malignant cells without medical intervention. Therefore, the development of a malignancy might also be seen as a failure of the immune system. Moreover, many malignancies are accompanied by various deregulations of the immune system that are harmful for the health of the patient but do not have a clear benefit for tumor cell survival. From this perspective, studying the immune system of cancer patients might not only give us more insight into the mechanism of tumor development but might also lead to the development of new therapeutic strategies. In this thesis, we have studied chronic lymphocytic leukemia (CLL), an example of a malignant disease that is accompanied by various immune disorders.

CLL: no curative treatment

CLL is the leukemic malignancy with the highest prevalence in Western Europe. At present however, there is no cure for the disease. Despite the fact that the first results of reduced intensity stem cell transplantation (RIST) look promising(19;58), this treatment has one major drawback: since CLL mainly affects elderly people many of them are not eligible for this procedure due to age limitations for this therapy. Thus, an urgent need exists for the development of new therapies that effectively attack tumor cells and that have few side effects. To develop these strategies, more knowledge on tumor biology needs to be acquired.

In most cases, CLL is marked by a slow accumulation of mature CD19/CD5/CD23 positive B-lymphocytes. It is thought that the accumulation of malignant B cells may result from disturbed apoptosis due to overexpression of anti-apoptotic B cell leukemia-2 (Bcl-2)(29), which was recently linked to mutations or deletions in a region on chromosome 13 containing microRNA
Besides Bcl-2 overexpression, CLL cells also use other mechanisms to protect themselves against apoptosis. Mutations or deletions of tumor-suppressor gene p53 (occurring in about 5-10% of all patients at diagnosis)(14) or ataxia-telangiectasia mutated gene (ATM; occurring in 10-15% of all patients at diagnosis)(2), which exerts its function upstream of p53, are correlated with an adverse prognosis and resistance to chemotherapy(26), since the most frequently used therapeutic agents depend on a functional p53 pathway in the tumor cells(54;63). Moreover, the prevalence of p53 mutations is even higher among patients that relapse after initial chemotherapeutic treatment, possibly due to prior DNA damage or selection for p53 dysfunctional clones(61;65). Thus, it goes without saying that there is an urgent need for therapeutic approaches that act in a p53 independent way. Besides protection against apoptosis, CLL has also found ways to protect itself against the immune system. Since the immune system is capable of detecting and eliminating malignant cells, altering host immunity can also offer a potential benefit for tumor cells. In CLL, most protection mechanisms against tumor surveillance affect the generation of adequate tumor-directed T cell responses. This is thought to be either mediated via changes within CLL cells which protect them against T cell-induced apoptosis, or to be contributed by changes within different T cell populations that may be induced by the tumor cells(25). Both aspects will be further addressed in the next paragraphs.

Tumor cells are protected against T cell attack

Altered expression of surface molecules
Tumor cells use various tricks to escape immune surveillance. One of these mechanisms is disabling recognition by immune cells through downmodulation of various surface molecules. The most obvious way to do this is to reduce MHC class I and II expression thereby preventing the presentation of peptides derived from tumor-specific antigens(38;53;70). That this is a very efficient way to prevent immune surveillance is supported by the fact that many viruses use the same strategy to hide from virus-specific immune cells(4;57). Therefore, it is not surprising that oncogenic transformation by viruses is sometimes accompanied by downmodulation of antigen presenting molecules (9;55). In CLL, there is no evidence for virus infection of tumor cells. Nevertheless, CLL cells have a lower surface expression of class I antigens than their non-malig-
nant counterparts. In addition, activation of anti-tumor immune responses is also prevented by reduced surface expression of costimulatory molecules on CLL cells. It has been postulated that a chronic deficiency of CD40 ligand on T cells in CLL patients is responsible for the downmodulation of immune-stimulatory molecules on the CLL cells(15). Studies by Kato et al(35) showed that CD40 ligation through adenoviral transduction of CLL cells with CD40L resulted in upregulation of surface expression of costimulatory molecules, MHC class I and death receptor molecules. Moreover, transduced tumor cells were able to induce autologous T cell responses in vitro. Later, promising results were obtained when CD40L transduced tumor cells were reinfused in CLL patients. This resulted in an increased tumor-specific T cell activity and, in some cases, reduction of the tumor burden(72). At present, more clinical studies with CD40L transduced tumor cell reinfusion are being performed. The results of these studies should point out whether resupplying CD40L is a feasible approach to enhance tumor cell immunogenicity in vivo.

Recently it has been discovered that CLL, as well as a subset of multiple myeloma, has increased surface expression of CD200, a membrane glycoprotein which shares extracellular domains with T cell regulation molecules like CD2, CD80 and CD86 (44;46). The function of this molecule was discovered only a few years ago. Animal studies pointed out that experimentally induced autoimmune disease had an earlier onset in CD200 -/- mice than in wild type animals(31). The results of this study suggest that CD200 has a down-modulatory effect on immune activation. To exert this effect, CD200 is dependent on interaction with its receptor, CD200R(74). This implies that, in order to have a down-modulating effect on immune activation, cells that express CD200 need to be in direct contact with cells that carry the receptor. Whereas CD200 is ubiquitously expressed, the expression of CD200R, is mainly restricted to cells of the myeloid lineage, like dendritic cells, monocytes and macrophages(73). Therefore, if CD200 indeed has an immune-modulatory effect in CLL, the most likely place for this to occur is in the lymph nodes, where dendritic cells are present. Recently, mouse studies have demonstrated that tumor cells with increased CD200 surface expression indeed appeared to be protected against immune attack(37), possibly due to an inhibitory effect on APC (which have high CD200R surface expression). Notably, CD200 blocking antibodies completely abrogated the immune-modulatory effect of CD200 on tumor cells, resulting in efficient tumor clearance. It is questionable however whether manipulating CD200 ligation can be used in a clinical setting, since it will have to
be highly tumor cell-specific in order to avoid massive immune activation and maybe even auto-immunity.

**Intrinsic factors protect CLL cells against CTL induced apoptosis**

When, despite all changes in the expression of surface molecules, a tumor cell is recognized by a T cell, subsequent killing of this tumor cell may be impaired *in vivo* because the tumor cells have adopted various ways to protect themselves against T cell-induced apoptosis. When a cytotoxic T cell (CTL) attacks a target cell, it releases the content of cytotoxic granules that is subsequently taken up by the target cell, possibly via endocytosis. Once inside the target cell, the granular content is released into the cytoplasm of the target cell and apoptosis of the target cell is induced(16). The initiators of apoptosis are the enzymes from the granzyme family(10), of which granzyme B is the best-studied member. Granzyme B is capable of cleaving the pro-apoptotic molecule Bid, which in turn translocates to the mitochondria and initiates apoptosis through mitochondrial depolarization and subsequent caspase activation(5). Tumor cells may protect themselves against granzyme B induced apoptosis via different mechanisms. First, many tumor cells express high levels of serpin protease inhibitor PI-9, which serves as an inhibitor of granzyme B(45). Recent data demonstrate that CLL cells also may contain very high levels of PI-9 (M. Bots, personal communication) and therefore may be more resistant to granzyme B induced apoptosis. Furthermore, the overexpression of anti-apoptotic Bcl-2 may prevent mitochondrial depolarization in CLL cells upon CLT attack(18), but whether this also protects them against CTL induced apoptosis *in vivo* remains elusive. Finally, CLL as well as other types of cancer also expresses high levels of x-chromosome-linked inhibitor of apoptosis protein (XIAP) (60;68), a molecule which can inhibit both mitochondrial-initiated apoptosis (through inhibition of caspase 9) and the activation of caspase-3. Recent research has demonstrated that XIAP indeed protects tumor cells against effector cell induced apoptosis(32). Moreover, the development of small molecule inhibitors of XIAP now enables interfering with the function of XIAP in a clinical setting(59). These inhibitors have demonstrated to sensitize tumor cells for both cytotoxic drugs(59) and Fas ligation *in vitro*(33). At present, the capacity of these compounds to sensitize tumor cells to CTL mediated killing is being explored (this thesis).
Chapter 1

Cytokine milieu inhibits Th1 responses
The production and secretion of cytokines allows cells to influence immune responses in their proximity but may also exert systemic effects. For example, the combination of the entity of the pathogen and the type of cytokines released can determine whether Th1 or Th2 type helper T cells will predominate in an immune activation. Thus, the balance between Th1 and Th2 cytokines affects the way the immune system is able to respond to certain antigenic stimuli. It has been observed that CLL patients have elevated serum levels of IL-6 and IL-10(20). Since both stimulatory and inhibitory influences of IL-10 on CLL tumor cell growth and survival have been described(21;36), the effect of increased levels of IL-10 on tumor cells in vivo remains uncertain. Besides through increased serum levels of IL-6 and IL-10, the cytokine balance may be further pivoted towards Th2 because of reduced availability of IL-2 for T cells, caused by absorption by CLL cells (which express the IL-2R)(22). It is tempting to speculate that the disturbed cytokine balance in CLL renders patients more susceptible to infections and provides a protective environment for tumor cells against tumor specific T cell responses, but at present there is no strong experimental evidence supporting this theory.

Various reports address the role of CLL cells in the production of above-mentioned cytokines. CLL cells are capable of producing IL-6(8) and IL-10(64), but whether they are the main source of serum IL-10 in vivo is not known. In contrast to these Th2 type cytokines, CLL cells have also been demonstrated to produce interferon gamma (IFN-γ), which subsequently may serve as an autocrine survival factor(12). This rather confusing finding raises the question whether altering the disturbed Th1/Th2 balance in CLL, for example through administration of cytokines, can have a beneficial effect on either malignant cells or on T cell dysfunctions. Identification of the cells responsible for the production of excessive amounts of Th2 cytokines in CLL however may give important clues as how to restore the Th1/Th2 balance.
T cells: the good or the bad guys?

**Tumor-specific T cells**
In healthy individuals, immune responses should only be elicited against non-self antigens. Despite this principle protects us against auto-immunity, this also implies that it may be difficult to raise tumor-specific immune responses, since tumor cells frequently highly resemble their non-malignant counterparts. To become a tumor cell, a cell has to acquire a number of mutations, deletions or duplications of genes to escape internal control mechanisms that normally prevent oncogenesis(48). These changes within the tumor cell lead to an altered protein content which may be noticeable for CD8 positive T cells because peptides of the mutated or differentially expressed proteins are presented on the cell surface in the context of MHC class I molecules(43;62). It has been postulated that in CLL, tumor-specific T cell clones are present that demonstrate cytotoxic potential against autologous tumor cells *in vitro*(42). Nevertheless, without *in vitro* manipulation, these clones are not capable of clearing a substantial amount of tumor cells *in vivo*, which may be explained in several ways. As described earlier in this chapter, CLL cells have a reduced expression of costimulatory molecules and increased surface expression of CD200, which may directly or indirectly affect the activation of tumor-specific T cell responses. This is supported by the finding that T cells from CLL patients may be impaired in their capacity to differentiate towards a Th1 phenotype, a feature which can also be induced in T cells from healthy donors through direct contact with CLL cells(25). The latter suggests a role for surface antigens in this process, but the molecules involved in this CLL-induced T cell suppression remain to be identified. Alternatively, the activation of tumor-directed T cell responses in CLL may be inhibited by regulatory T cells. These cells are discussed in the next paragraph.

**Increased number of regulatory T cells**
It has been described that in CLL, as well as in other malignancies, there are increased numbers of regulatory T cells (Treg)(6). Since animal studies have indicated that Treg depletion can result in augmented tumor-rejection(66), it is possible that the increased amount of Treg in CLL patients may interfere with adequate tumor surveillance by tumor-specific T cells. At present, it is not clear what causes the increased amount of Treg in cancer patients. The results from a study by Beyer *et al* show that this T cell population in multi-
ple myeloma patients is largely expanded after emerging from the thymus(7), suggesting that the expansion of this population is antigen-driven(71). This may imply the involvement of a tumor antigen in the formation and expansion of these Treg, but in the case of CLL may also very well be the result of the long-term exposure to chronic herpes viruses, like cytomegalovirus (CMV) or Epstein-Barr virus (EBV) that are carried by a large fraction of the CLL patient population(34). However, if Treg protect tumor cells against T cell attack, they may form an interesting target for therapy. Various studies suggest that Treg are very sensitive to apoptosis-inducing agents like agonistic Fas antibodies(23) and chemotherapeutic drugs(67). The latter is confirmed by the
finding that in CLL, treatment with fludarabine results in a reduced number of Treg that are less suppressive than before treatment(6). Thus, the treatment of CLL with chemotherapeutic agents may serve as a double-edged sword.

**Virus-specific T cells**

The knowledge of all above described mechanisms of defense against CTL attack and the possible ways to manipulate them remains trivial if a powerful tumor-specific T cell population is lacking. In CLL however there is one specific T cell population that might be assigned to fulfill this role.

During more advanced stages of the disease, CLL patients may suffer from various upper airway bacterial infections. The reactivation of CMV and EBV however is rarely observed in CLL patients. Nevertheless, the prevalence of CMV and EBV seropositivity within the CLL patient population is high(40), mainly because of the age of these patients. This demonstrates that the immunity against this chronic herpes virus is very well maintained amidst all immune deregulations that occur in CLL. The efficiency of the suppression of CMV reactivation is supported by the observations that treatment of CLL patients with alemtuzumab frequently results in exacerbations of CMV(39). Since alemtuzumab targets all lymphocytes, it is likely that the observed reactivations of CMV under alemtuzumab treatment are caused by T cell depletion(11).

The CD8 positive CTL population that is associated with chronic CMV infection is very unique in that it possesses an effector-memory (EM) phenotype. This EM phenotype is marked by surface expression of CD45Ra and the lack of both CD27 and CD28 expression combined with high content of granzyme B and perforin(1;28). This allows CMV specific CTL to exert their cytotoxic function without the need of antigen presenting cell interference and makes them independent of costimulatory signals. During chronic CMV infection, the total CD8 positive T cell pool frequently consists for an important part of these EM T cells(24). Remarkably, in CMV seropositive CLL patients, this population is highly increased compared to healthy CMV seropositive individuals(40). It is not clear why the CMV specific CD8 population is expanded in CLL, but apparently these T cells manage to prevent CMV reactivation.

The capacity to induce target cell death without pre-stimulation and independence of costimulation makes CMV specific CTL an ideal weapon for active anti-tumor immunotherapy. Moreover, the use of CMV specific CTL in a therapeutic setting might provide a solution to the problem of therapy resis-
tance due to p53 dysfunction since these cells induce apoptosis of target cells in a p53-independent manner. Different groups have appreciated the quality of these T cells and have developed strategies to redirect the attention of these T cells towards tumor cells(30;34). Unfortunately, one major drawback of most active anti-tumor immunotherapy approaches is the requirement of \textit{ex vivo} manipulation of patient cells. Recent studies have indicated that this may be circumvented through targeting of viral peptides presented in MHC class I (MHC-I) molecules to tumor cells \textit{in vivo}(50;56). These peptide-loaded MHC-I molecules can be targeted to tumor cells via a single-chain antibody fragment that is specific for a desired tumor-antigen (figure 2). In case of CLL,

\begin{figure}
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\includegraphics[width=\textwidth]{figure2.png}
\caption{CMV specific CTL can be redirected against CLL. A streptavidin bound CD20 single chain antibody fragment (scFvCD20) is used to target CLL cells with biotinylated MHC class I molecules containing peptides from the immunodominant CMVpp65 protein. CMV specific cytotoxic T lymphocytes can recognize these targeted tumor cells and will be triggered to excrete cytotoxic molecules (of which granzyme B and perforin are the most important). These molecules subsequently induce death of the targeted CLL cell. CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; GrB, granzyme B; MHC-I, Major Histocompatibility Complex class I; TCR, T cell receptor.}
\end{figure}
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it was demonstrated that the cytolytic capacity of CMV-specific CTL could be efficiently redirected against autologous CLL cells that were targeted with MHC-I molecules containing CMVpp65 peptide via a CD20 single-chain antibody fragment(47). Although further testing should provide insight in the efficiency of tumor cell targeting and killing in vivo, this strategy looks promising for the development of an active immunotherapy for CLL, as the CMV specific CTL may not be hampered in their cytolytic response by the lack of costimulatory molecules on CLL. Moreover, as this antigen-specific targeting method avoids ex vivo manipulation, it seems perfectly applicable in a clinical setting. With growing knowledge on tumor-specific antigens, it should be possible to adapt this approach to other tumor types via tailor-made single-chain antibody fragments(17;51;52). Therefore, redirecting the power of CMV specific CTL towards tumor cells has the potency to become a feasible clinical approach towards active immunotherapy beyond CLL.

Concluding remarks

CLL is an example of a tumor that manages to escape immune surveillance through various mechanisms. On one hand, changes in the expression of apoptosis-regulating molecules protect the tumor cells against either CTL- or drug-induced cell death. On the other hand, both the tumor cells themselves and changes in the immune system of CLL patients may suppress the induction of tumor-directed T cell responses. This combination unfortunately also hampers the development of successful immunotherapeutic strategies towards CLL. Recent research has focused on manipulating CLL cells to facilitate killing by tumor-specific CTL or cytostatic drugs. Alternatively, attention is directed to manipulating T cell populations to elicit T cell driven anti-tumor responses. Possibly, combining both strategies may have a synergistic effect and therefore result in a therapy that is highly tumor-cell specific at cost of low side effects.
Chapter 1

Aim of this thesis

CLL is a malignancy that is accompanied by various immune disorders and drug-resistance mechanisms that severely complicate the treatment of the disease. The objectives of this thesis are (1) to provide more insight into CLL associated immune dysfunction, (2) to improve the understanding of the genes involved in apoptosis regulation that leads to drug-resistance and (3) to develop new strategies that either circumvent or overcome resistance to treatment.

In chapter 2 of this thesis, we address the increased number of regulatory T cells (Treg) in CLL, which might protect CLL cells against tumor surveillance. In this perspective, two possible mechanisms that might facilitate Treg accumulation are explored: antigen stimulation and resistance to apoptosis.

Chapter 3 of this thesis addresses another important problem complicating the treatment of CLL: drug resistance due to dysfunction of the p53 pathway. Unfortunately, the current available technique (FISH) often fails to detect p53 dysfunction in clinical samples at diagnosis. It is therefore explored in chapter 3 whether MLPA (a semi-quantitative PCR assay) may serve as a tool to increase the detection rate of p53 dysfunction in CLL samples.

The last chapters of this thesis focus on the development of a therapeutic strategy that acts in a p53-independent manner. It is investigated whether the redirection of virus-specific cytotoxic T lymphocytes towards CLL cells may serve as an active immunotherapy for this leukemic malignancy. In chapter 4, we describe a targeted complex that can be used to redirect CMV specific CTL towards tumor cells. Subsequently, the efficacy of this complex to activate virus-specific CTL and induce tumor cell killing is investigated by a detailed study of (mechanisms) of T cell activation (chapter 5). In chapter 6, we address the use of small molecule inhibitors of XIAP as therapeutic agent in combination with immunotherapy. To this end, it is investigated whether CLL cells (which are resistant to apoptosis) can be sensitized to CTL mediated killing via small molecule inhibitors of XIAP.

Finally, in chapter 7, we discuss the relevance of the data described in chapters 2-6 as to the future approach towards treatment of CLL.
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