Introduction to the Thesis

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CHAPTER 1

HEMATOPOIESIS AND LEUKEMIA

The continuous formation of new blood cells is driven by hematopoietic stem cells (HSCs), which reside in specialized niches in the bone marrow [1]. In these niches the HSCs are nurtured and nourished. Fundamental to the biology of HSCs is their ability to self-renew via asymmetrical cell division, in which a new HSC and a Hematopoietic Progenitor Cell (HPC) are generated. This self-renewal ensures that the HSC pool is protected from exhaustion [2]. Although, all HSCs divide via asymmetrical cell division, the frequency of these cell divisions varies amongst the HSCs. In this respect, the most primitive HSCs divide only rarely, whereas other HSCs divide more frequently [3].

The formation of mature and functional blood cells by the HSCs occurs via several consecutive cell divisions and maturation stages. The initial asymmetrical cell division is followed by several symmetrical cell divisions, during which two identical daughter cells are generated. These newly formed cells are more differentiated than their parental cells and become increasingly committed to either the myeloid or the lymphoid lineage. In the myeloid lineage of hematopoiesis erythrocytes, platelets and certain white blood cell types, such as monocytes and granulocytes are formed. In the lymphoid lineage, T cells, B cells and NK cells are generated.

It may be obvious that this intricate process of hematopoiesis is highly regulated to ensure the proper generation of sufficient blood cells. Therefore, aberrations in this process can severely hamper normal hematopoiesis and even result in the onset of leukemia. In the case of leukemia, these aberrations are often the result of inherited or acquired genetic alterations [4;5]. The aberrant hematopoiesis in leukemia typically results in an enormous overproduction of a certain blood cell type or in an impairment of immature cells to differentiate into their mature and functional status.

The acute leukemia’s are characterized by the rapid increase of large numbers of immature blood cells. This enormous production of immature blood cells impairs the production of normal cells. Furthermore, due to overcrowding of the bone marrow these immature blood cells can enter the circulation and spread throughout the body. Typical for chronic leukemia’s is the accumulation of more mature blood cells and the longer progression time as compared with the acute leukemia’s. Since acute and chronic leukemia’s can affect cells belonging the myeloid or the lymphoid lineage, four main categories of leukemia can be defined: Acute Myeloid Leukemia (AML), Chronic Myeloid Leukemia (CML), Acute Lymphocytic Leukemia (ALL) and Chronic Lymphocytic Leukemia (CLL). In addition, leukemia’s can be further classified based upon the cell type involved e.g. B cell Chronic Lymphocytic Leukemia (B-CLL) or T cell Acute Lymphocytic Leukemia (T-ALL).

Therapeutic strategies for leukemia mainly comprise of intensive radiotherapy and chemotherapeutics. Unfortunately, these therapeutics have severe and sometimes life-threatening side effects as the result of toxicity towards normal cells. Furthermore, all too often, therapy-resistant relapses after seemingly successful therapy occur. Together, this indicates that effective and leukemia-targeted therapies, with reduced toxicity are urgently warranted. This leukemia-targeted therapy can be achieved using antibody-based therapeutic strategies. By this means a tumoricidal agent can be delivered selectively to the leukemia cells. In this respect, the targeted delivery of apoptosis inducing agents seems a particular promising approach for the elimination of leukemia cells. This targeted
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apoptosis induction in leukemia cells is in fact the main focus of this thesis and the studies described herein.

APOPTOSIS

To ensure normal homeostasis, diseased cells are removed continuously from the human body by apoptosis induction. Apoptosis is a highly regulated process which forces targeted cells to commit suicide. Furthermore, to prevent collateral damage of the apoptotic cells towards healthy surrounding cells, the cells are carefully removed. The diseased cells that are removed by apoptosis typically include aged cells at the end of their life-cycle as well as (pre-)cancerous and virus infected cells. The initiation of apoptosis in targeted cells can occur via specialized immune cells such as NK and activated T-cells, which are designed to recognize diseased cells. Thereto, the NK and activated T-cells express pro-apoptotic proteins on their cell surface which enables them to initiate the apoptotic cascade in the targeted cells [6]. The subsequent execution of apoptosis is the final result of the activity of an intricate network of pro-apoptotic proteins in the targeted cells.

Key players in the apoptotic cascade are the cysteine-aspartic acid proteases (caspases) and members of the Bcl2 family of proteins [7]. The pro-apoptotic caspases can be divided, based upon their position in the cascade, in the initiator caspases and the effector caspases. As their name implies the initiator caspases are at the apex of the apoptotic signaling cascade and mainly serve to relay and amplify the apoptotic signal. The effector caspases are more downstream in the cascade and they are able to cleave numerous proteins, finally resulting in DNA fragmentation and apoptotic cell death. The Bcl2 family of proteins consists of pro-apoptotic molecules such as Bax, Bad and Bid as well as anti-apoptotic molecules such as Bcl2 and Bcl-XL [8;9]. The balance between the activation status of pro-apoptotic and anti-apoptotic proteins determines the fate of the cell. When pro-apoptotic proteins dominate the cell will go into apoptosis, however when the anti-apoptotic proteins dominate the cell will survive. In cancer cells this balance is often deregulated and dominated by anti-apoptotic proteins which results in a disturbed apoptosis and enhanced cell survival. Therefore, tipping the balance in favor of the pro-apoptotic proteins specifically in cancer cells is of particular interest for therapeutic strategies.

There are two main apoptotic pathways which are referred to as the intrinsic and the extrinsic apoptotic pathway. The intrinsic apoptotic pathway is regulated by the mitochondria and is activated upon intracellular stress, which triggers the mitochondria to release pro-apoptotic proteins [10]. In this respect, DNA damage induced by chemotherapeutics can activate the intrinsic apoptotic pathway. The extrinsic apoptotic pathway is initiated by binding of so-called death ligands to their cognate death receptors [11]. Since these death receptors convey a potent apoptotic signal and are relatively easy accessible, the targeted activation of these death receptors has generated enormous enthusiasm for novel cancer therapies [12;13].

APOPTOSIS INDUCTION BY THE DEATH LIGANDS TRAIL AND FASL

Two prominent death ligands are the tumor necrosis factor (TNF) family members, TNF Related Apoptosis Inducing Ligand (TRAIL) and Fibroblast associated cell surface Ligand
(FasL). Like the other TNF family members, TRAIL and FasL are self-assembling trimeric proteins. Both, TRAIL and FasL are expressed on the cell surface of NK- and activated T-cells. Activation of the corresponding death receptors by TRAIL and FasL, results in the intracellular formation of the Death Inducing Signaling Complex (DISC). This assembly of the DISC results in the sequential activation of the initiator caspase-8 or -10 and effector caspases, ultimately followed by apoptotic cell death. In addition, in some cell types, the death-receptor pathway relies on a mitochondrial amplification loop that is activated by caspase-8-mediated cleavage of pro-apoptotic Bid to a truncated form. Next, truncated Bid (tBid) mediates the release of pro-apoptotic cytochrome C and caspase-9 from the mitochondria [14], thereby connecting the extrinsic to the intrinsic apoptotic pathway.

Although TRAIL and FasL are expressed on the cell surface of NK- and T-cells, they can be proteolytically cleaved off, yielding soluble forms of TRAIL (sTRAIL) and FasL (sFasL). Intriguingly, sTRAIL potently induces apoptosis in variety of malignant cells, whereas normal cells appear to be resistant to sTRAIL mediated apoptosis [15-17]. The mode of action of this apparent tumor-selectivity of sTRAIL-induced apoptosis is still enigmatic. Nevertheless, this tumor-restricted activity has sparked the potential use of TRAIL for the treatment of human malignancies, including leukemia. In contrast, the application of sFasL for the treatment of cancer was originally deemed impossible due to severe hepatotoxicity observed in sFasL treated mice [18;19]. However, it became apparent that this toxicity was due to high molecular weight aggregates present in the used sFasL preparations. Additional studies demonstrated that homotrimeric sFasL is in fact not toxic at all [20;21]. This observation renewed the potential of sFasL for the effective and safe treatment of cancer.

Despite, that the use of homotrimeric sTRAIL and sFasL preparations for the treatment of leukemia seems very promising, several limitations may hamper their use. A fundamental problem is the widespread expression of the various cognate death receptors throughout the human body. This unfavorable surplus of potential binding sites will impede the leukemia-selective accretion of sTRAIL and sFasL. In addition, the apoptotic activity of sTRAIL and sFasL is less pronounced than the activity of their corresponding membrane-bound forms [20;22].

TARGETED DELIVERY OF TRAIL AND FASL TO LEUKEMIA CELLS

To overcome the limitations associated with sTRAIL and sFasL we developed a method for the targeted delivery sTRAIL and sFasL to leukemia cells. To this end we genetically fused a single chain fragment of the variable region (scFv) antibody fragment with either sTRAIL or sFasL. The scFv’s have the same specificity as the parental antibodies, however they are much smaller [23]. In our studies we have used several scFv’s which specifically recognize leukemia-associated antigens such as CD7, CD20, CD33 and CLL-1. Thus, these fusion proteins are designed to selectively deliver sTRAIL or sFasL to the cell surface of target antigen-positive leukemia cells. By using this fusion protein format the limitation associated with the wide spread expression of the death receptors on normal cells can be circumvented. In addition, the high affinity binding of the scFv to the target antigen is characterized by fast on/slow off binding kinetics. Therefore, the fusion proteins accrete at the cell surface of target antigen-positive cells. Importantly, this cell surface accreted
sTRAIL and sFasL resemble their membrane bound form, resulting in a strongly enhanced apoptotic activity [24;25].

To accelerate the potential clinical application we expressed the fusion proteins in the eukaryotic Chinese Hamster Ovary (CHO) cells, an industry-favored production cell line [26]. Production of the fusion proteins in the CHO cells benefits from the stringent quality control mechanism and post-translational modifications typical for eukaryotic cells. This CHO-based production platform resulted in the production of stable and homotrimeric fusion proteins, in the absence of detectable high-molecular-weight protein aggregates.

An obvious potential limitation to antibody-based approaches is the escape of target antigen-negative tumor cells from therapy. In this respect, a heterogeneous expression of selected target antigens has been demonstrated in leukemia. As a result, target antigen-negative leukemia cells may exist, which consequently can escape from antibody-based therapeutic approaches [27;28]. Furthermore, target antigen-negative leukemia cells can arise during or after therapy due to therapy-induced down-regulation. In this respect, in chapters 2, 4, 6 and 8 we demonstrated the so-called bystander effect, which is an essential part of the concept of targeted apoptosis induction by scFv:sTRAIL and scFv:sFasL fusion proteins. The bystander effect is based on the principle that targeted tumor cells are not only eliminated, but are also exploited to convey a therapeutic effect towards neighboring tumor cells which can be devoid of the target antigen.

**CD7 AS A TARGET ANTIGEN ON MALIGNANT T-CELLS**

CD7 is an antigen which is highly expressed on T cell Acute Lymphocytic Leukemia (T-ALL) [29]. Although, the function of CD7 is not yet fully understood it is known that is normally expressed on T and myeloid cells in early hematopoietic-cell ontogeny, thymocytes, natural killer (NK) cells, and to a distinct subset of peripheral-blood T cells. Due to the high expression on T-ALL, CD7 has been used for antibody-based therapeutic strategies in both preclinical studies and clinical trials [30;31]. In chapter 2, we report on scFv:sFasL fusion protein designed to induce apoptosis in CD7+ leukemia’s. Thereto, we produced a homotrimeric fusion protein designated scFvCD7:sFasL. In our study we assessed the CD7-restricted apoptosis induction of this fusion protein towards CD7+ leukemic T-cells lines and patient-derived CD7+ leukemia cells.

**CD20 AS A TARGET ANTIGEN ON MALIGNANT B-CELLS**

Malignant B-cells typically express high levels of normal B-cell surface antigens, such as CD20. This elevated expression of CD20 on malignant B cells has resulted in the development of the CD20-targeted monoclonal antibody Rituximab. The anti-leukemia effect of Rituximab is the result of antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) upon binding to CD20[32]. In addition, part of the tumoricidal activity of Rituximab is the result of the direct activation of apoptosis via cross-linking of cell surface-expressed CD20 on malignant B cells [33]. However, the exact mechanism by which Rituximab induces apoptosis via the cross-linking of CD20 is currently not known. In chapter 3, we report a novel mechanism by which Rituximab induces apoptosis.
Although, Rituximab has improved the therapy outcome for patients its application is often not curative [34]. Thus, the development of alternative Rituximab-based approaches with enhanced tumoricidal activity is warranted, especially towards Rituximab-resistant malignant B cells that currently escape from therapy. Therefore, in chapter 4 we generated scFvRit:sFasL, a fusion protein in which the specificity and apoptosis-inducing activity of Rituximab is retained and combined with the potent apoptosis inducing activity of FasL.

CD33 AS A TARGET ANTIGEN ON ACUTE MYELOID LEUKEMIA (AML) CELLS

In the next chapters we have turned our focus to the targeted elimination of AML cells. The prognosis for AML patients is dismal with a 5-year survival rate of 20-30% [35;36]. Like T-ALL and B-CLL cells, AML cells express several lineage-specific antigens that may be exploited for antibody-based therapy. The best characterized of the AML target antigens is CD33. The expression of CD33 on AML cells has resulted in the clinical development of Gemtuzumab Ozogamicin (GO), a CD33-targeted immunotoxin. GO comprises an anti-CD33 antibody chemically coupled to a highly cytotoxic calicheamicin derivative via a hydrolysable linker. After CD33-selective binding of GO, the CD33-GO-complex internalizes and ends up lysosomes. In the acidic milieu of the lysosomes the calicheamicin moiety is hydrolytically released from the antibody moiety. Subsequently, free calicheamicin can translocate to the nucleus and intercalate with the DNA, causing site-specific double-strand breaks resulting in apoptotic cell death [37;38]. Although, GO has a generally acceptable safety profile, its application is associated with serious and dose-limiting toxicity in some patients, including hepatotoxicity and severe myelosuppression [35;39-41]. Therefore, the rational design of combinatorial approaches of GO with other therapeutics in order to achieve optimal tumoricidal activity with reduced toxicity is warranted. In chapter 5, we demonstrate that a promising candidate for such a combinatorial approach is valproic acid (VPA). For over 20 years VPA is being used safely for the treatment of seizures. Recently however, it was shown that VPA acts as a histone deacetylase inhibitor (HDACi) with promising anti-AML activity [42;43]. Treatment of cells with an HDACi results in a hyper-acetylated state of their histones, which translates in a more open chromatin structure. Consequently, the DNA becomes more accessible to intercalating agents. Therefore, we hypothesized that treatment of AML cells with VPA should augment calicheamicin binding capacity and potentiate the apoptotic activity of GO.

Furthermore, to utilize CD33 for the targeted delivery of sTRAIL we have generated a novel fusion protein designated scFvCD33:sTRAIL, which is described in chapter 6.

In this study we compared the in vitro activity of scFvCD33:sTRAIL with the activity of GO towards CD33+ AML cells. Furthermore, we compared the toxicity profile of scFvCD33:sTRAIL and GO.

TARGETING LEUKEMIA STEM CELLS (LSCS)

To a varying degree most leukemia types initially respond well to therapy with partial or even complete remissions. However, after a period of minimal residual disease many patients succumb to refractory relapses of the disease. Recent insights indicate that the development of these relapses may be due to the selective continued survival of a small, but distinct population of therapy-resistant tumor-initiating cells, commonly referred
to as Leukemia Stem Cells (LSCs) [44;45]. These LSCs are thought to originate either from normal hematopoietic stem cells (HSCs) or from more differentiated progenitor cells that have acquired malignant features [46]. In the latter case, the progenitor cells have de-differentiated and re-acquired stem cell-like characteristics via as yet undefined pathways. Both LSCs and HSCs possess self-renewal capacity but are relative quiescent compared to more mature progenitor’s cells. However, LSCs typically have a much stronger capacity for cellular expansion than normal HSCs, probably due to an increase in symmetric self-renewal activity of LSCs [44;47]. Although LSCs are typically resistant to conventional therapy, several possibilities for the targeted elimination of these LSCs seem feasible. In chapter 7, we review several promising possibilities for the elimination of LSCs and provide perspectives and recommendations for future therapeutic strategies.

Since the expression of death receptors appears to be upregulated on LSCs as compared with HSCs, one of those promising possibilities appears to be the targeted delivery of sTRAIL and sFasL to the LSCs [48;49].

Unfortunately, not all AML patients are eligible for CD33-targeted therapy and therefore the exploitation of additional AML cell surface antigens is warranted. In this respect, C-type Lectin Like Molecule-1 (CLL-1) appears to be an attractive alternative target antigen to CD33. CLL-1 is expressed in >90% of the AML patients [50;51] in which expression appears to be retained under all conditions of treatment and disease [52]. In peripheral blood, both monocytes and granulocytes show moderate CLL-1 expression, while CLL-1 is completely absent in any other tissue. Importantly, CLL-1 is also expressed on CD34+/CD38- AML LSCs, whereas it is not expressed on normal hematopoietic stem cells [51-53]. The targeted elimination of CD34+/CD38- AML LSCs is of particular therapeutic interest, since they are thought to be responsible for the frequent therapy-resistant relapses in AML [47]. In chapter 8 the anti-AML LSCs activity of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL is reported. Together, the targeted delivery of sTRAIL and sFasL to leukemia cells including the LSCs might offer a step towards curative leukemia therapy.

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