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A Modified P450 Cytochrome as Safety Mechanism in Adoptive T-cell Therapy

Inaugural dissertation

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Zusammenfassung

Adaptive T-Zelltherapien mit unveränderten allogenen oder genetisch veränderten autogenen T-Zellen kann eine Immunität gegen maligne Zellen vermitteln. Um die Sicherheit dieser zellulären Therapien zu erhöhen, ist es wichtig, die T-Zellen vor der Infusion mit einem Suizidgen auszustatten, welches somit eine spezifische Kontrolle *in vivo* ermöglicht. Das Ziel dieser Arbeit war es, einen klinisch einsetzbaren selbst-inaktivierenden (SIN) lentiviralen Vektor zur effizienten Koexpression von zwei Transgenen zu entwickeln, der für die Anwendung einer Donor-Lymphozyten-Infusion nach allogener Stammzelltransplantation oder für die spezifische Eliminierung autogener T-Zellen, die einen chimären Antigenrezeptor (CAR) exprimieren, geeignet ist. Als neuartiges Suizidgen habe ich eine aktivitäts-optimierte Variante des humanen CYP4B1 Enzyms verwendet, das eine ER-membranständige Monooxygenase ist, die spezifisch das natürlich vorkommende Prodrug 4-Ipomeanol zu einer hochgiftigen alkylierenden Substanz katalysiert. Diese modifizierte CYP4B1-Variante sollte entweder mit dem trunkierten CD34 oder CD271/p75 nerve growth factor receptor als Selektionsmarker für die Donor-Lymphozyten-Infusion oder mit CARs gegen CD19, CD22 und CD33 als Immuntherapie gegen ALL und AML durch autologe T- Zellen koexprimiert werden. Hierzu wurden die Komponenten eines lentiviralen Expressionsvektor systematisch getestet. Diese umfassen sowohl verschiedene virale oder menschliche Housekeeping Promotoren, mit oder ohne aktiven Spleiß-Stellen, als auch Strategien zur Koexpression zweier Transgene durch zwei Promotoren, ein IRES-Element oder einem CHYSEL wirkenden Element, der 2A-Seite. Funktionell erfolgreiche Parameter für die verschiedenen Konstrukte waren die MACS Selektion, die Induktion der Apoptose und die CAR-vermittelte Zytotoxizität gegen Leukämiezellen.

Im zweiten Teil dieser Arbeit wurden systematisch unterschiedliche Struktur-Analoga von 4-Ipomenol in menschlichen Leberzellen und die Induktion von Apoptose in primären T-Zellen analysiert. Eines der Substrate ist Perilla keton, ein natürlich vorkommendes Lungentoxin. Perilla keton induziert Apoptose in MACS selektionierten humanen T-Zellen, die das modifizierte CYP4B1 exprimieren, bei gleicher Konzentration doppelt so effektiv wie 4-Ipomeanol.

In dieser Arbeit wurden mehrere klinisch relevante Expressionskonstrukte entwickelt, die potentiell adaptive T-Zelltherapien bei Menschen sicherer gestalten können. Dies muss in präklinischen Tiermodellen *in vivo* weiter verfolgt werden. Zudem wurde ein neues wirksameres Prodrug für das CYP4B1 Suizidgen identifiziert, das durch *in vivo* Studien in einer *Cyp4b1*- Knock-out Maus für den potentiellen klinischen Gebrauch weiter getestet werden muss.

Summary

Adoptive T-cell therapies with unmodified allogeneic or genetically engineered autologous T-cells can transfer immunity against malignant cells. An important safety strategy for these cellular therapies is to equip the T-cells prior to reinfusion with a suicide gene that facilitates specific *in vivo* control. A major focus of this thesis was to develop clinically applicable self-inactivating (SIN) lentiviral vectors with efficient co-expression of two transgenes, either for donor lymphocyte infusion after allogeneic stem cell transplantation or for eliminating autologous T-cells expressing chimeric antigen receptors (CARs). As a novel suicide gene, I used an activity-optimized human CYP4B1 enzyme that is an ER-expressed monooxygenase and specifically bio-catalyzes a natural occurring pro-drug, 4-ipomeanol, towards a highly toxic alkylating substance. Systematically testing of the best modules for co-expression of modified CYP4B1 either with truncated CD34 or CD271/p75 nerve growth factor as selection markers for donor lymphocyte infusions or with CARs targeting CD19, CD22 and CD33 for immunotherapy of ALL and AML by autologous T-cells included different viral or human housekeeping promoters with or without splice-active sites and co-expression strategies with two promoters, IRES or 2A sites, a CHYSEL-acting element. Successful functional read-outs for the different constructs were MACS selection, apoptosis induction and CAR-mediated cytotoxicity against leukemia cells.

In the second part of the thesis, I systematically analyzed different structure analogues of 4-ipomenol in human liver cells and the induction of apoptosis in primary T-cells. Importantly, one of these substrates, perilla ketone, which is a naturally occurring pneumotoxin, was twice as efficient at similar concentrations as 4-ipomeanol to induce apoptosis in mutant CYP4B1 expressing genetically modified affinity selected human T-cells.

In summary, I developed several clinically suitable constructs in this thesis that will potentially make adoptive T-cell therapies in humans safer and that will be further tested in preclinical animal models *in vivo*. In addition, I also identified a novel more potent prodrug for the CYP4B1 suicide gene that needs to be further tested *in vivo* in Cyp4b1-/- mice for potential clinical usage.

1. Introduction

The idea of exploiting the immune system to target cancer cells dates back into the 19th century, when William Coley inoculated repeatedly live bacteria into malignant tumors as stimulation of the immune system [7]. Immunotherapies against cancers can be divided into 3 divergent strategies. Active immunotherapies enhance the patient's immune system to provoke an immune response, recognition of tumor-associated antigens and elimination of malignant cells. Passive immunotherapies induce immune responses by administration of exogenously produced components such as lymphocytes and antibodies. Modulatory immunotherapies are thought to amplify immune responses against cancer cells by a general enhancement of the immune responsiveness. Active immunotherapy can involve either polyvalent vaccines that are derived from whole tumor cells and/or dendritic cells or antigen-specific vaccines. The development of active immunotherapy is shown in figure 1 in form of a timeline.

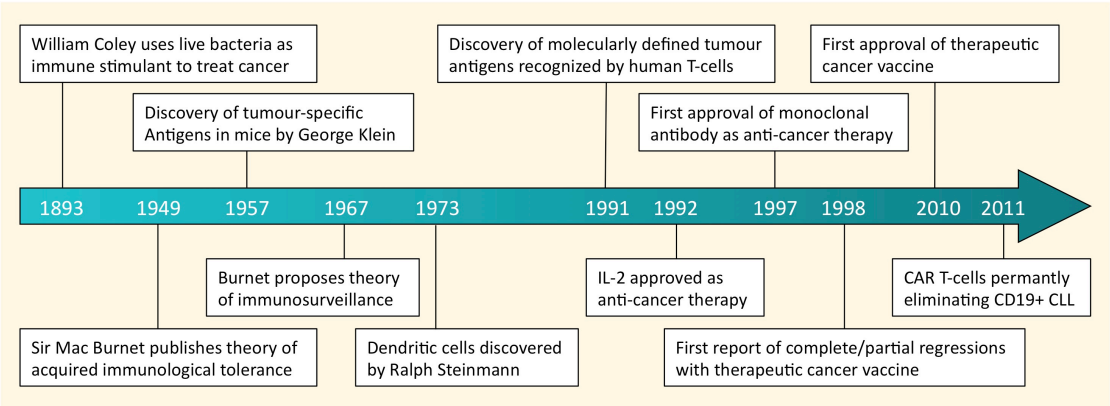

Figure 1: Milestones in the development of active immuntherapy. Modified after Melero *et al.* 2014 [1].

1.1 Adoptive Cell Therapy

The term adoptive cell therapy (ACT) describes the transfer of immune cells (T-cells, NK-cell, B-cells) into a patient with cancer to give their immune system the ability to recognize and kill tumor cells. The transferred immune cells may have originated from the patient or from another individual.

The *ex vivo* expansion of specific T-cells, which were able to recognize cancer antigens and infiltrate the stroma of growing, transplantable tumors, without a loss of function was only possible due to the discovery of interleukin-2 (IL-2) as T-cell growth factor in 1976 [8]. Infiltrating T-cells could be easily expanded from tumors by excising the tumor mass, dissociating it into a single cell suspensions and incubating the non-

adherant cells with high doses of IL-2. After *ex vivo* expansion in IL-2 and transplantation of these tumor-infiltrating lymphocytes (TILs) into patients, they induced regression of lung and liver tumors [9] as well as metastatic melanoma after all available therapies have failed [10]. Another critical milestone in ACT was the establishment of a non-myeloablative chemotherapy regimen administered prior to the TIL administration. In contrast to the myeloablative conditioning, the doses of chemotherapy agents like cyclophosphamide used for a non-myeloablative regimen are too low to eliminate all bone marrow cells including stem cells while it still enhances engraftment and *in vivo* survival. This reduced-intensity conditioning by chemotherapy led to increased cancer regression and a persistent oligoclonal repopulation of the host [11]. Within the tumor, tumor-specific T-cells experience, despite chronic activation, and an excessive flow of overexpressed immunosuppressive factors such as T-cell immunoglobulin domain and mucin domain-containing protein 3 (TIM3), lymphocyte activation gene-3 (LAG3), programmed death 1 (PD1) and cytotoxic T-Lymphocyte-associated protein 4 (CTLA4) [12, 13]. The *ex vivo* expansion of TILs and transfer back into the patient can also lead to complete eradication of the tumor and hence a durable and complete remission in patients with metastatic melanoma [14]. Objective response rates of some clinical studies are up to 48% [15, 16].

Interestingly, ACT with TILs is most effective for targeting highly especially metastasized immunogenic cancers such as melanoma despite of several escape mechanisms of tumors [17]. T-cells can infiltrate also other tumors, for example derived from the colon or breast, but the function/anergy and specificity of these infiltrating T-cells are not understood [18]. Importantly, principally any target cell can be recognized and lysed if T-cells are engineered with specific receptors recognizing the antigen.

1.2 Genetic engineering of tumor specific T-cells

Naturally occurring T-cell receptors (TCR) can be specific for antigens that are also present on cancer cells, but thymic selection usually leads to low affinity of these TCRs towards endogenous antigens and thereby limits the ability to detect low levels of HLA restricted antigens that are typically expressed on cancer cells [19]. The α- and β-chain of a TCR can be enhanced in affinity and introduced with transfer vectors into recipient T-cells [20]. A high affinity is critical for improved functioning of TCR-modified T-cells, however a major concern within this therapy is the mispairing of the introduced α- or β-chain with endogenous TCRs resulting in TCRs with unknown specificity and potential autoimmunity. Recently, two different therapies using engineered α-chains with increased affinity for the melanoma antigen family A3 (MAGE-A3), which is abundantly expressed in multiple tumor entities and only little present in normal tissue, led to severe and unpredictable toxicities in patients treated with autologous TCR-expressing T-cells [21, 22].

Redirecting and engineering T-cells with a synthetic receptor specific for an antigen present on malignant and non-malignant cells can mediate tumor-specificity, which have been naturally occurred in TILs. The first attempt was made by Gideon Gross *et al.* in 1989, who combined either one of the C-region gene segments of the α- or β-chain of a TCR with the heavy (V_H) and light chains (V_L) of the variable domain of an antibody against TNP hapten into a fusion protein [23]. By this, the group developed the first chimeric antigen receptor (CAR) that unites the antigen binding property of a monoclonal antibody with the lytic capacity of T-cells [24, 25]. The design of a CAR is depicted in Figure 2. The light and heavy chains of the single-chain variable fragment (scFv) of a monoclonal antibody with a hinge region compose the ectodomain. This part is responsible for the antigen recognition. The affinity of scFv is essential for the functionality and efficacy of a CAR [26, 27]. The hinge region links the antigen-recognizing part and the transmembrane domain, while the transmembrane domain anchors the CAR within the plasmamembrane and connects the ectodomain with the endodomain. First, the transmembrane domain was derived from CD3ζ, but these CARs were less stable than CARs with a transmembrane domain derived from CD28 [28]. Today, transmembrane domains from IgG4, CD8 or CD28 were used.

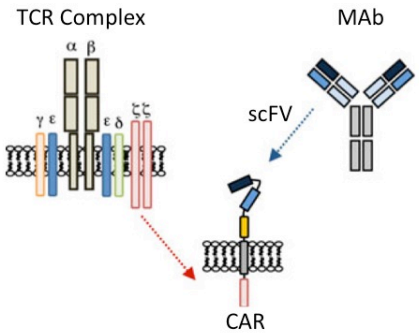

Figure 2: Design of a CAR. The ectodomain of the CAR is composed of the light and heavy chain (scFv) of a monoclonal antibody (MAb), the endodomain of stimulatory and co-stimulatory molecules of TCR chains. Both domains are linked via a transmembrane region. The TCR complex includes α, β, γ, δ, and ζ chains. The image is obtained from Dotti *et al.* 2014 [3].

The endodomain mediates activation and co-stimulatory signals upon antigen binding of the ectodomainby incorporating regions of different signaling or stimulatory chains. The first designed CAR contained only one signaling domain; the ζ-chain of CD3 of TCRs. Brentjens and his collagues transduced primary T-cells with CD19 CAR containing a TCR ζ-chain and infused them in SCID-Beige mice where Raji tumor cells had been stably engrafted [29]. The anti-tumor efficacy of this 1st generation CAR was enhanced due to the expression of CD80 on Raji cells as an additional stimulus to adoptively transferred T-cells. Otherwise, the engagement of just the TCR ζ-chain without costimulatory domains in the constructs leads to T-cell anergy and impaired engraftment. Therefore, 2nd generation CARs were constructed by incorporating a co-stimulatory domain. Initially CD28 was added as co-stimulatory domain to the signaling

ζ-domain into the CAR and thus drastically increased the autocrine production of IL-2 as well as the cytotoxicity [30, 31]. Other co-stimulatory domains were derived from CD27, OX40, 4-1BB, CD244 and ICOS. All these domains can enhance the proliferation and persistence of T-cells [32-34]. 3rd generation CARs incorporate two co-stimulatory domains, for example from CD28 and 4-1BB Although CAR T-cells successfully treat hematologic malignancies, targeting solid tumors is limited by antigen-loss tumor cell variants, increased resistance of the tumor cells to T-cell mediated cytolysis due to an defective apoptosis pathway [35], less penetrable tumor stroma and a repressive tumor microenviroment [36, 37].

To address these problems, 4th generation CARs (also called TRUCKs) added another module. CAR T-cells were co-transduced with a vector encoding for a cytokine expression cassette whose expression is linked to the CAR activity [38, 39]. This enables a local production of cytokines within the tumor microenvironment, which is important for T-cell persistence in solid tumors.

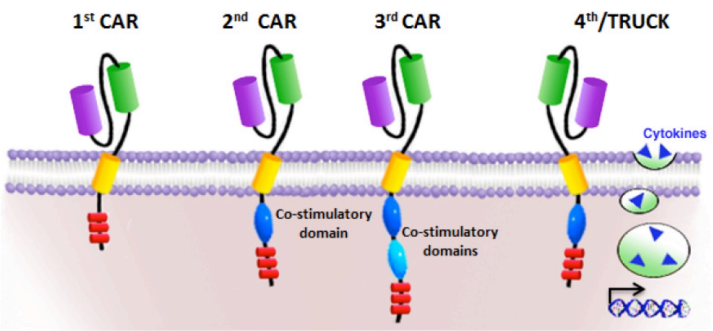

Figure 3: The 'evolution' of CARs. 1st generation CARs have one signaling domain incorporated, 2nd generation and 3rd generation CARs have added one or more co-stimulatory domains to the signaling domain. 4th generation CARs utilizes a CAR-responsive promoter for cytokine production. The image was obtained from Cheadle *et al.* 2012 [2].

The efficacy of a CAR seems to be dependent on the number/density of the CAR molecules and on the affinity of scFv fragment to the antigen. However, this affinity can reach a plateau where an additional increase does not further enhance T-cell activation [26]. Also, the differences in length and flexibility of the CARs mediated by the hinge region can affect the efficacy and function of a CAR [40]. This may be partly due to the location of the antigen epitope on the target cell, partly a consequence of antigen 3D structure. Since there are so many reciprocal actions between scFv, hinge, transmembrane domain and endodomain, the optimal design for any CARs has to be determined individually.

Major histocompatibility complex (MHC) molecules are highly polymorphic surface antigens, marking the body's own cells as 'self'. A main function is to present processed peptides and antigens via antigen presenting cells (APC) to CD4 positive helper T-cells for developing specific immunity (MHC class II restricted) and to CD8 positive T-cells for mediating cellular cytotoxicity and cell lysis (MHC class I restricted). Paramount for the specificity of CARs is that by engaging the single-chain variable fragment of an

antibody, CARs recognize tumor antigens independently from MHC presentation. Therefore, CARs can only detect epitopes on intact surface proteins, but they are also unaffected by common tumor escape mechanisms like the downregulation of human leukocyte antigens class I and defective antigen processing [41].

Beside these major advantages some disadvantages of CAR T-cell therapies have been identified. Importantly, one major problem is unexpected toxicities, which can be divided into three groups [42]:

1) Off-target toxicity: as artificial molecules that contain parts of a monoclonal antibody and a TCR and have not undergone positive/negative selection in the thymus, CARs themselves theoretically hold a risk of off-target recognition. This problem occurs more often with affinity enhanced TCRs than CARs, but it has been recently shown that CARs incorporating the hinge region derived from IgG1 CH2CH3 activate cells of innate immunity by interacting with their Fc receptor [43].
2) On-target, off-tumor toxicity: Tumor-associated antigens may be expressed in variable degrees on non-malignant cells in the body. Therefore reactivity of a CAR against non-malignant cells can occur.
3) Cytokine-release syndrome (CRS): This syndrome describes a rapid immune response characterized by a massive release of a group of acute phase cytokines such as IFNγ and TNF [44-47]. Especially IL-6 is associated with the macrophage activation syndrome (MAS). For this critical clinical situaion the administration of tocilizumab, an IL-6 receptor antagonist, was shown to be an effective treatment [48].

1.2.1 CAR T-cells to treat B-lineage malignancies

Malignancies of B-cell lineages can be targeted specifically by a CAR specific for an antigen, that is restricted to the B-lineage and not expressed in other tissues. Hence, attractive targets for B-cell malignancies are the cell surface antigens CD19, CD20 and CD22 (Figure 4), where highly specific antibodies are available for each antigen. Expression of CD19 and CD22 begins in Pro-B-cell and is maintained until maturation to plasma cells. Expression of CD20 starts later, in Pre-B-cells. Therefore, CD19 and CD22 can be used to treat preB-acute lymphatic leukemia and B-cell leukemias and lymphomas. In contrast, CD20 is not expressed at high densities in malignant pre-B-cells; hence targeting of CARs using this antigen is restricted to B-cell leukemias and lymphomas. Therapies with B-lineage specific CARs lead to complete B-cell aplasia as these CARs recognize both normal and malignant B-cells. Here infusion of immunoglobulin can rescue the humoral deficiency.

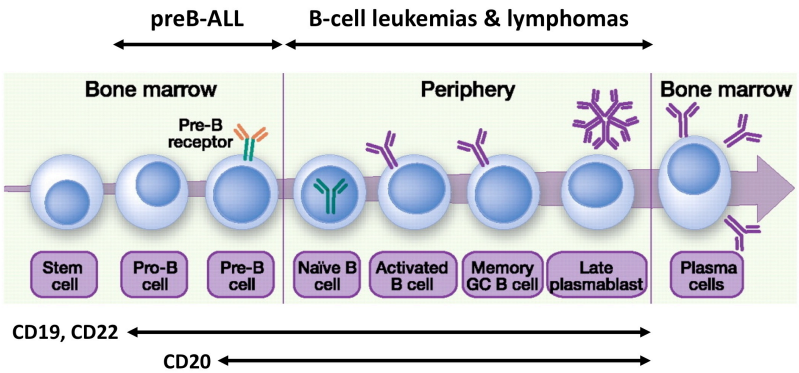

Figure 4: B-linage antigen representation and association with B-lineage malignancies. CD19 and CD22 are expressed from Pro-B-cell until maturation into plasma cells. CD20 expression starts in Pre-B-cells and lasts until maturation into plasma cells.

The most success has been achieved with CARs targeting CD19. Preclinical studies testing a 1st generation CD19 CAR have shown that lymphodepletion through non-myeloablative conditioning is necessary for engraftment of CD19 CAR positive T-cells [49]. These engrafted T-cells were capable to eradicate established tumors [50], but that they rapidly declined within 3-4 months [50]. Recent clinical phase I/II studies targeting relapsed or refractory acute lymphocytic leukemia (ALL) [44, 45, 51] or chronic lymphocytic leukemia (CLL) [44, 47] employ 4-1BB [45, 47, 51] or CD28 [44] as costimulatory domains. Here, the transduced autologous T-cells were infused after chemotherapy for lymphodepletion. Davila and Brentjens used a CD19-CD28-CD3ζ CAR construct to treat patients with refractory or relapsed B-ALL and achieved 88% complete response rate as preparation for allogeneic hematopoietic stem cell transplantation [52]. From the collective experience from over 100 patients treated in different settings for B-cell malignancies, Gill *et* June concluded that 1) acute lymphoid leukemia is more feasible for the treatment than chronic lymphoid leukemia, 2) that a dramatic response is associated with the cytokine release syndrome, 3) that there is no clear dose-response relationship of the numbers of T-cells administered to patients, and 4) that there seems to be no correlation between tumor burden and response rate [53].

1.2.1 CAR T-cells to treat AML

Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults. AML originates in the myeloid lineage of hematopoietic cells and often has a poor prognosis [54]. Depending on the subtype, usually most AML patients achieve remission after 6-8 months of intensive chemotherapy, but more than the half experience disease reoccurrence [55]. Allogeneic stem cell transplantation (ASCT) is an important potentially curative treatment, but is associated with severe morbidity and mortality and not all patients are healed from their AML [56]. Also here, CAR T-cell therapy is an attractive strategy for a new approach in this disease. While CD19, CD20 and CD22 are B-lineage restricted antigens and expressed on malignant and non-malignant B-cells, myeloid blasts do express a myeloid specific but not AML specific

target surface antigen. A CAR T-cell therapy targeting myeloid antigens such as CD33 and CD123 leads to severe hematopoietic toxicity and reduction of all myeloid cells including normal cells and AML blasts [57]. Such a constant ablation of myeloid cells would be a lethal consequence of this therapy. Tumor-specific immunotherapies using antibodies against CD33, a receptor of the SIGLEC family and expressed on myeloid cells, 90% of AML blasts and normal myeloid progenitors [58-60], has been shown effective in clinical trials [61, 62]. However, these effects were only transient. CD44 is a hyaluronate receptor and ubiquitously expressed, but overexpressed in hematologic and epithelial tumors. Importantly, certain isoforms of CD44 can be found relatively tumor specific on malignant cells. Especially CD44v6 is overexpressed in AML [63], among other cancers, but not in hematopoietic stem cells [64]. For AML, preclinical studies using CD33-CARs with 4-1BB and CD3ζ targeting AML cell lines and AML samples [57, 65] as well as CD44v6-CAR with CD28 and CD3ζ targeting AML samples and control cells [66] have been conducted, but results are not as impressive as in B-cell malignancies.

1.3 Allogeneic hematopoietic stem cell transplantation and donor lymphocyte infusion

Principally, the most successful T-cell immunotherapy to date is the infusion of donor T-cells present in the graft in allogeneic hematopoietic stem cell transplantation (HSCT). Engrafting a new blood and immune system after myeloablative chemotherapy is a form of adoptive cell therapy used to treat malignancies of the hematopoietic system, immunodeficiencies, metabolic diseases and neuroblastoma [67, 68]. A major achievement in transplant biology was the discovery of human leukocyte antigens (HLA), also known as major histocompatibility complex (MHC). Prior to this discovery, only syngeneic transplantations could be performed with patients surviving [69-71]. The first successful syngeneic bone marrow stem cell transplantation was performed in 1959 [72] and the first successful allogeneic transplantations in 1968, the latter in patients suffering from immunodeficiency disorders such as Wiscott-Aldrich-syndrome or X-linked severe combined immunodeficiency [73, 74]. Today, for allogeneic HSCT, the donor and the host should match within the major HLA antigens (A, B, C, DR and DQ) [75], although a partial match with a difference in one of these antigens can also be used for HSCT. However, also a 'perfect' match may still have differences within the minor HLA antigens. Host T-cells are the main meditators of the immune reactions against the transplant and can induce the alloreactive rejection against the graft. Stem cells for HSCT can be obtained from bone marrow and from peripheral blood after consecutive administration of a growth factor (G-SF), which facilitates the release of stem cells from the bone marrow into the blood [76-79].

1.3.1 Graft-versus-Host-Disease versus Graft-versus-Leukemia

In their seminal paper, Kolb *et al.* described that relapsed AML after allogeneic HSCT can be treated by infusions of mature donor lymphocytes from peripheral blood (DLI), which can induce long-term complete remission [80]. Here, through a reaction called graft-versus-leukemia effect (GvL), the mature donor lymphocytes/T-cells from peripheral blood (PB) recognize and kill the leukemia cells in an HLA-dependent fashion. This activity can at least partially be mediated by the hematopoiesis-restricted minor histocompatibility antigens HA-1 or HA-2, which are expressed on malignant cells and can serve as targets for the donated T-cells [81, 82]. The success of DLI treatment is also influenced by a reaction called graft-versus-host disease (GvHD), which 40-60% of patients receiving DLIs develop [83, 84]. This immunologic consequence of DLIs is one of the most important complications with high morbidity and mortality in allogeneic HSCT [85-87] and was first described in 1955 as 'secondary disease' [88]. Mechanistically, the immune competent donor T-cells recognize HLA positive cells of the host as foreign and mount immunological reaction against them. (The same activity of donor T-cells mediates the alloreactive rejection of the transplant.) There are two forms of GvHD: acute and chronic. The acute GvHD develops within the first days up to two months post HSCT depending among other factors on the number of infused T-cells and HLA compatibility [89]. Clinical symptoms are damages to the liver, skin, mucosa and the gastrointestinal tract caused by severe lymphocyte inflammations; weak manifestation can also be diffuse erythematous maculopapular rashes of the skin. Based on the extent of the symptoms, the GvHD is systematically classified into grade I-IV [90]. A chronic GvHD can develop after an acute GvHD or without any acute GvHD symptoms as 'de novo' disease [91]. Here, manifestations of an acute GvHD are added to damages to eyes, joints and lungs. In general, GvHD is comparable to an autoimmune disease and can further be divided into limited or extensive chronic GvHD, again based on the extent of the symptoms [92].

GvHD is usually treated with immunosuppressive agents such as cortisone, cyclosporine A and/or methotrexate [93], all inhibiting the function and activation status of the T-cells. Another possible precaution to prevent a GvHD is the T-cell depletion of the transplant prior to HSCT. However, any reduction in T-cell activity can also reduce the graft-versus-leukemia effect [83, 94-96]. This has been shown by the association of chronic GvHD with a better response and a lower risk of relapse [97], because both reactions, GvL and GvHD, depend on the alloreactivity of the donor T-cells.

1.4 Suicide gene therapy

The concept of a suicide gene clinically emerged about three decades ago [98]. It describes a genetically encoded mechanism allowing selective killing of positively transduced cells in the light of unwanted toxicity. Basically, the protein resulting from the suicide gene converts a non-toxic substance, called prodrug, into a cell toxin and this

reaction only occurs in transduced cells. This activity enables to control the natural limitations of GvHD following HSCT or DLI. T-cell depleted stem cell grafts engraft worse as T-cells facilitate engraftment. Instead of depleting T-cells from a transplant, they can be added back after being transduced with a suicide gene to control unwanted alloreactivity. In case of a life-threatening GvHD the prodrug can be administered and the GvHD stopped without interfering with the immune reconstitution that follows HSCT (figure 5) [99, 100].

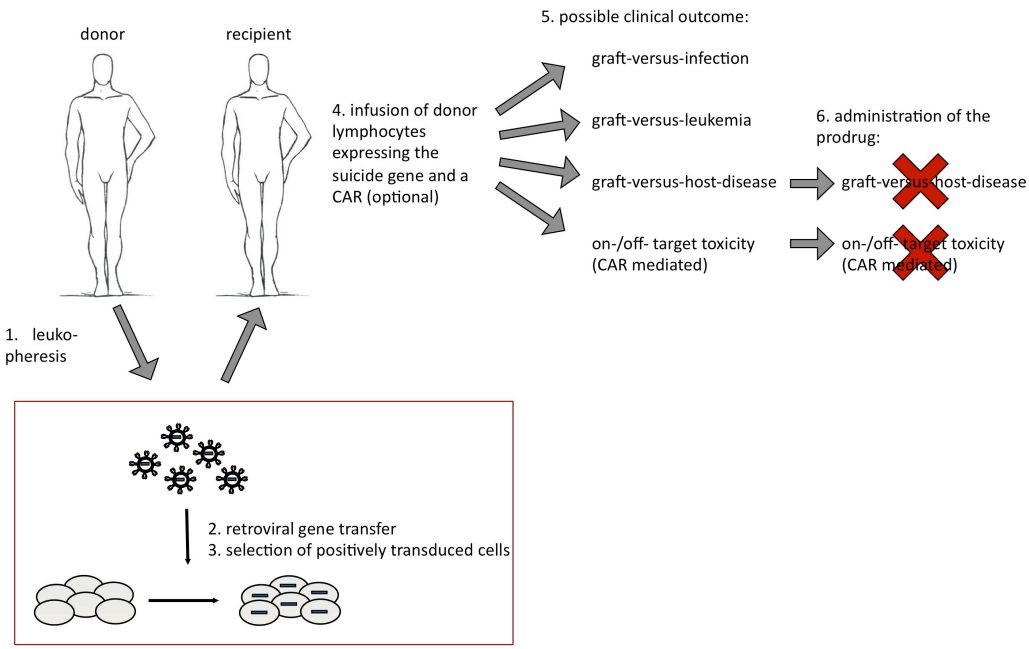

Figure 5: The principle of a suicide gene therapy. After a leukopheresis the T-cells are prestimulated and retrovirally transduced with transgene cassette consisting of a suicide gene and a CAR (optional). After selection of the transduced T-cells they are infused into the recipient. Should a graft-versus-host-disease or unwanted toxicities develop, the prodrug can be administered and the toxic side effects stopped. Modified after Bonini *et al.*, 2007.

One of the most prominent and widely tested suicide genes is the human *Herpes simplex virus* thymidine kinase (HSVtk) in combination with its prodrug, ganciclovir (GCV) [101]. The HSVtk phosphorylates ganciclovir into ganciclovir monophosphate, which is subsequently phosphorylated by other cellular kinases into a triphosphate. As a synthetic analogue to 2'-desoxy-guanosin, ganciclovir triphosphate is incorporated into the DNA in replicating cells and thereby inhibits the DNA synthesis and inducing apoptosis. Although the HSVtk/ganciclovir system has been proven effective in several clinical studies [102, 103] involving transduced T-cells in a DLI setting after allo-HSCT, major disadvantages have been described: 1) cell death induction depends on cell replication, 2) ganciclovir is very important during transplantation for infections with viruses such as the *cytomegalovirus* or HSV, and 3) the viral tk protein is strongly immunogenic [104, 105]. A newer suicide gene already tested in preclinical and clinical settings [106, 107] is the iCasp9 suicide gene. Here, the sequence of the human caspase 9

effector domain is fused with the sequence of the CID-binding dimerizing domain from the human FK506-binding protein 12 [108, 109]. The prodrug AP1903, a small synthetic and non-toxic molecule, binds to the CID-binding dimerizing domain, thereby inducing the dimerization of iCasp9 and the initiation of apoptosis. Other suicide gene systems are summarized in table 1 and can be categorized in 3 distinct classes due to their mechanism of action: metabolic (HSVtk – rabbit CYP4B1), dimerization inducing (FAS, iCasp9) and therapeutic mAb mediated (EGFR, CD20) [110].

Table 1: Comparison of suicide genes (modified after Bonini et al. 2007 [111]).

Suicide gene/ prodrug	Mechanism	Advantages	Disadvantages
HSVtk/GCV	Inhibition of DNA synthesis	Safe in several HSCT clinical settings Effective abrogation of GvHD	Viral origin GCV used to treat viral infections
mTMPK/AZT [112]	Inhibition of DNA replication	Human origin	Low enzymatic efficiency AZT used for viral infections No clinical trials
Cytosine desaminase/5'-FC [113-115]	Inhibition of nucleotid synthesis	Significant anti-tumor effects in preclinical studies	Fungal origin 5'-FC used as antifungal agent No clinical trials
Nitroreductase [116]	DNA crosslinking	Proliferation-independent	Bacterial origin No clinical trials
Carboxylesterase [117, 118]	Inhibition of topoisomerase	Can activate some clinical anticancer drugs like irinotecan	Rodent origin No clinical trials
Purin-nucleosid-phosphorylase [119]	Inhibition of DNA synthesis	Rapid cell killing Less cell cycle dependent	Bacterial origin No clinical trials
Rabbit CYP4B1/4-IPO [120, 121]	DNA alkylation	Specific and efficient cell killing Human isoform not active	Rodent origin No preclinical studies
EGFR/Cetuximab [122]	Inhibition of cell growth Blockade of EGFR receptors	Human origin	No clinical trials On-target toxicity from mAb
CD20/ Rituximab [123, 124]	Antibody dependent cytolysis and cellular cytotoxicity	Human origin Also as chimeric protein with CD34 sequences for selection Rituximab is highly lytic and easy available Immediate cell death induction	No clinical trials Unwanted depletion of B-cells by rituximab (On-target toxicity from mAb) High expression levels for cell death required
FAS/AP20187 [125]	Activation of pro-apoptotic molecules	Human origin Specific elimination Immediate cell death induction	Failure in case of over-expresssion of anti-apoptotic molecules Limited clinical experience with the dimerizer No clinical trials
iCasp9/AP1903 [106, 107]	Activation of pro-apoptotic molecules	Human origin Specific elimination Immediate cell death induction Safe and efficient in clinical trials	Limited clinical experience with the dimerizer Incomplete elimination (≥90%)

In addition to the use in allo-HSCT, a suicide gene system can generally function as a safety switch for adoptive T-cell therapies. A very popular indication is the use of such a system to control the potential toxicities following CAR T-cell therapy. Co-expression of a suicide gene together with the CAR facilitates *in vivo* control of the genetic modified

cells and thereby improves the safety of this therapy. Another benefit appears to be that, if no relapse occurs within 5 years after treatment and the patient is considered as cured, the genetically modified T-cells can be easily removed from the patient. For example patients having received CD19 CAR T-cell therapy do not have any B-cells due to the constant elimination of also normal B-cells and require therefore the administration of substituting immunoglobulin for humoral immunity. By inclusion of a suicide gene these long-term consequences can be abrogated. A recent clinical trial combines a GD2-CAR against neuroblastoma with the iCasp9 suicide gene (NCT01822652).

1.4.1 A novel human suicide gene system

In humans, P450 cytochromes are primarily membrane-associated hemoproteins located in the inner membrane of mitochondria or in the endoplasmic reticulum and responsible for the metabolism of endogenous and exogenous chemicals [126]. The term P450 is derived from the characteristic profile during spectrophotometry. When a P450 enzyme is in a reduced state and in a complex with carbon monoxide, the absorption maximum is at 450 nm [127]. There are 57 P450 enzymes known in humans, which can be grouped according to their substrate specificity. Based on homology in sequence and structure, P450 enzymes are clustered in 18 families with 45 subfamilies.

Like the other CYP proteins of the CYP4 family, CYP4B1 can ω-hydroxylate medium-chain fatty acids. Unlike his CYP4 family members, CYP4B1 is also responsible for the bioactivation of a wide range of xenobiotics [128-130]. One of the xenobiotics bioactivated by CYP4B1 was identified after cattle died in the 70's due to respiratory distress. The cattle had ingested sweet potatoes (*Ipomea batatas*) which were infected with the common mold *Fusarium solani* [131]. Sweet potatoes produced the furan-derived toxin 4-ipomeanol (4-IPO) as a defense mechanism [132, 133]. Subsequent animal experiments in rodents, dogs or cows among others confirmed the pulmonary toxicity of 4-ipomeanol without the histological lesions or toxicities in other organs expect the kidneys of male mice [134-136]. This almost exclusive lung toxicity occurs as

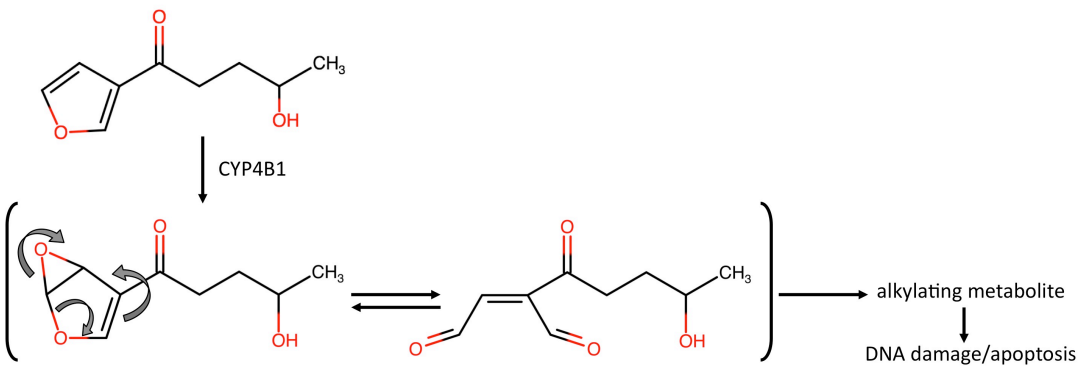

Figure 6: Bioactivation of 4-IPO. CYP4B1 metabolizes 4-ipomeanol into a highly alkylating metabolite by transferring oxygen to the furan ring. Thereby DNA damage and apoptosis is induced.

more than 70% of CYP4B1 is expressed in the lungs. CYP4B1 transfers oxygen to the furan ring of 4-ipomeanol and thus generating a highly toxic alkylating metabolite, which in turn provokes DNA-protein cross-links and DNA strand breaks resulting in the introduction of apoptosis [137].

Based on the fact that some human non-small cell lung cancer cell lines are sensitive to 4-ipomeanol [138] and that CYP4B1 is highly expressed in Clara cells and to a lesser degree in type II pneumocytes, oncologists thought 4-ipomeanol would be an agent for lung-targeted chemotherapy regimens [135]. Surprisingly, there was no anti-tumor effect in lung or liver tumors in humans observed in three clinical phase I/II studies [139-141]. Biochemical characterization then revealed that the human CYP4B1 enzyme contained a serine within the meander region at position 427 whereas the CYP4B1 of the rabbit and other species contained a proline at the corresponding position [142, 143]. This amino acid exchange renders the human protein incapable of metabolizing 4-ipomeanol [144]. Based on the fact that the human protein is inactive, Rainov and his colleagues suggested to use rabbit CYP4B1 in combination with 4-ipomeanol as a suicide gene system to treat brain tumors [121]. They fused the rabbit CYP4B1 cDNA 3' with EGFP, stably expressed it *via* a retroviral gene transfer in human and rat glioma cell lines and observed efficient tumor cell killing through 4-ipomeanol incubation *in vitro*. However, although they infused Fisher 344 rats with rat glioma cell line cells to establish an epidural tumor, they could not show specific 4-ipomeanol mediate tumor cell killing *in vivo* due to the active rat CYP4B1 and the respiratory distress resulting from 4-ipomeanol administration [121].

Our group has recently developed a novel human suicide gene, CYP4B1P+12, by systematically rendering the inactive human CYP4B1 enzyme capable of efficiently converting the inert natural lung-toxic furan 4-ipomeanol into highly toxic DNA alkylating metabolites [144]. The serine to proline exchange at position 427 not only stabilizes the protein and improves the protein half-life; it also partially restores the enzymes capability to bioactivate 4-ipomeanol. By substituting 12 more amino acids with the corresponding amino acids in the rabbit enzyme, the human protein was as active as the rabbit in the bioactivation of 4-ipomeanol. Since all of the changed amino acid residues were present in other human P450 enzymes, the likelihood of inducing immunogenicity *in vivo* is probably low. *In vitro* data showed a strong cytotoxicity of the human CYP4B1P+12 upon incubation with 4-ipomeanol similar to that mediated by the rabbit isoform [144]. By the generation of a *Cyp4B1* null mouse [145] further *in vivo* testing of the suicide gene system is now possible.

1.5 Magnetic activated cell sorting

Magnetic activated cell sorting (MACS) is a 3-step process - labeling, separation and eluation - to isolate cell subsets. Within the last 30 years, utilizing magnetic particles has become a standard tool. The paramagnetic microbeads from Miltenyi Biotech are

usually 0.5-5 μm in diameter and can be bind directly to an antigen-specific antibody or to anti-fluorochromes, anti-biotin, and anti-immunoglobulin and thereby indirectly labeling cells. After labeling the cells, they are applied to a column filled with ferromagnetic spheres, which is placed on a magnet field. By magnetic force, beads labeled cells are not actually bound to the column but rather 'hold' within the spheres. Unlabeled cells are not 'hold' and flow through the column. By removing the column from the magnet, labeled cells can be eluted. If the target cells are the labeled cells, the process is called positive selection. In contrast, a negative selection process targets the unlabeled cell subset in the flow through.

1.6 Retroviruses

The Retroviridae family, retrovirus, include seven genera: α-, β-, γ-, δ- and ε-retrovirus, spumavirus and lentivirus [146]. Retroviruses were first described in 1908, when Ellermann and Bay were able to induce leukemia in mice by injecting ultra filtrated extracts of leukemia cells of other mice's. Reyton Rous was 1911 able to reproduce this principle with ultra filtrated extracts of chicken sarcomas and honored with the Nobel price in 1966. J.J. Bittner found that the infection could not only take place when infectious particles were set free (horizontal transfer) but also as endogenous part of the genome and in the germlines (vertical transfer). Due to their ability to induce tumors, they were called *Oncornavirus*. After Howard Termin, S. Mituzami and David Baltimore found the reverse transcriptase that enabled retroviruses to transcript RNA in double stranded DNA in 1970, the term retrovirus (reverse transcriptase oncornavirus) was born. In general, retroviruses are characterized by their specific genomic structure, their capacity for reverse transcription, integration into the genome, and their double stranded RNA genome. That the induction of tumors could be linked to specific oncogenes was described in 1976 [147]. In 1980, the first retrovirus was described that can induce T-cell leukemia in humans, the human T-cell-leukemia virus, HTLV [148].

A typical retroviral particle (Figure 7) is 100-120 nm in diameter [149, 150]. The genome, double stranded RNA, is in a complex with nucleocapsid proteins (NC). Together with enzymatic proteins, reverse transcriptase (RT), integrase (IN) and proteases (PR), the genome is enclosed in a proteic capsid (CA), which is encircled by matrix proteins (MA) that interact with the envelope. The envelope is a lipid bilayer derived from the host cellular membrane. Within it, viral envelope glycoproteins (env) are anchored. These proteins recognize specific receptors on the surface of host's cells and initiate the infection. They are composed of two subunits, the transmembrane (TM) and the surface unit (SU). TM is anchored within the membrane via 20 hydrophobic amino acids and non-covalent bound to SU, which binds to cellular receptor. The envelope proteins of retroviruses are often modified by glycosylation. The proteins MA, CA, LI, and NC comprise the group-specific antigene (gag) and the enzymes within the proteic capsid comprise the pro-pol proteins.

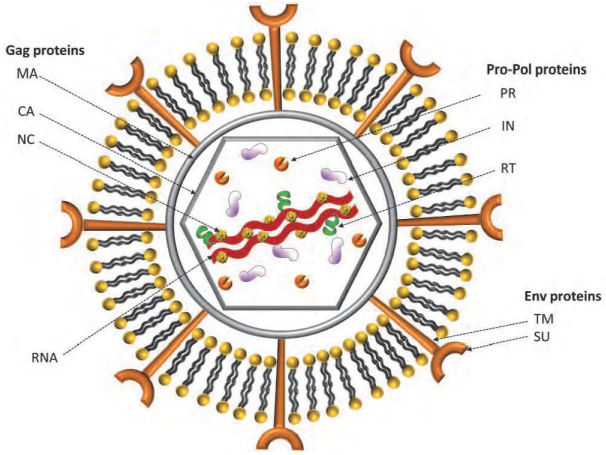

Figure 7: Schematic representation of a retrovirus particle structure. The lipid membrane anchoring the envelope proteins harbors a proteic capsid (CA), which is covered in matrix proteins, containing the genome in a complex with nucleocapsid proteins (NC) and pro-pol-proteins. The image is taken from Rodriguez *et al.* 2011 **[5].**

1.6.1 The lentiviral genome

Lentiviruses are a subfamily within *retroviridae*. The genome of a lentivirus is classified as a "complex" genome, meaning that beside the genes encoding for env, pro-pol and gag proteins, genes for regulatory and accessory proteins exist (Figure 8) [150, 151]. It can be 7-12 kb long and has a 5' cap and a 3' polyadenylation signal [4]. At both ends the genes coding for proteins are enclosed with control sequences important for the reverse transcription as well as integration into the host genome [4]. Gag proteins encompass matrix, capsid and nucleocapsid proteins and link protein p6 [152, 153]. The HIV matrix protein p17 supports the transport of the translated, viral genome into the nucleus. Importantly, thereby lentiviruses are able to infect quiescent cells [4]. The enzymes of prot-pol are the protease, reverse transcriptase and integrase [4]. The envelope proteins are synthesized as a precursor protein. The Golgi apparatus transports the precursor protein to the cell membrane. Cellular proteases associated with the Golgi apparatus cleave the precursor protein into the transmembrane and the surface unit. gp120 of HIV has a very high variability in specific regions of the protein through insertion, deletion and modified glycosylation muster. Receptors of gp120 are CD4 and CXCR4 or CCR5 expressed on T-cells and macrophages [153]. Regulatory proteins are tat (trans-activators of transcription), tev, and rev (regulator of expression of virion proteins). Translation of almost all viral proteins (excluding rev, tat, tev and nef) is rev-dependent. Accumulating within the nucleus it binds to the RRE (rev-responsive element) within the env gene and enhances the transport into the cytoplasm [4]. Vif (viral infectivity factor), vpr (viral production rapid), vpu (viral protein out) and nef (negative factor) are accessory proteins [153-156].

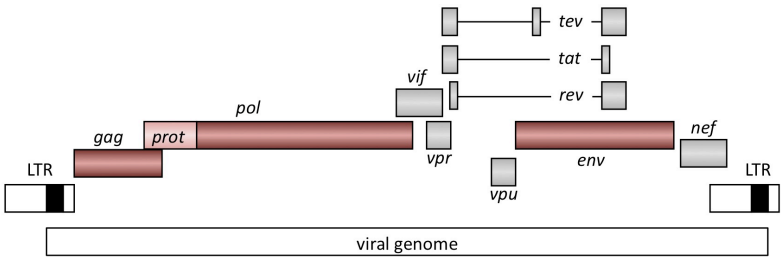

Figure 8: Schematic representation of the lentiviral genome. Genes encoding for gag, prot, pol and env are marked in red, accessory proteins are marked in grey. Modified after Modrow, 2003 [4].

1.6.2 Replication cycles

The replication cycle of lentiviruses will be explained on the basis of HIV-1 (see Figure 9 for numbers in text). Upon attachment (1) of gp120 to CD4 and secondary to CXCR4 or CCR5, the virion merges with the cell membrane (2) and the capsid is released into the cytoplasma [157-159]. Uncoating makes the capsid permeable for nucleotides (3), thus the reverse transcription (4) can begin. The dsDNA remains bonded with the viral protein as pre-integration complex, which is transported into the nucleus (5) [160, 161]. Since the complex is about 20-30 nm big, the import is dependent of adjuvant factors. There, the integrase cuts randomly the cellular DNA open and ligates the viral DNA into it (6) [149]. By the integration, cellular genes can be destroyed or trans-activated by the U3 promoter in the LTR region. The viral proteins are transcribed (7), translated (9) and transported to the cell membrane, where they assemble (10). Here, infectious virions are generated. Unspliced mRNA contains a packaging signal, Ψ, within their leader region, which binds to the nucleocapsid protein region of gag and gag/pol precursor proteins. Through the contact with the RNA molecule, the membrane starts

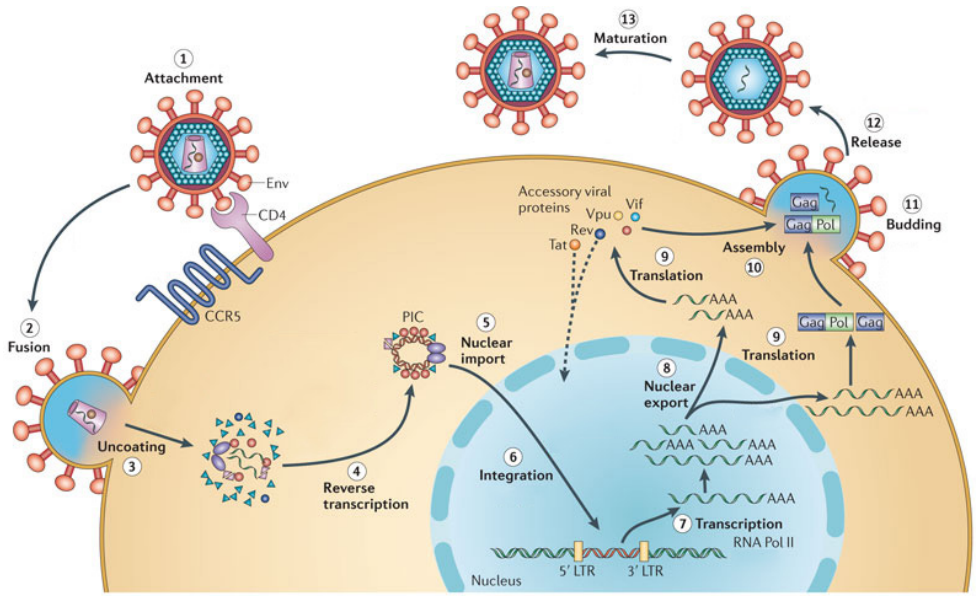

Figure 9: Replication cycle of HIV-1. The 13 steps from attachment to maturation are described here. Modified after Engelman, 2012 [6].

budding (11) and building vesicle, which are soon released (12). In these unmature virions the protease domain dimerizes and autocatalysis its own cleavage. Subsequently they cleave matrix, capsid, nucleocapsid and link proteins as well as the reverse transcriptase and integrase from the precursor proteins. Simultaneously the capsid is formed (13) [4].

1.6.3 Lentiviral vectors

The first retroviral vector was based on murine leukemia viruses (MLV) since those viruses have rather simple genomes. Thus it was utilized to study human gene marking and gene therapy [162-164]. Three important principles have been developed from from the complex lentiviral and foamyviral vectors systems: 1) a split-genome design to avoid the formation of replication-competent viral particles [165], 2) the deletion of enhancer and promoter sequences within the LTR to generate a self-inactivating (SIN) vector [166] and 3) the ability of specific cell-targeting by pseudotyping [167].

A split genome design leads to the removal of all viral genes but LTRs, the packaging signal Ψ and splice donor and acceptor sites in the vector. Thereby all sequences important for efficient packaging and integration remain. Here, the interaction between the packaging signal, Ψ, and nucleocapsid proteins is crucial, but also additional sequences within the trans-activating-response element (tar), U5, PBS, gag and rev are needed for efficient packaging, vector production and integration. Also, retroviral proteins are encoded on separate vectors, called helper plasmids.

The generation of lentiviral vectors [168] required a helper plasmid expressing HIV-1 core proteins, enzymes and auxiliary factors from heterologous transcription signals. The envelope protein was expressed on a separate vector. Further development led to the generation of lentiviral vectors where the HIV-derived packaging components were reduced to gag, pol, tat and rev [169]. Both approaches needed the vector with the transgene cassette to express HIV-derived cis-acting sequences required for transcription, encapsidation, reverse transcription and integration [170-176]. Hence, the vector contained the full HIV 5'LTR. By replacing the enhancer/promoter of HIV 5'LTR with a heterologous, constitutively active promoter and thus generating a third generation vector, genomic RNA is expressed independent of *tat* in high levels in producer cells [177].

The third generation lentiviral vector used here is depicted in Figure 10. The chimeric 5'LTR contains the promoter of a heterologous virus, e.g. the human cytomegalovirus (CMV) or respiratory-syncytial virus (RSV), followed by the R region und U5, the leader sequence with the packaging signal and packaging relevant sequences within the gag and env genes. The RRE element allows high expression and packaging and also suppresses aberrant splice events thus enabling lentiviral vectors to comprise more complex transgene cassettes. The central polypurine tract (cPPT) is derived from the pol

gene and increases the efficacy of reverse transcription. Modification of the 3'UTR includes the introduction of the *Woodchuck hepatitis virus* posttranscriptional element (WPRE) improving nuclear export, mRNA stability, translation efficacy and reduction of read-through transcription into cellular sequences [178]. Here, an optimized form of WPRE is used, WPRO, where the promoter sequences and the endogenous ORF for the X protein and by mutating residual ATGs of other ORFs larger than 25 nucleotides are removed [179]. Another modification is the deletion of the promoter/enhancer sequences within the U3 region. After the reverse transcription within the target cell, the deleted U3 region serves as a template and is copied into the 5'LTR region. Thereby, the generated 5'LTR is inactive. This kind of vector is called self-inactivating (SIN) vector [180-182].

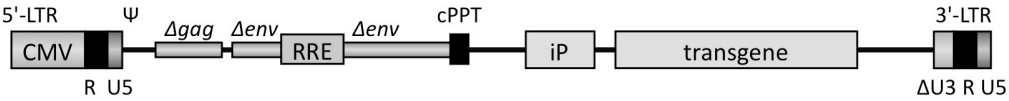

Figure 10: Schematic representation of a 3rd generation SIN lentiviral vector. A heterologous CMV promoter replaces the enhancer/promoter region of the 5'LTR of the HIV-1 derived vector and the U3 region is deleted. Sequences required for packaging remained while other for viral proteins were deleted. The transgene is driven by an internal promoter.

In order to avoid cryptic splice sites and reduce the G/C content, transgenes can be optimized for human codon usage. This can increase the mRNA stability and export as well as the translation efficacy [183-186]. Other parameters that modify the transgene expression include the choice of the internal promoter. Constitutively active promoters mediate constant expression, activation-dependent promoters induce transgene expression upon cell activation and inducible promoters such as tetracycline-induced Tet-on/off promoters induce the transgene expression upon the supplement of tetracycline or one of its derivate.

More than one transgenes can be expressed by insertion of a second internal promoter, an internal ribosomal entry site (IRES) or a 2A-derived sequence. IRES sites are initiate cap independent translation, especially when located in the middle of an mRNA [187]. The food and mouth disease virus (FMDV), member of the Picornavirinidae, expresses its polyproteins as one open reading frame, which subsequently gets 'cleaved' into 2 polyproteins. This cleavage is mediated by a CHYSEL acting element called 2A site. The characteristics featured of 2A sites are:

1) The cleavage mediated by a 2A site occurs cotranslational, uncleaved protein is not subsequently cleaved [188].
2) The cleavage is actually not a proteolysis but a translational effect [189].
3) The upstream sequence is not critical but influential [190, 191].
4) 2A sites function in eukaryotic but not in prokaryotic expression systems [188, 190].
5) The consensus motif responsible for cleavage is DxExNPG/P [192].

Implementation of chromatin opening elements such as UCOE [193] can prevent heterochromatin formation and gene silencing. Other elements like insulator sequences that shield genes against position effects of surrounding chromatin (e.g. chicken hypersensitive site-4 [194]) are also possible targets for vector optimization.

1.6.4 Pseudotyping of lentivirus

Lentiviral core particle can form and bud in the absence of an envelope protein, therefore any transmembrane protein can become incorporated into the virion. The incorporation of a heterologous envelope protein is called pseudotyping. Critical for gaining high titers is that the envelope protein is efficiently processed by the viral protease and highly expressed on the cell surface [195, 196]. Dependent on the envelope protein, the new tropism can be broad or restricted. A widely used envelope protein for pseudotyping is the glycoprotein of the *Vesicular stomatitis virus* (VSV-G) [197, 198], which receptor is the LDL receptor family ubiquitously expressed on all cells [199]. However, usage of the VSV-G protein for pseudotyping can be associated with cytotoxicity [200, 201].

For some pseudotypes, modifications within the cytoplasmic tail of the envelope protein are necessary [202-205]. The envelope protein of the *Gibbon ape leukemia virus* (Galv) has been used for human gene therapy application due to its host cell tropism and non-pathogenicity [206-210]. The receptor is a sodium-dependent phosphate transporter protein, which is highly expressed on human T-lymphocytes [211, 212]. The glycoprotein is a single transmembrane surface protein and gets cleaved into a SU and TM domains, but the TM domain contains a 30 amino acid long C-terminal cytoplasmic tail, which prevents the incorporation of Galv envelope protein into the virion [205, 213]. Exchanging the TM of Galv with the one of the *amphotrophic murine leukemia virus* results in the chimeric envelope protein GalvTM that is fully compatible with HIV-1 derived vectors [213].

Another envelope protein of a heterologous virus for pseudotyping HIV-1 derived vectors is the envelope protein of a spumaretrovirus, also known as foamy virus (FV), a member of this subfamily of retroviruses [214]. First identified in 1954 in cultures of rhesus macaque cells [215], it was later shown that humans are not their natural hosts; infections occur through zoonosis [216, 217]. Ubiquitination sites in the N-terminal of the TM unit of the envelope protein (K_{14} and K_{15}) were shown to reduce cell surface expression and suppress the release of subviral particles [218]. The mutated envelope protein PE01 is derived of the ubiquitination sites K_{14} and K_{15} by point mutations [218].

Due to the accessory proteins of lentiviruses like vpu, a broad tropism by pseudotyping and the fact that the pre-integration complex is stable for several days, it is possible to infect miscellaneous cells with lentiviral vectors without the requirement of cell division like neurons [168], hepatocytes and muscle cells [219], undifferentiated stem cells [213, 220], T-lymphocytes [213, 221] and embryonic cells [222].

1.6 Objective

Adoptive T-cell therapies with unmodified allogeneic or genetically engineered autologous T-cells can transfer immunity against malignant cells. An important safety strategy for cellular therapies is to equip T-cells prior to infusion with a suicide gene that enables specific elimination of the gene modified cells *in vivo*. A human CYP4B1 variant that was engineered to bio-activate the natural occurring prodrug, 4-ipomeanol, towards a highly toxic alkylating substance by systematically exchanging 13 amino acid residues, was utilized as novel suicide gene in combination with other transgenes.

The first part of the thesis focused on establishing a clinical applicable SIN lentiviral vector that facilitates efficient co-expression of two transgenes with different cellular locations. This was necessary since the suicide gene is an ER membrane bound monooxygenase and CARs or truncated versions of CD34 and CD271 as MACS selection markers are cell surface molecules. To develop clinically applicable SIN lentiviral vectors for either DLI or CAR therapies, different viral or human housekeeping promoters with or without splice-active sites and co-expression strategies with two promoters, IRES or 2A sites needed to be systematically tested. In order to determine what is the best/most efficient combination of modules in the vector, important functional read-outs for the different constructs would be MACS selection, apoptosis induction and CAR-mediated cytotoxicity against leukemia cells.

In the second part of the thesis, it was attempted to identify an alternative prodrug by systematically characterizing the enzymatic activity of different CYP4B1 isoforms towards 4-ipomeanol structurally similar substances. To this end, human liver cells would be transduced with different CYP4B1 isoforms and the cytotoxic potential of several structure analogues of 4-ipomeanol were to be tested in order to find an activity of CYP4B1s towards these drugs. The induction of apoptosis in primary T-cells expressing the CYP4B1 isoforms upon incubation with the different prodrugs also needed to be tested.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Unless noted separately, suppliers for chemicals were Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Applichem (Darmstadt, Germany).

2.1.2 Buffers, solutions and media

2.1.2.1 cell culture media and solutions

DMEM	Dulbecco's Modified Eagle Medium, 4.5 g/l D-glucose, Pyruvate, GlutaMAX™-I (#31966-047, Gibco® by life technology™, Darmstadt, Germany)
RPMI-1640	Roswell Park memorial Institute 1640 Medium (#31870-074, Gibco® by life technology™)
IMDM	Iscove's Modified Dulbecco's Media, (#I3390, Sigma Adrich)
Media supplements:	10% fetal calf serum, heat inactivated (#10500-064,Gibco® by life technology™) 100 U/ml penicillin and 100 µg/ml streptomycin (#15140-122, Gibco® by life technology™) 2 mM L-Glutamin (#25030,Gibco® by life technology™) IL-2 for primary T-lymphcytes, (Chiron, Marburg)
DPBS	Dulbecco's Phosphate Buffered Saline (# 14190-169, Gibco® by life technology™)
Trypsin-EDTA	0.05% trypsin, 0.02% EDTA (#L11-004, PAA)
0.1% gelatin	1 g gelatin from porcine skin (#G1890, Sigma Aldrich) ad. 1 l A. dest
Red Blood Cell Lysis Buffer	8.99 g NH_4CL 1 g KHCO3 40 µl 0.5 M EDTA ad. 1 l A. dest, pH 7.3

Propidium iodide stain	0.5 µg/ml propidium iodide (#P4864, Sigma Aldrich) in PBS
MACS buffer	0.5% Albumine from Bovine Serum (#A7906-10G, Sigma Aldrich) 2 mM EDTA in PBS, pH 7.2

2.1.2.2 solutions for molecular biology

LB medium	Luria Bertani Broth (#L3022, Sigma Aldrich)
LB Agar	Luria Bertani Agar (#L2897, Sigma Aldrich)
TAE buffer (50x)	242 g Tris base 57.1 ml acetic acid 100 ml 0.5 M EDTA ad. 1l A. dest, pH 8.5
loading buffer (10x)	250 mg Bromphenolblau 250 mg Xylene Cyanol 33 ml 150 mM Tris, pH 7.6 60 ml Glycerin ad. 100 ml A. dest

2.1.2.3 solutions for protein biochemical analysis

lysis buffer (10x)	10 ml 1M Tris/HCl; pH 8,0 8,2 g NaCl 0,25 g NaN3 10 ml Triton®X-100
loading dye	NuPage® LDS Sample Buffer 4x (#NP0007, novex® by life technologies) NuPage® Sample reducing agent 10x (#NP0009, novex® by life technologies)
Running Buffer	NuPAGE® MES SDS Running Buffer 20x (#NP0002, novex® by life technologies)
Transfer Buffer	NuPAGE® Transfer Buffer 20x (#NP0006-1, novex® by life technologies) 5% methanol
Supplements:	NuPAGE® Antioxidant, (#NP0005, novex® by life technologies)

Ponceau S Staining solution	1 g Ponceau S 50 ml acetic acid ad. 1 l A. dest
PBS-T	PBS with 0.1% Tween®20 (#1379, Sigma Aldrich)
Blocking solution	PBS-T with 5% milk powder (#T145, Roth)

2.1.3 Substrates

Table 2: List of Substrates

Substrates	Manufacturer
4-IPO	NIH, Washington, USA
PK	Synthesized
2-AA	#A38800, Sigma Aldrich
2-AF	#A55500, Sigma Aldrich
3-MI	#M51458, Sigma Aldrich
PenF	#AB113695, abcr (Karlsruhe, Deutschland)
HexF	#AB212272, abcr
HepF	#AB131522, abcr
PFK	#AB131568, abcr

2.1.4 Enzymes and kits

Table 3: enzymes and kits

Enzymes and kits	Manufacturer
Pwo polymerase	#11644955001, Roche (Grenzach-Wyhlen, Germany)
restriction enzymes (RE)	NEB (Frankfurt a.M., Germany)
T4 DNA Ligase	#M0202M, NEB
FastPlasmid Mini Kit	#2300010, 5 prime (Hilden, Germany)
NucleoBond® Xtra Maxi	#740414.100, Macherey-Nagel (Düren, Germany)
HP PCR Product Purification Kit	#11732676001, Roche
Big Dye® Terminator v1.1 cycle sequencing Kit	#4337451, Thermo Scientific (Rockford, IL)
Dye Ex™ 2.0 Spin Kit	#63206, Quiagen (Hilden, Germany)
Super Signal West Pico Chemiluminescent Substrate	#34079, Thermo Scientific
CD271 MicroBead Kit (APC)	#130-099-023, Miltenyi Biotec (Bergisch Gladbach, Germany)

CD271 MicroBead Kit	#130-092-283, Miltenyi Biotec
CD34 MicroBead Kit	#130-046-702, Miltenyi Biotec
Anti Biotin MicroBead Kit	#130-090-485, Miltenyi Biotec

2.1.5 Antibiotics

Table 4: antibiotics

Antibiotic	Manufacturer	Concentration
Ampicillin	Sigma Aldrich	100 µg/ml
Puromycin	Gibco® by life technology™	1-2 µg/ml
Penecillin, Streptomycin	Gibco® by life technology™	10 µg/ml

2.1.6 Ladders

GeneRuler 1 kb Plus DNA Ladder, ready-to-use (#SM1333, Thermo Scientific)

PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (#26619, Thermo Scientific)

2.1.7 Oligonucleotides

Table 5: List of oligonucleotides

#	Sequence 5' to 3'	Function
1037	GTACAGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGG	forward primer for T2A, encodes for RE *BsrG*I
1038	GTCCGGGATTCTCCTCCACGTCACCGCATGTTAGAAGACTTCCTC	reverse primer for T2A, encodes for RE *Rsr*II
1039	CACAGCTGGATATCGATCCGAGAGATCTCGTGCGAAGCGCTTGCG	forward primer, binds to 5' of SFFV, encodes the RE *Pvu*I, *Bgl*II and *Cla*I
1040	ATCCCCFFFATATCGATCCGAGAGATCTCGTGCGAAGCGCTTGCG	forward primer, binds to 5' of hPGK, encodes the RE *Xma*I, *Bgl*II and *Cla*I
1058	ATATCGATGAGCCTCGAGAAGCTTGATATCG	forward primer, binds to 5' of hPGK, encodes the RE *Cla*I
1059	CGAGATCTCTACTGGAACAGGTGGTGGC	reverse primer, binds to 3' of E2 Crimson, encodes the RE *Bgl*II
1060	CGCGGACCGATGGATAGCACTGAGAACGTCATCAAGCC	forward primer, binds to 5' of E2 Crimson, encodes the RE *Rsr*II
1061	CGCGGACCGATGGTGAGCAAGGGCGAGGAGC	forward primer, binds to 5' of EGFP, encodes the RE *Rsr*II
1062	CGAAGCTTATGGATAGCACTGAGAACG	forward primer, binds to 5' of E2 Crimson, encodes the RE *Hind*III
1339	CACCGCGGACATGGTGAGCAAGG	forward primer, binds to 5' of

	GCGAGGAGG	dTOMATO, encodes the RE *Sac*II
1387	CAACCGGTATGGTGAGCAAGGGC GAGGAGGTCATCAAAGAGTTCAT GCG	forward primer, binds to 5' of dTOMATO, encodes the RE *Age*I
1341	CGCGGACCGATGGTGAGCAAGGG CGAGGAGG	forward primer, binds to 5' of dTOMATO, encodes the RE *Rsr*II
1342	CGAAGCTTATGGTGAGCAAGGGC GAGGAGG	forward primer, binds to 5' of dTOMATO, encodes the RE *Hind*III
1389	GTGCGGCCGCCTACAGCTCGTCC ATGCCGTACAGGAACAGGTGGTG G	reverse primer, binds to 3' of dTOMATO, encodes the RE *Not*I
1390	CGTGTACACTACAGCTCGTCCATG CCG	reverse primer, binds to 3' of dTOMATO, encodes the RE *Bsr*GI, stop codon deleted
1345	CGACCGGTCGTTAATTAAGCATG GTGAGCAAGGGCGAGGAGG	forward primer, binds to 5' of dTOMATO, encodes the RE *Age*I and *Pac*I
1384	GCAGATCTTACAGCTCGTCCATGC CGTACAGGAACAGGTGGTGG	reverse primer, binds to 3' of dTOMATO, encodes the RE *Bgl*II
1347	CAGCGGCCGCACTACTGGAACAG GTGGTGGCGGGCCTCGGCGCG	reverse primer, binds to 3' of E2 Crimson, encodes the RE *Not*I
1386	CGACCGGTATGGTGAGCAAGGGC GAGGAGC	forward primer, binds to 5' of EGFP, encodes the RE *Age*I
1349	GCGTACATCAAGCACCCCGC	forward primer for sequencing of E2 Crimson
1350	GCGGGGTGCTTGATGTACGC	reverse primer for sequencing of E2 Crimson
1388	GTGCGGCCGCTTACTTGTACAGCT CGTCCATGCCGAGAGTGATCCCG GCGGC	reverse primer, binds to 3' of EGFP, encodes the RE *Not*I
1385	CGACCGGTATGGATAGCACTGAG AACG	forward primer, binds to 5' of E2 Crimson, encodes the RE *Age*I
1354	ACAAGAAGCTGTCCTTCCCC	forward primer for sequencing of dTOMATO
1355	ACCTTGTAGATCAGCGTGCC	reverse primer for sequencing of dTOMATO
1356	CAGAATTCATGGTGAGCAAGGGC GAGG	forward primer, binds to 5' of dTOMATO and EGFP, encodes the RE *Eco*RI
1357	CAGAATTCATGGATAGCACTGAG AACG	forward primer, binds to 5' of E2 Crimson, encodes the RE *Eco*RI
1358	CAGGATCCTTACTTGTACAGCTCG TCC	reverse primer, binds to 3' of EGFP, encodes the RE *Bam*HI
1392	CAGGATCCCTACAGCTCGTCCATG CCG	reverse primer, binds to 3' of dTOMATO, encodes the RE *Bam*HI
1360	CAGGATCCCTACTGGAACAGGTG GTGGC	reverse primer, binds to 3' of EGFP, encodes the RE *Bam*HI

1361	CACCATGGATAGCACTGAGAACG	forward primer, binds to 5' of E2 Crimson, encodes the RE *Nco*I
1362	CACCATGGTGAGCAAGGGCGAGG AGG	forward primer, binds to 5' of dTOMATO, encodes the RE *Nco*I
1371	CACTCGAGATGGTGAGCAAGGGC GAGGAGC	forward primer, binds to 5' of EGFP, encodes the RE *Xho*I
1372	GTGCTAGCTTACTTGTACAGCTCG TCC	reverse primer, binds to 3' of EGFP, encodes the RE *Nhe*I
1373	GCCTCGAGATGGTGAGCAAGGGC GAGGAGG	forward primer, binds to 5' of dTOMATO, encodes the RE *Xho*I
1383	GTGCTAGCCTACAGCTCGTCCATG CCG	reverse primer, binds to 3' of dTOMATO, encodes the RE *Nhe*I
1375	CACTCGAGATGGATAGCACTGAG AACG	forward primer, binds to 5' of E2 Crimson, encodes the RE *Xho*I
1376	GTGCTAGCCTACTGGAACAGGTG GTGGC	reverse primer, binds to 3' of E2 Crimson, encodes the RE *Nhe*I
1377	CACCCGGGCCGGCATGGTGAGCA AGGGCGAGGAGC	forward primer, binds to 5' of EGFP, encodes the RE *Sma*I
1378	CACCCGGGCCGGCATGGTGAGCA AGGGCGAGGAGG	forward primer, binds to 5' of dTOMATO, encodes the RE *Sma*I
1380	CACCCGGGCCGGCATGGATAGCA CTGAGAACG	forward primer, binds to 5' of E2 Crimson, encodes the RE *Sma*I
1381	GCTGTACACTACTGGAACAGGTG GTGGC	reverse primer, binds to 3' of E2 Crimson, encodes the RE *Bsr*GI
1053	CGAATTGGGTACCTGTACAAGGA AGCCCGGCACAAGC	forward primer, binds to 5' of 14+2A site, encodes *Kpn*I
1054	GCTGGAGCTCGGATCCCCGGTCC GGGATTGCTCTCCACG	reverse primer, binds to 3' of 14+2A site, encodes *Sac*II
1863	GCACCGGTCGCCACCATGGTGCC CAGCTTTCTGAGCCTGAGC	forward primer, binds to 5' of h4B1, encodes for RE *Age*I
1864	GAGCGGCCGCTCACTTGCCGCTG CCAGGGCCCAGG	reverse primer, binds to 5' of h4B1, encodes for RE *Not*I

2.1.8 Plasmids

2.1.8.1 Helper plasmids and expression vectors for envelope proteins

pCD/NL-BH — Expression vector for gag/pol and accessory genes of HIV driven by a CMV enhancer/promoter. Deletion inactivated the packaging signal [223]. The plasmid was kindly provided by Dr. Jakob Reiser, Lousinana State University School of Medicine, New Orleans, USA.

pczVSV-G — Expression vector for the glycoprotein G of the *Vesiculostomatits virus* (VSV)[224]. The plasmid was kindly provided by Prof. Dirk Lindemann, Institute for Virology, Dresden, Germany.

pczPE01 — Expression vector with a CMV enhancer/promoter for a mutated form of a mutated envelope protein of the *Human foamy virus* (HFV). The plasmid was kindly provided by Prof. Dirk Lindemann, Institute for Virology, Dresden, Germany.

pcoPE01 — Based on pczPE01, this expression vector contains the cDNA of PE01 optimized for human codon usage. It was ordered at GeneArt (Regensburg, Germany) by Dr. Wade Clapp (Indiana University, Indianapolis, USA), Prof. Helmut Hanenberg (University Children's Hospital Essen), Prof. Dirk Lindemann (Institute for Virology, Dresden) and Prof. Axel Rethwilm (Institute for Virology and Immunology, Würzburg, Germany).

pALVGalvTM — Expression vector for chimeric envelope protein consisting of the transmembrane and cytoplasmic domain of the *amphotropic murine leukemia virus* (aMLV) and the extracellular domain of the envelope protein of the *gibbon ape leukemia virus* (Galv) [205]. The plasmid was kindly provided by Prof. Klaus Cichutek, Paul-Ehrlich-Institute, Langen, Germany.

pcoGalvTM — Based on pALVGalvTM this expression vector contains the cDNA of GalvTM optimized for human codon usage by GeneArt.

2.1.8.2 Expression vectors

puc2CL6IEGwo — A lentiviral expression vector with a SFFV U3 promoter and a multiple cloning site (mcs) followed by an IRES-EGFP cassette. The IRES site enables the expression of a second transgene, here EGFP, from a single mRNA [225-228]. Behind EGFP, an optimized version of the woodchock element (WPRO) is placed to stabilize the mRNA. This expression vector carried the cDNA of CYP4B1 variants:

r4B1 (puc2CL6r4B1IEGwo),

h4B1 (puc2CL6coh4B1IEGwo),

h4B1P427 (puc2CL6coh4B1P427IEGwo) and

h4B1P+12 (puc2CL6coh4B1P+12IEGwo).

The human cDNAs were codon optimized by GeneArt.

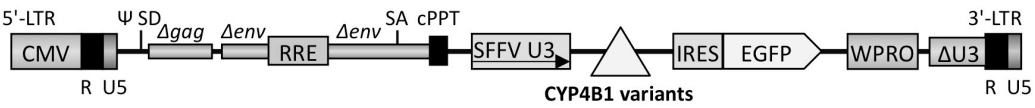

Figure 11: Schematic figure of the expression vector puc2CL6IEGwo

puc2CL6IPwo Designed like puc2CL6IEGwo this expression vector contains a resistance gene against puromycin instead of EGFP.

puc2CL24EGNwo This vector served as template to construct the expression vectors for the comparative analysis of the promoter strength. Using the restriction enzymes *Xho*I and *Pac*I, the promoter sequences have been exchanged. The HLA-DR promoter drives an EGFP-NeoR fusion protein.

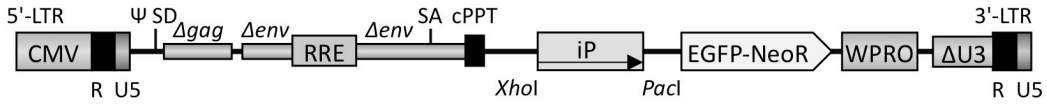

Figure 12: Scheme of teh expression vector puc2CL24EGNwo

The promoters, which have been exchanged, are summarized in Table 6.

Table 6: List of puc2CL24EGNwo variants.

Plasmid name	Internal promoter
puc2CL7EGNwo	SFFV
puc2CL10EGNwo	hEF1a
puc2CL11EGNwo	hPGK
puc2CL20EGNwo	hEF1a + Intron
puc2CL21EGNwo	MPSV
puc2CL22EGNwo	MPSV + Intron
puc2CL24EGNwo	HLADR
puc2CL25EGNwo	HLADR + Intron
puc2CL26EGNwo	rGlut1 -500 +134
puc2CL27EGNwo	rGlut1 -104 +134
puc2CL28EGNwo	rGlut1 -273 +134
puc2CL29EGNwo	hGlut1 -500 +210
puc2CL30EGNwo	hGlut1 -89 +11
puc2CL31EGNwo	hGlut1 -200 +210
puc2CL32EGNwo	hAsct2 -653 +210
puc2CL33EGNwo	hUbiC + Intron

puc2CL11EGwo This expression vector contains the human PGK promoter which drives EGFP. There are also vectors expressing E2 Crimson (puc2CL11CRwo) and dTOMATO (puc2CL11TOwo).

puc2CL7EGwo — Organized like puc2CL11EGwo this vector has the SFFV U3 promoter to drive EGFP. There are also vectors expressing E2 Crimson (puc2CL7CRwo) and dTOMATO (puc2CL7TOwo).

pSL1180 — This is a plasmid designed for cloning carrying a mcs with more than 50 unique restriction enzyme sites. An ampicillin resistance allows selection in bacteria. The plasmid was obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

S11IEG2 — This γ-retroviral expression vector contains a mcs before an IRES-EGFP cassette and was solely used as a cloning vector.

2.1.8.3 Expression vectors designed within this study

I. Expression vectors used for comparative analysis of different co-expression approaches

Using fluorescent proteins as a read-out for expression, expression vectors with EGFP and E2 Crimson/dTOMATO have been designed. Since they will be further discussed within the results, there will be only briefly summarized here.

Dual promoter expression vectors:

Primary, transgene cassettes with two promoters were constructed in foamyviral expression vectors. Due to low titers, the transgene cassette was transferred into lentiviral vectors. The foamyviral vectors were generated using PCR amplification of the human PGK promoter (SFFV U3 promoter) with a fluorescent protein and cloned into a foamyviral expression vector equivalent to a puc2CL7 vector (puc2CL11) using unique restriction sites.

The transgene cassette CR/TO-hPGK-EGFP (EGFP-hPGK-CR/TO) was cut out of the foamyviral vector and cloned into puc2CL7EGwo, the cassette hPGK-CR/TO-SFFV U3-EGFP (hPGK-EGFP-SFFV U3-CR/TO) was cloned into puc2CL11EGwo using unique restriction enzymes.

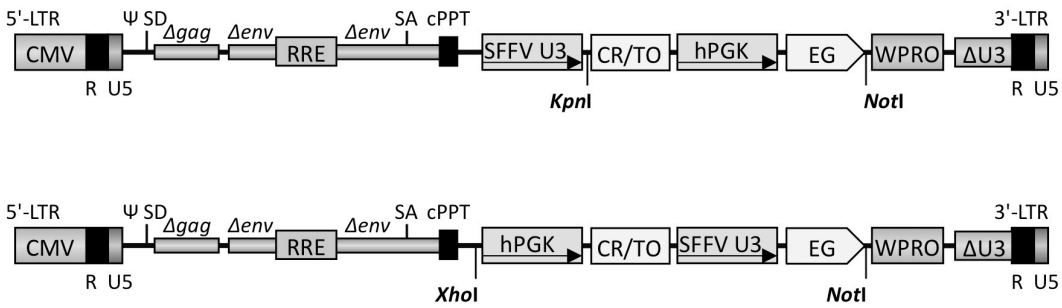

Figure 13: Scheme of dual promoter expression vectors. Transgene cassettes were cloned using KpnI and NotI into puc2CL7EGwo and with *Xho*I and *Not*I into puc2CL11EGwo.

Table 7: List of expression vectors containing a dual promoter system

Plasmid name
p2CL7CRhPGKEGwo
p2CL7TOhPGKEGwo
p2CL7EGhPGKCRwo
p2CL7EGhPGKTOwo
p2CL11CRSFFVEGwo
p2CL11TOSFFVEGwo
p2CL11EGSFFVCRwo
p2CL11EGSFFVCRwo

Expression vectors with 2A sites:

The T2A site was generated by annealing ordered oligonucleotides (#1037 and #1038). Both T2A and PCR amplified 14+2A sites were cloned into pSL1180. After insertion of EGFP and E2 Crimson/dTOMATO 3' and 5' to the 2A site, respectively, the whole cassette was PCR amplified and cloned into puc2CL11EGwo cut with the same restriction enzymes, *Age*I and *Not*I (Figure 14).

Table 8: List of expression vectors containing a 2A site

Plasmid name
p2CL11CR14+2AEGwo
p2CL11TO14+2AEGwo
p2CL11EG14+2ACRwo
p2CL11EG14+2ATOwo
p2CL11CRT2AEGwo
p2CL11TOT2AEGwo
p2CL11EGT2ACRwo
p2CL11EGT2ATOwo

Expression vectors with IRES site:

The transgene cassettes EGFP-IRES-E2 Crimson/dTOMATO and E2 Crimson/ dTOMATO-IRES-EGFP were initially generated in S11IEG2. Using PCR amplification to add restriction sites for *Age*I and *Not*I the cassettes were transferred into puc2CL11EGwo cut with the same enzymes.

Table 9: List of expression vectors containing the IRES site

Plasmid name
p2CL11CRIEGwo
p2CL11TOIEGwo
p2CL11EGICRwo
p2CL11EGITOwo

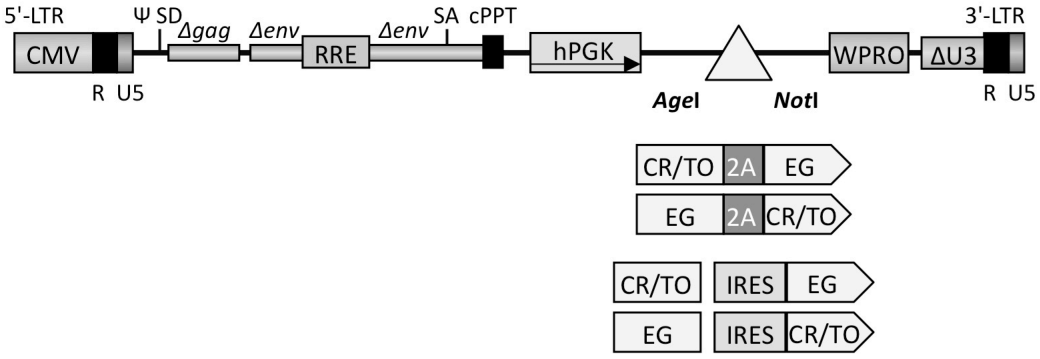

Figure 14: Scheme of an expression vector. Using AgeI and NotI restriction sites, transgene cassettes containing two fluorescent proteins co-expressed via an IRES or an 2A site, were cloned into puc2CL11EGwo.

II. Expression vectors for establishing a selection marker and cytotoxicity assays

Truncated versions of CD34 (ΔCD34) and the low affinity nerve growth factor receptor p75 (ΔNGFR) were PCR amplified and cloned into p2CL21P+12coT2AEGwo (Figure 15) and p2CL21EGT2AP+12cowo, respectively, using the unique restriction sites of *Nhe*I, *Sac*II, *Rsr*II and *Not*I. The same way, expression vectors for cytotoxicity assays were generated: EGFP was cut out of p2CL21EGT2AJ.CD19cowo, p2CL21EGT2AJ.CD33E7wo and p2CL21EGT2AJ.CD22wo using *Nhe*I and *Sac*II and replaced by h4B1P+12.

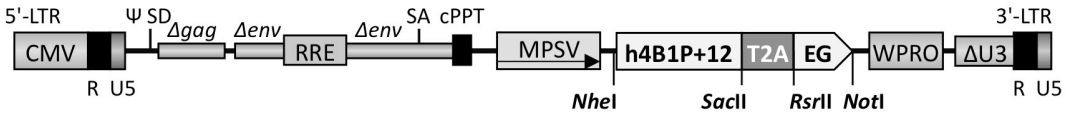

Figure 15: Scheme of p2CL21P+12coT2AEGwo. Using the unique restriction site displayed below the tansgenes, ΔNGFR and ΔCD34 were cloned into the vector.

Table 10: List of expression vectors with selection markers and CARs

Plasmid name
p2CL21ΔNGFRT2AEGwo
p2CL21ΔNGFRT2AP+12wo
p2CL21EGT2AΔNGFRwo
p2CL21P+12T2AΔNGFRwo
p2CL21ΔNGFRT2Ah4B1wo
p2CL21ΔCD34T2AEGwo
p2CL21ΔCD34T2AP+12wo
p2CL21EGT2AΔCD34wo
p2CL21P+12T2AΔCD34wo
p2CL21ΔCD34T2AP+12wo
p2CL21P+12T2AJCD19cowo
p2CL21P+12T2AJCD22Bcowo
p2CL21P+12T2AJCD33cowo

III. Expression vectors for analysis of potential bystander killing

The cDNA of r4B1, h4B1P427 and h4B1P+12 was cut out of puc2CL6IEGwo vectors using *Nhe*I and *Bam*HI restriction enzymes and cloned into puc2CL6IPwo.

Table 11: List of expression vectors used to analyze bystander activity

Plasmid name
puc2CL6r4B1IPwo
puc2CL6coh4B1P427IPwo
puc2CL6coh4B1P+12IPwo

2.1.9 Bacterial strains

Transformation of ligation products was performed with One shot® Top10 Competent cells (Invitrogen).

2.1.10 Cell lines

Cell lines were obtained from DSMZ.

Table 12: Cell lines

Cell line	Characteristics	Reference
HEK293T	Derived from HEK293 cells, which are human embryonic kidney cells. They stably express the SV40 large T antigen that can bind to SV40 enhancers of expression vectors thus increasing protein production. They also contain a neomycin resistance.	[229-231]
HT1080	A human fibrosarcoma cell line.	[232]
HepG2	A human liver carcinoma cell line.	[233, 234]
Jurkat	A human immortalized T-cell leukemia cell line.	[235]
Molm14	An acute myeloid leukemia cell line.	[236-238]
REH	A B-cell precursor cell lin.	[239, 240]

2.1.11 Primary cells

Primary human T-lymphocytes from peripheral blood of healthy donors have been isolated using Ficoll plaque as described in 2.2.2.3. Volunteers provided written consent. The study was approved by the local ethics committee of the Heinrich-Heine-University of Düsseldorf (#4687).

2.1.12 Antibodies

Following antibodies were used for western blot and FACS analysis as well as coating of cell culture vessels.

Table 13: antibodies

Antibody	Manufacturer	Concentration
Western Blot		
Anti-EGFP Monoclonal ab	Clontech, #632375	1:10000
Anti-CYP4B1 polyclonal ab	Abcam, #ab83224	1:1000
Anti-β-Aktin monoclonal ab	Sigma, #A2228	1:20000
Sheep anti-mouse IgG horseradish peroxidase	GE Healthcare, #RPN4201V	1:10000
Goat anti-rabbit IgG horseradish peroxidase	GE healthcare, #RPN4301	1:10000
FACS		
CD3-PE	Miltenyi Biotec, #130-098-159	
CD34-PE	Miltenyi Biotec, #130-081-002	
CD34-FITC	Miltenyi Biotec, #130-081-001	
CD271-PE	Miltenyi Biotec, #130-091-885	
CD271-FITC	Miltenyi Biotec, #130-091-917	
CD271-APC	Miltenyi Biotec, #130-098-112	
AnnexinV-PE	Miltenyi Biotec, #130-092-052	
CD2-vioblue	Miltenyi Biotec, #130-098-708	
CD4-PE	Miltenyi Biotec, #130-098-134	
CD8-PerCP	Miltenyi Biotec, #130-098-057	
HLA-DR-Fitc	Miltenyi Biotec, #130-098-176	
Cell culture		
CD3 (OKT-3)	eBioscience, #19-0037-85	1 µg/cm^2
CD28	BDPharmingen™, #555725	1 µg/cm^2

2.1.13 Software

FACS measurements were evaluated using CellQuest Pro and FCS Express 5 (De Novo Software) and the arithmetical mean and standard error of the mean were calculates with Microsoft Excel. Contrast and saturation of Western Blot images were prepared using Adobe Photoshop CS6.

2.2 Cell culture

2.2.1 Cell culture of eukaryotic cells

Human embryonic kidney 293T cells, HT1080 cells and HepG2 cells were grown in Dulbecco's modified Eagle's medium with 4.5 mM glucose and 2 mM GlutaMAX supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. These cells are adherent and form monolayers; when the monolayers have been confluent, they've been split. Therefore, cells were incubated with Trypsin-EDTA that dissociates cell monolayers. The reaction was stopped by adding cell culture medium and the dissociated cells were transferred into a new cell culture dish according to the proliferation rate. Cell culture dishes were coated with 0.1% gelatin.

Jurkat, REH and Molm14 cells were grown in RPMI with 2 mM L-Glutamin, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Jurkat as well as REH cells grow in suspension.

All cells were grown at 37°C and 5% CO_2.

2.2.2 Cryoconservation of cells

For long-term storage, cells were frozen at -80°C and stored in tanks with liquid nitrogen. Therefore, cells were pelleted and resuspended in the appropriate media with 10% (v/v) DMSO and then transferred into cryogenic vials.

2.2.3 Isolation of T-lymphocytes from peripheral blood

Primary human T-lymphocytes were collected from peripheral blood of healthy adult volunteers and separated by Ficoll-Paque centrifugation according to the manufacturers protocol. Briefly, peripheral blood was diluted in PBS and carefully added to a Ficoll layer. Centrifugation lead to 4 layers: red blood cells, Ficoll solution, lymphocytes and monocytes and finally plasma with thrombocytes. The layer containing the lymphocytes was extracted, washed with PBS and incubated with red blood cells lysis buffer.

The cells were stimulated with CD3 and CD28 coated cell culture wells in combination with IL-2 and were grown in Isocove´s Modified Dulbecco´s supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin and 2 mM L-Glutamin. After two days, more than 95% of the living cells were T-cells and used for transduction.

2.2.4 Lentiviral vector production and transduction

$6x10^6$ HEK293T cells were seeded in 10 cm cell culture dishes. Following day, the HEK293T cells were transfected with 6 µg of each plasmid- the helper plasmids pCD/NL-BH for HIV1 gag/pol/rev, a vector for the envelope protein, and the vector containing the transgene cassette. As transfection reagent polythyleneimine (45 µg / transfection) was used. 24 hours after transfection the medium was changed to IMDM supplemented with 2 mM L-Glutamine, 10% FCS and 100 U/ml penicillin and 100 µg/ml streptomycin. 48 hours after transfection the viral supernatant was harvested and filtered through 0.45 µm filter (Sarstedt, Germany).

For titration of infectious viral particles, HT1080 cells were transduced using a serial dilution of the viral supernatant in DMEM medium including supplements. The media was changed the day after transduction. If the transgene cassette contained a resistance gene against an antibiotic, the selection started the day after transduction with medium containing antibiotics and continued until visible colonies were formed and the selection process was completed. The colonies per each dilution were counted and the transducing units per millimeter (TU/ml) calculated.

If the transgene cassette contained EGFP instead of a resistance gene towards an antibiotic, the titer was determined using FACS analysis. 4 days after transduction cells were harvested and analyzed. The titer was calculated as following:

$$TU/ml = \frac{number\ of\ cells\ during\ transduction}{100\%} \times \%\ pos.EGFP\ cells \times dilution\ factor$$

The day before HepG2 cells were transduced, they were seeded in a density of $3x10^6$ cells per 10 cm cell culture dish. For transduction, medium was replaced with 8 ml freshly harvested viral supernatant. 24 hours after transduction medium was changed. Depending on the experimental setting, a selection with an antibiotic started.

Human primary T-cells were transduced on the fibronectin fragment CH296 (Takara Bio Inc., Otsu, Japan) in 6-well-plates. $1\text{-}5x10^5$ T-cells were added with 2 ml viral supernatant. After 24 hours, 3 ml IMDM medium was added. Jurkat cells were transduced without the fibronectin fragment CH296.

All transductions were performed with 10 µg/ml protamine phosphate.

2.2.5 MACS Miltenyi microbead seletion

For positive selection of primary T-cells transduced with either truncated versions of CD34 or NGFR, the MACS microbead selection system was used. Briefly, T-cells were incubated with antibodies against CD34 or NGFR according to the manufacturer's protocol. The cells were washed with MACS buffer, pelleted and separated with the

OctoMACS Separator (Miltenyi, #130-042-109), MACS MultiStand (Miltenyi, #130-042-303) and MACS® Seperation Columns MS (Miltenyi, #130-042-201). The positive cells were held by the magnet within the columns whereas negative cells pass the column. For elution, the column was removed from the separator.

2.2.6 Cell proliferation assays

1-2x10^5 HepG2 cells were seeded in 12-well plates coated with 0.1% gelatine and incubated with different substrates in concentrations ranging from 2.9 to 900 µM. After 24 and 48 hours the cells were harvested and stained with propidium iodide (PI, 50 µg/ml). FACS analysis allowed discrimination between living and dead cells, EGFP between transduced and non-transduced cells.

Transduced primary T-cells were selected 3 days after infection using NGFR and CD34 antibodies coupled to microbeads. The next day cells were seeded in 24-well plates (at least 0.5x10^5 cells/well) and challenged with different concentrations of 4-ipomeanol (2.9, 9, 29, 90 and 290 µM) and perilla ketone (0.09, 0.9, 2.9, 9, 29, 90 and 290 µM) for 24 and 48 hours. Cells were pelleted and stained with PI to discriminate between live and dead cells. Detection of apoptosis was performed using Annexin V-Fitc and PI staining.

2.2.7 Bystander killing

HepG2 cells were transduced with CYP4B1 isoforms and a resistance gene against puromycin. After the selection was complete, a total of 2x10^4 HepG2 cells/well were seeded in 12-well plates in various ratios of transduced and non-transduced cells. Cells were incubated with 90 µM 4-IPO, PK and 2-AA. After 24 and 48 hours cells were harvested, stained with PI and analyzed by FACS.

2.2.8 Cytotoxicity assays

The cytotoxicity of CAR transduced human primary T-cells against a B-cell or AML cell line was determined by flow cytometry. T-cells were incubated over night with 10^4 $EGFP^+$ B-cells/AML cells in different effector to target cell ratios in U-bottom 96 well plate in a final volume of 200 µl. The next day three wells of each ratio were harvested and combined for further analysis. PI staining was used to discriminate between living and dead cells. Following the gating of viable cells the number $EGFP^+$ cells were determined.

2.2.9 FACS analysis

Flow cytometry is used to determine the size, morphology and fluorescence emitted by the cells enabling to detect different cell populations with marker genes such as EGFP or fluorescence labeled antibodies in a heterogeneous fluid. Briefly, the cells are suspended in a stream of fluid and pass a laser beam, the hereby generated scattered light and fluorescence is detected by different diodes. Here, the FACS Calibur (Becton Dickinson) was used.

2.3 Molecular biology

2.3.1 Plasmid preparation

Preparation of purified plasmid DNA in small quantities was performed with the FastPlamid Mini Kit (5Prime) to control positive transformed bacterial clones during cloning according to manufacturer's protocol.

For isolation of purified plasmid DNA in high quantities the NucleoBond® Xtra Maxi Kit (Macherey und Nagel) was used according to manufacturer's instructions. Plasmid DNA was solved in TE buffer (#A0386, AppliChem).

2.3.2 Agarose gel electrophoresis

Gel electrophoresis was used for analysis and separation of DNA. Agarose concentration was 1% and supplemented with 0.2 µg/ml ethidiumbromide. DNA was detected with a UV trans illuminator.

2.3.3 DNA restriction and dephosphorylation

DNA was digested with type II restriction enzyme, which recognize a specific sequence and cleave within it, to prepare DNA for traditional cloning. Usually, 2-5 U restriction enzyme was used for up to 5 µg DNA with it's appropriate buffer in a volume of 20 µl. After digestion the cleavage ends can be blunt or sticky dependent of the restriction enzyme. 3'-ends contains a hydroxyl group, 5'-ends a phosphate.

Plasmid DNA, digested for inserting a new DNA fragment, was dephosphorylated to prevent self-ligation. Calf intestinal alkaline phosphatase dephosphorylates the phosphate at the 5' end of DNA. Mostly, 10 U of CIAP was added to the restriction and incubated at 37°C for 1h.

2.3.4 DNA ligation

To insert a new DNA fragment into plasmid DNA, both DNA's were cut with restriction enzymes, which produce compatible ends. Here, T4 DNA ligase was used, which ligates both DNA fragments by connecting the 3'-hydroxyl group and the 5'-phosphate group and rebuilding phosphodiester bounds. DNA fragments and DNA plasmids was mixed in a molar ratio of 3:1 and incubated with 2 U T4 DNA ligase at room temperature for 3 h.

2.3.5 Transformation of DNA into *E. coli*

In order to amplify plasmid DNA fast and efficient DNA is inserted into bacteria. Therefore, the natural competence of bacteria to take up DNA is utilized. It is enhanced by a chemical treatment with calcium chloride.

Frozen stocks of competent bacteria is thawed on ice, 50 µl are incubated with the ligation reaction for 30 min on ice. A short heat shock of 45 s at 42°C enhances the permeability to take up DNA. Afterwards, the bacteria are incubated for 1 h at 37°C with gentle rocking and medium free of antibiotics. Then, bacteria are seeded on LB agar plates supplemented with the appropriate antibiotic and incubated over night at 37°C.

2.3.6 PCR

Amplification of DNA fragments for cloning was performed with Pwo DNA Polymerase because due to it's proofreading capacity- the 3'-5' exonuclease activity the error rate is extremely low.

Table 14: PCR reaction

Components	Concentration	Quantity
DNA template	200 ng	2 µl (100 ng/µl)
buffer	1x	5 µl (10x)
ddNTP	0.2 mM	1 µl (10 mM)
Primer forward	100 pmol	1 µl (100 pmol/µl)
Primer reverse	100 pmol	1 µl (100 pmol/µl)
Pwo DNA Polymerase	2 U	2 µl (1 U/µl)
ad. A. dest 50 µl		

PCR reaction was conducted with Biometra T3 Thermocycler.

Table 15: PCR program

Step	Temperature	Duration	Cycle
denaturation	94°C	3 min	1
denaturation	94°C	30 s	
annealing	50-65°C	1 min	30
elongation	72°C	30 s/1kb	
elongation	72°C	10 min	1
cooling	4°C	∞	1

2.3.7 DNA sequencing

In order to control the sequence of PCR amplified DNA fragments and cloning products, the sequencing service of the BMFZ Düsseldorf was used. Therefore, a PCR reaction with DNA template, a primer and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem) was started. The kit contains a DNA polymerase and fluorescence labeled ddNTPs. A typical reaction was made of 0.5 to 1 µg DNA, 4 µl BigDye® Terminator 1x, 10 pmol primer and H_2O in a volume of 20 µl.

The PCR reaction was afterwards purified using the DyeEx Spin Kit (Quiagen), Hi-Di™ Formamid (Applied Biosystem) was added and the PCR reaction was handed to the BMFZ for sequencing.

Table 16: PCR program for sequencing

Step	Temperature	Duration	Cycle
denaturation	96°C	1 min	1
denaturation	96°C	10 s	
annealing	55°C	50 s	25
elongation	60°C	4 min	
elongation	60°C	7 min	1
cooling	4°C	∞	1

2.4 Western Blot

2.4.1 Lysis of eucaryotic cells

Cells were washed with PBS and centrifuged at 1600 rpm for 10 min. The pellet was resuspended in lysis buffer and incubated on ice for 30 min. Centrifuging at 4°C and 13000 rpm for 10 min pelleted cell debris leaving proteins and cytoplasm within the supernatant, which was transferred into a new reaction tube and could be stored at -20°C.

2.4.2 SDS PAGE

Whole cell lysates were incubated for 5 min at 95°C and loading buffer added. A 4-12% NuPAGE Bis-Tris polyacrylamide gel in combination with NuPAGE-MES-SDS-running buffer was used to separate the proteins. For 30 min 60 V was applied followed by 110 V for 2 h.

2.4.3 Western Blot

The polyacrylamide gels were blotted on a Hybond-P PVDF membrane (GE Healthcare, #RPN303F) for 1.5 h at 20 V. Afterwards, the membrane was stained with Ponceau S to visualize and confirm protein transfer. The membrane was destained, blocked in 5% milk for 1 h and probed with primary antibodies over night. Next day, the membrane was washed with PBS-T and probed with a secondary antibody. For detection of the chemiluminescence signal we used the ECL system (Pierce, Thermo Fisher Scientific) and the Image Quant LAS 4000 mini (GE Healthcare).

3. Results

Both DLI and CAR therapies require robust expression of a second transgene in addition to the suicide gene, a clinically usable transgene for selection or the CAR, respectively. For the development of an integrating vector with high-level co-expression of two transgenes, a third generation SIN-lentiviral vector was brought into the focus. This type of vector is currently used in several clinical trials for two principle indications, hematopoietic stem cell gene therapy for monogenetic disorders, and CAR T-cell therapies. Beside 4-ipomeanol, other substrates for CYP4B1 such as 2-aminoanthracene are described. Different substrates might exhibit different isoform specificities, toxicities and kinetics. Changing the pseudotype can enhance transduction efficiency while reducing the stress for T-cells and therefore form another possibility to improve the lentiviral vector design.

3.1. Vector optimization

The SIN-lentiviral vector design allows to choose an internal promoter and to include a complex transgene cassette that will be stably integrated into the DNA of target cells. In addition, the possible cargo to be inserted into the vector can be quite big (<10 kb), hence it is possible to express more than one transgene in a single vector. Therefore, this chapter focuses on the optimal design of lentiviral vector for preclinical and clinical studies.

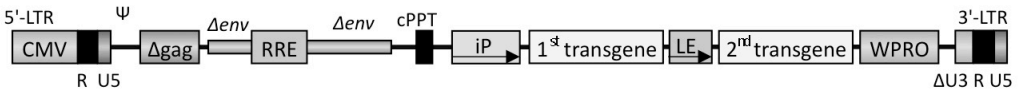

Figure 16: Schematic representation of a lentiviral SIN-vector. iP: internal promoter, LE: linking element, WPRO: optimized woodchuck posttranscriptional regulatory element.

3.1.1 Co-expression strategies of two transgenes

Several approaches to co-express two transgenes have been presented, for example in a context for an optimal co-expression of the α- and β-chain of a TCR [241, 242]. Here, the most common clinically usable strategies, a 2-promoter system, a virus-derived internal ribosomal entry site, and two 2A-like sites were compared in respect of their reliability and expression levels in the Jurkat T-cell line. To test the dual promoter approach, the U3 region of the *spleen focus-forming* virus (SFFV) and the promoter of the human phosphoglycerol kinase (hPGK) as widely used promoters were cloned in the

same orientation into the lentiviral vector (Figure 17). The most commonly used IRES site for hematopoietic cells is the IRES site from the *encephalomyelocarditis virus* (EMCV). Expression of the downstream cDNA starts at the 11th ATG. Two 2A sites were tested here. One is the 2A site of *foot-and-mouth-disease* virus (FMDV), which is N-terminally expanded by 14 aa from the 1D region according to Donnelly *et al.* [189]. This 2A site had a final length of 33 aa and was termed 14+2A site. The other 2A site was derived from the *Thosea asigna* virus (TAV) with a length of 18 aa and called T2A [243]. As transgenes for testing the different co-expression strategies, two spectrally non-overlapping fluorescent proteins, E2 Crimson and EGFP, were expressed using the IRES site as well as both 2A sites driven by the hPGK promoter (Figure 17).

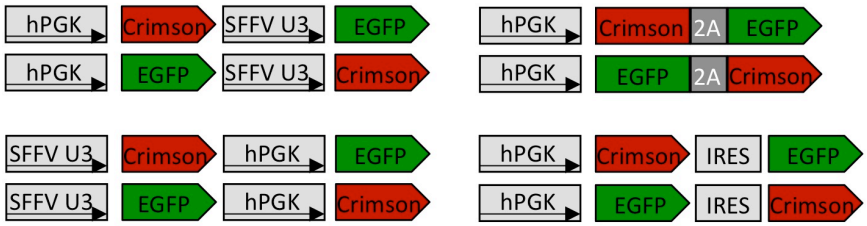

Figure 17: Schematic representation of the transgene cassettes. EGFP and E2 Crimson were placed on both positions. The promoters within the dual promoter vectors (left side) have also switched the positions.

Jurkat cells were transduced at similar MOI with VSV-G pseudotyped LV vectors shown in Figure 17 and then analyzed by flow cytometry one week post transduction. As demonstrated in Figure 18, the dual promoter system did not lead to comparable high-level expression of both transgenes. When hPGK and EGFP were placed first, 87.3% of the cells were positive for EGFP and only 2.3% of the cells for both EGFP and E2 Crimson. Similar was observed when hPGK is placed first in combination with E2 Crimson. Placing the SFFV promoter with one of the fluorescent proteins first also led to cell subsets expressing only one transgene. The other approaches seemed to lead to a more equal expression but the overall fluorescence signal of E2 Crimson was too weak.

Due to the low fluorescence intensity of E2 Crimson, all constructs were recloned by replacing E2 Crimson with dTOMATO. Jurkat cells were transduced with VSV-G pseudotyped LV vectors at similar MOIs of ≈5. As shown in Figure 19, dTOMATO proved to be brighter and better measurable than E2 Crimson. The tendencies, shown in the experiments with E2 Crimson, could be reproduced with dTOMATO (Figure 19). Using the dual promoter strategy, promoter interferences could be observed in every promoter/transgene combination. The difference between double and single positively expressed fluorescent proteins was quite distinct thus generating two cell subsets. For example, when hPGK and dTOMATO were placed first (Figure 19, 2nd in first row), 98% of the cells were positive for dTOMATO but only 61% for EGFP. Similar results were obtained when hPGK and EGFP were placed first.

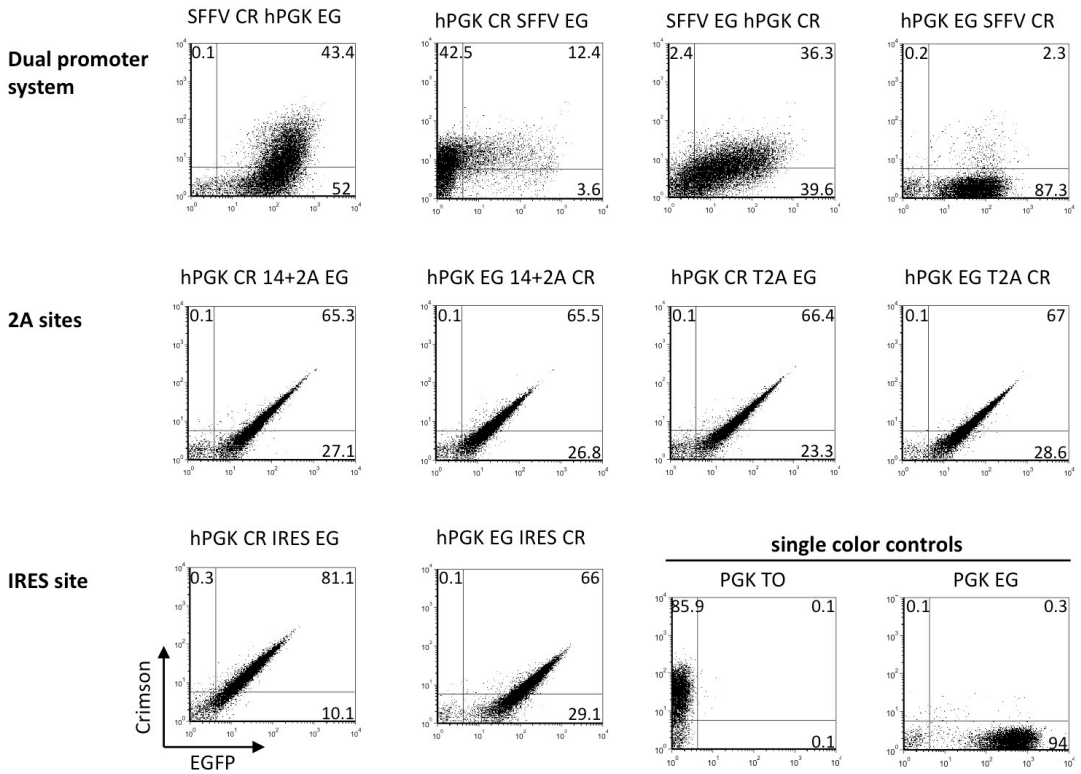

Figure 18: FACS analysis of Jurkat cells expressing E2 Crimson (CR) and EGFP (EG). First row shows the expression pattern using a dual promoter system, the second row the 2A sites and the third row the IRES site. EGFP and E2 Crimson were either placed at the first or at the second position. The position of the promoters in the vectors was also changed. The percentages of cells positive for EGFP and/or E2 Crimson are shown in the dot blots for each quadrant. Representative blots of two independent experiments were shown.

Using the IRES site showed a correlation between the expression levels of the two transgenes. However, in general, the transgene in the second position had a much weaker expression compared to in the first place. For example, the mean fluorescence intensity (MFI) of EGFP decreased a fourfold being positioned behind the IRES site (data not shown). Finally, the use of both 2A site leads to the most robust and equal expression of both transgenes, either with EGFP and dTOMATO or EGFP and E2 Crimson. There was no difference in the efficacy and expression levels between the 2A sites from FMDV and TAV.

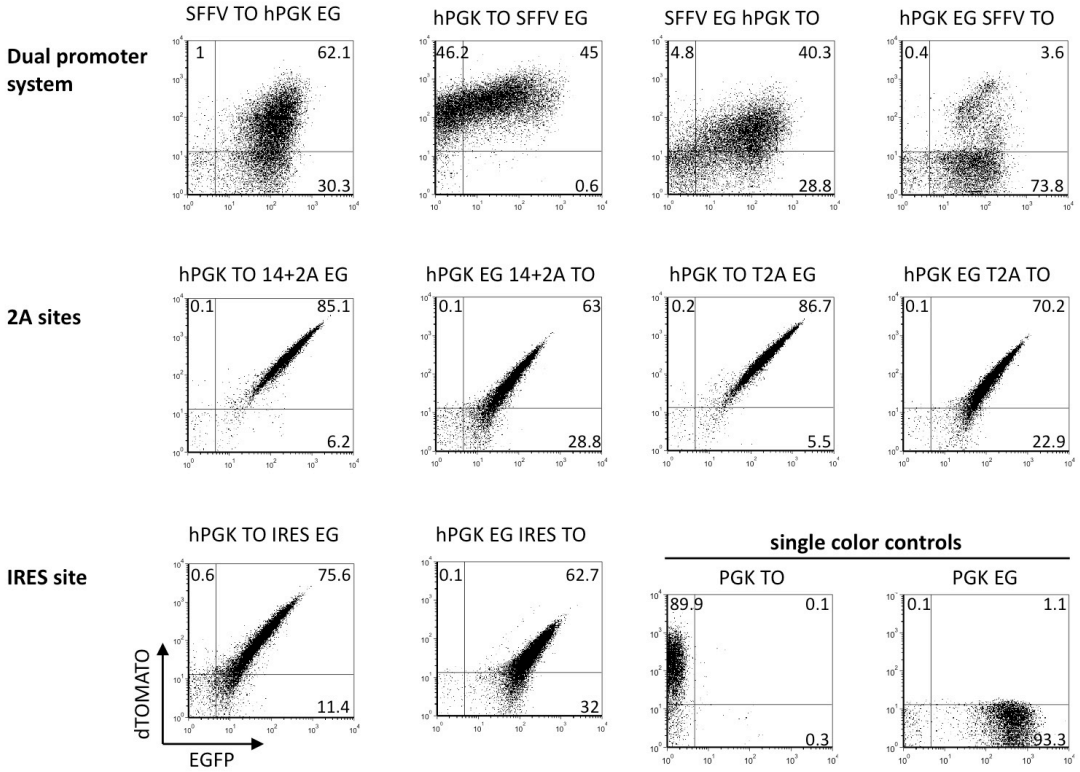

Figure 19: FACS analysis of Jurkat cells expressing dTOMATO and EGFP. First row shows the expression pattern using a dual promoter system, the second row the 2A sites and the third row the IRES site. EGFP and E2 Crimson were each placed at the first and at the second position. The promoters were equally changed. The percentages of cells positive for EGFP and/or dTOMATO are shown in the dot blots for each quadrant. Representative blots of two independent experiments were shown.

Western Blot analysis with a commercially available monoclonal antibody against EGFP confirmed a robust expression of EGFP with the IRES site and both 2A sites (Figure 20.A). However, the Western Blots also demonstrated a common problem that 2A sites exhibit: 2A sites mediate a process that is called “ribosomal skipping” [188, 189]. Here, the viral peptide blocks the ribosome from connecting a newly inserted amino acid to the already translated protein while the ribosome keeps translating the rest of the mRNA. Thereby, the already synthesized protein is co-translationally cleaved and clears the ribosome while the downstream protein is still synthesized. This cleavage occurs at a specific sequence, the D(V/I)ExNPGP motif at the end of all 2A sites, between glycine and the last proline. Because of this mechanism of action, the transgene placed before the 2A site has the viral peptide as a tail at the 3’-end, whereas the transgene placed behind the 2A site has a proline added to the 5’-end. The different viral peptides fused to EGFP were reflected within the Western Blot analysis. EGFP placed before the 14+2A site contained additional 34 aa, EGFP placed before T2A 17 aa. Also, the protein was not always cleaved, whereby fusion proteins of EGFP and dTOMATO connected by the 2A site were generated. They were also present in the Western Blot and detectable at 55 kDa and 57 kDa in lanes 3-6, respectively. Moreover, when analyzing the transgene positions, EGFP placed upstream of the 2A site seemed to lead to a higher ‘cleavage’ rate than the downstream position (compare lanes 3 and 5 with lanes 4 and 6). In Jurkat

cells, where both fluorescent proteins were expressed via a dual promoter system, position dependent expression of EGFP was observed (Figure 20 B).

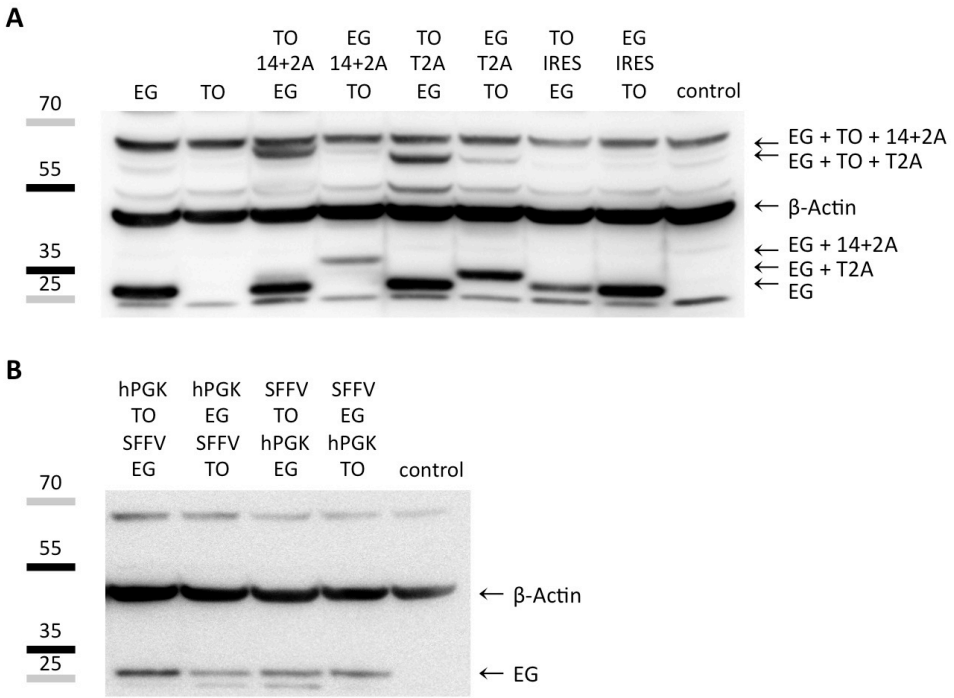

Figure 20: Western Blot analysis of the different co-expression strategies. EGFP detection of Jurkat cells expressing Tomato and EGFP via an IRES site, a 2A site (**A**) or a dual promoter system (**B**). As control non-transduced cells were used. β-Actin staining was used to visualize equal protein loading.

Based on these results, the two 2A sites are clearly the best way to co-express two transgenes at high levels. As there was no difference in the expression level and the cleavage efficacy between the two 2A sites, the shorter T2A with 18 amino acids was chosen for all experiments.

3.1.2 Comparative analysis of promoter strength

As mentioned before, the lentiviral SIN-vector design allows to use a wide variety of transcriptionally active elements as an internal promoter to drive the transgene cassette. In order to identify a promoter, which facilitates high-level expression in primary T-cells, several promoter elements were tested. These different promoters were easily exchanged using two unique restriction sites, *Xho*I and *Pac*I, in the vector backbone designed for this purpose.

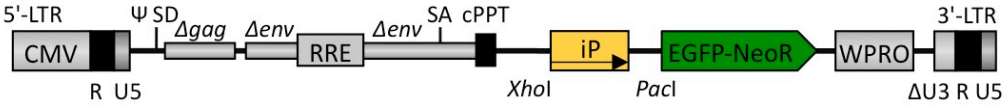

Figure 21: Schematic representation of the lentiviral expression vector. The internal promoter can be exchanged using *Xho*I and *Pac*I restriction enzymes and drives an EGFP-NeoR fusion gene.

Using the LV vector shown in Figure 21, two viral U3 promoters from SFFV and MPSV as well as promoters of human cellular house keeping genes, PGK, EF1α and UbiquitinC (UbiC) were PCR amplified adding *Xho*I and *Pac*I sites. Also, the inducible-promoters of members of the solute carrier families were tested, glucose transporter 1 (Glut1) and amino acid transporter 2 (Asct2), derived from humans or rats in different lengths. The HLA-DR promoter was tested as an activation-dependent promoter. In addition, the U3 MPSV, HLA-DR and hEF1α promoters were tested with and without the inclusion of an intron, because splice sites and an appropriate intron located in the 5'UTR of transgenic RNA [244, 245] can promote mRNA export [244, 246] and translation [245].

The MFI of the EGFP-NeoR fusion gene served as a reporter of the expression strength. The lentiviral particles were VSV-G pseudotyped and the titer of infectious particles assessed on HT1080 cells infected with a serial dilution of the viral supernatant. Stably transduced cells were analyzed after 5 days by flow cytometry. As shown in Figure 22, the insertion of an intron clearly impacted the quantity of produced infectious particles since every promoter with an intron (HLA-DR + intron, MPSV + intron, hEF1α + intron and hUbiC + intron) had a lower titer compared to the same promoter without an intron.

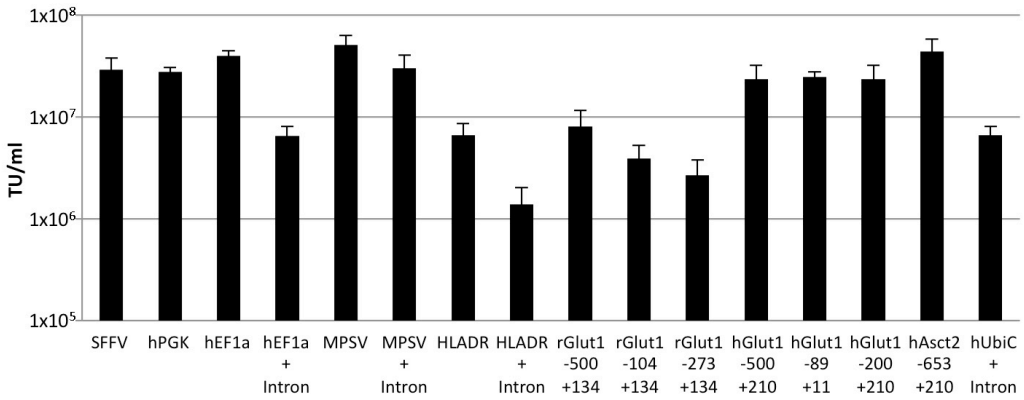

Figure 22: Infectious titer of LV vectors assessed by serial dilution of lentiviral supernatant for transduction of HT1080 cells. EGFP positive cells were analyzed by flow cytometry. For each construct, mean ± SEM is shown from at least three experiments.

Next, primary T-cells from 3-5 volunteers were transduced with similar MOI to generate less than 30% positive T-cells thereby limiting the number of proviral integrations [247]. 5 days later, the number of positive cells and expression strength of EGFP were assessed by flow cytometry.

A shown in Figure 23, the viral promoters SFFV and MPSV facilitated strong expression of EGFP, whereas the mammalian promoters facilitated a weak expression. Interestingly, the addition of an intron to the promoter MPSV led to a slight increase in EGFP expression, while the addition of an intron to the hEF1α promoter led to a dramatically increase resulting in an expression level comparable to the viral promoters induced levels. However, the addition of an intron to HLA-DR led to a decrease. The promoters HLA-DR, Glut1 and Asct2 are activation-dependent and inducible promoters; all facilitate a weak EGFP expression. Therefore, either these promoters generally facilitate a weak expression or the activation status of the T-cells, which were stimulated with CD3/CD28 coating prior to transduction and constant IL-2 supplement, was not high enough to induce a strong expression.

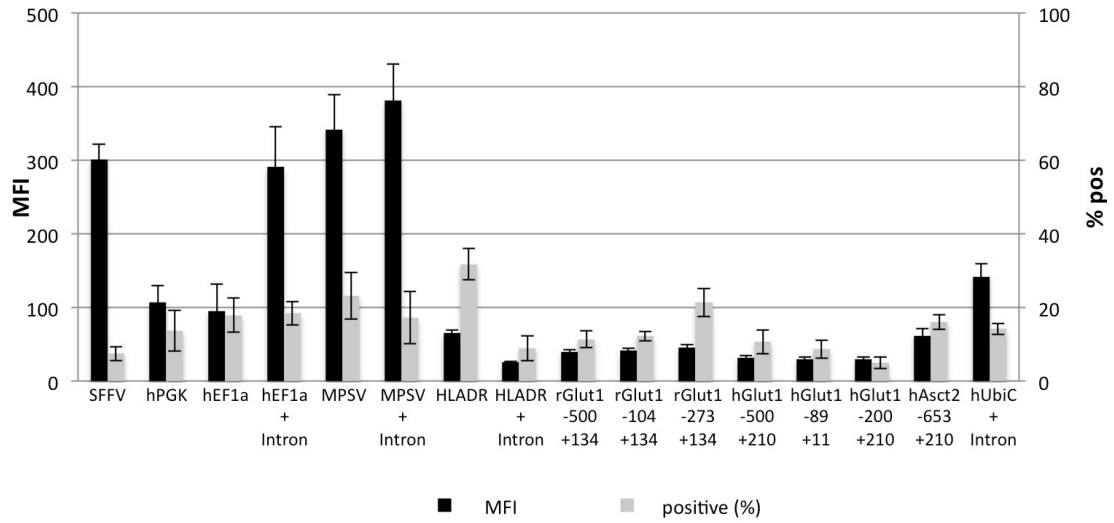

Figure 23: Comparison of EGFP fluorescence in transduced primary T-cells with 16 different EGFP LV vectors varying only in the internal promoter. Transduction occurred with similar MOI. Black bars indicate the mean fluorescence intensity (MFI), grey bars the percentage of positive T-cells assessed by flow cytometry. For each construct, mean ± SEM is shown from at least three experiments.

For both applications, DLI and CAR T-cell therapy, a strong transgene expression is required. The virus-derived promoters SFFV and MPSV as well the hEF1α + intron meet this demand, but the intron added to hEF1α induces a loss of 90% viral infectious particles and SFFV is associated with insertional mutagenesis [248-250], although not in mature T-cells [251]. Since the inclusion of an intron only mildly affected either the MFI of EGFP driven by MPSV or the infectious titer, the MPSV promoter without an intron was chosen for a clinically applicable vector.

3.1.3 Pseudotyping of lentiviral vectors

During the production of lentiviral particles a large variety of transmembrane protein can become incorporated as envelope protein into the virion. By exploiting this amazing flexibility of LV vectors, the tropism of the infectious particles can be strongly

influenced. In order to identify an alternative envelope protein that facilitates gentle and efficient transduction of primary T-cells, the envelope proteins GalvTM and PE01 were tested. GalvTM is a chimeric envelope protein build from the SU unit of the *gibbon ape leukemia virus* (Galv) and the TM units of the *ampophotrophic murine leukemia virus* (aMLV) [213]. The TM unit of the Galv glycoprotein contains critical amino acid residues proximal to the R peptide cleavage site that inhibit cleavage by HIV-1 proteases [205]. PE01 is the envelope protein of the *human* foamy virus with point mutations within the leader region to avoid ubiquitination sites, which results in an increase of subviral particle release. Without these modifications, pseudotyping with both proteins is not efficient [205, 218]. They were also tested before and after codon optimization for human codon usage. As reference, the VSV-G envelope was used, which had been used earlier to transduce primary T-cells.

The 2nd generation helper plasmid, pCD/NL-BH, was cotransfected in HEK293T cells for virus production. In addition to the necessary proteins gag, pol, rev and RRE, it also encodes for regulatory and accessory proteins of the lentiviral genome like vif, vpr, vpu and tat. EGFP was used as reporter gene encoded on the transfer vector. For a typical transfection with polyethylen (PEI) as reagent, 15 µg DNA was used, 5 µg of each plasmid. Importantly, since optimization for human codon-usage of viral genes can lead to an extraordinary increase of protein expression levels, every envelope plasmid DNA except the one of VSV-G was titred. A mock plasmid (cloning vector pSL1180) was used to keep the amount of transfected DNA constant at 15 µg DNA (table 11).

Table 17: Amount of DNA used for transfection based on PEI

Mock plasmid	Envelope plasmid	Helper plasmid	Transfer plasmid
0 µg	5 µg		
4 µg	1 µg		
4.5 µg	0.5 µg	5 µg	5 µg
4.9 µg	0.1 µg		
4.95 µg	0.05 µg		

First, HT1080 cells were transduced with serial dilutions of the viral supernatant to determine the infectious titer (Figure 24). With standard condition (5+5+5 µg DNA), VSV-G pseudotyped lentiviral particles had titers of $4.23*10^7 \pm 1.2*10^7$ TU/ml. The titer of the lentiviral particles pseudotyped with the codon-optimized FV PE01 (coop PE01) envelope was almost as high as the one with VSV-G and constant with a slight peak at 0.5 µg envelope DNA per 10 cm dish and a small decline at 0.1 and 0.05 µg. The same envelope without the codon-optimization only had a 10-fold lower titer. Remarkably, the titer of codon-optimized GalvTM (coop GalvTM) pseudotyped particles was almost

constant with a peak at 1 µg envelope DNA per 10 cm dish, albeit with $6*10^5 \pm 3.1*10^5$ TU/ml considerably lower than PE01. Also here, the non-codon optimized GalvTM only reached a 10-fold lower titer compared to the codon-optimized plasmid, demonstrating the potential of human codon-optimization.

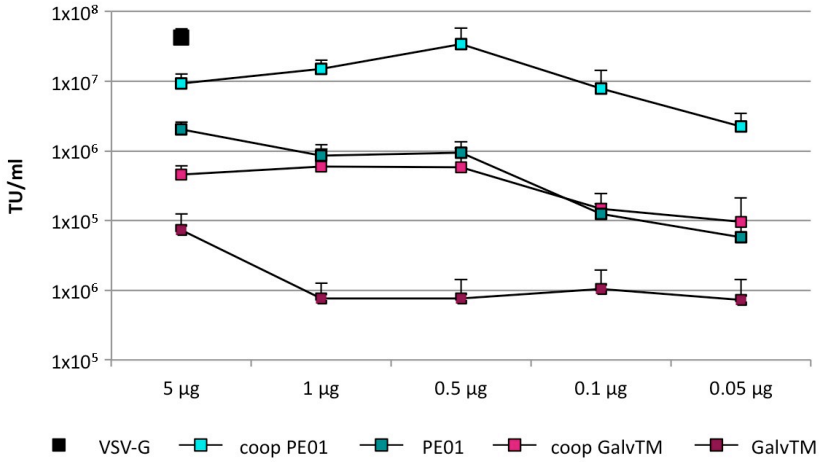

Figure 24: Infectious titer of LV vectors assessed by serial dilution of lentiviral supernatant and transduction of HT1080 cells. Positive transduction was evaluated by flow cytometry counting EGFP positive cells. For each construct, mean + SEM is shown from at least four independent experiments.

Next, primary T-cells were transduced at similar MOIs of ≈ 5 and EGFP expression assessed 4 days post transduction by flow cytometry (Figure 25). VSV-G pseudotyped lentiviral particles efficiently transduced 50.3 ± 5.4% T-cells. Interestingly, using 0.1 µg coop PE01 DNA led to the most transduced T-cells with 62.7 ± 9.8%., while 5 µg coop PE01 resulted in 19.3 ± 4.8% and 5 µg of non-optimized PE01 in 38.2 ± 5.7% EGFP positive T-cells. In summary, increasing the amount of PE01 DNA led to a decrease of positive transduced cells. Codon-optimized GalvTM had a plateau between 1 µg and 0.1 µg DNA (36.7 ± 6.1& to 35.1 ± 3.3%). Also here, increasing the amount of DNA resulted

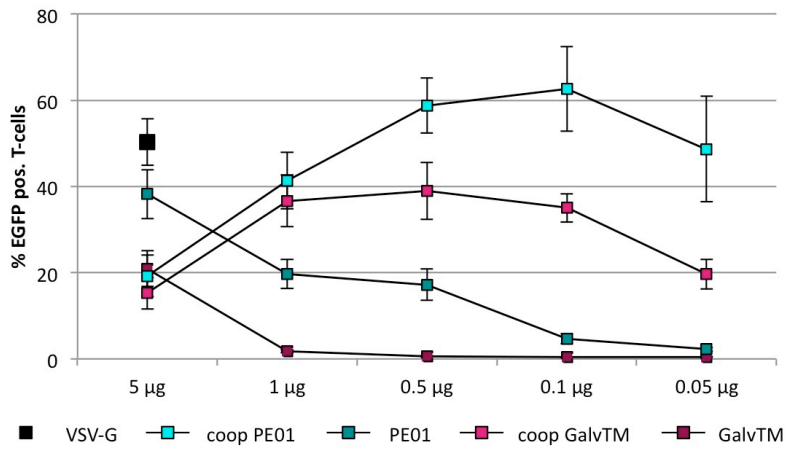

Figure 25: Comparison of transduction efficacy of differently pseudotyped LV in primary T-cells assessed by flow cytometry. Transduction occurred with similar MOIs. For each envelope, mean ± SEM is shown from 6-8 independent experiments.

in less positive transduced cells (15.3 ± 3.7%) in contrast to non-optimized GalvTM, where using 5 µg DNA led to the highest number of transduced T-cells (20.9 ± 4.1%). Decreasing the DNA quantity during transfection led to a rapid decline against zero.

3.1.4 Selection marker

An important prerequisite for suicide gene therapy to control GvHD following DLI is that only genetically engineered T-cells are infused into the patient. Hence, the transduced cells have to be separated from the non-transduced. Therefore, in this paragraph the best position for the expression of two selection markers relative to the T2A site was investigated. Ideally, the selection marker does not require a long-term *ex vivo* cultivation and the marker should be of human origin to avoid potential immunogenicity and not naturally expressed on mature T-cells.

First, it was analyzed whether the expression levels and the activity of the suicide gene CYP4B1P+12 is influenced, if it is expressed before or after the T2A site. This was important as previous studies have shown that 5'-fusions of EGFP with CYP4B1 enzymes lead to a reduction of the enzymatic activity [144, 252] and placing CYP4B1 5' of T2A leaves 17 aa on the 3'-end of the protein. CYP4B1 in second positions adds a proline in front of methionine. The general structure of the expression vector used here is depicted in Figure 26.

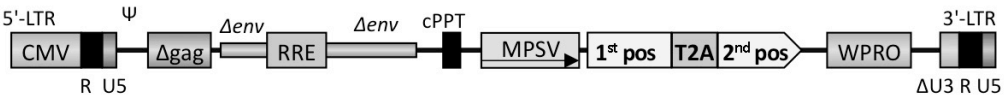

Figure 26: Schematic outline of the lentiviral vector used. The MPSV drives the expression of two transgenes connected by a T2A site.

For the following experiments, EGFP and the human CYP4B1P+12 (h4B1P+12) were co-expressed via the T2A site in both positions. The activity of the suicide gene was assessed by incubating primary T-cells 3 days after transduction with increasing concentrations of 4-ipomeanol and then analyzing the cultures after 24h and 48h by flow cytometry [144, 252]. Viable cells were readily detected using propidium iodide (PI) staining. As shown in Figure 27, incubation with 290 µM 4-ipomeanol led to only 22.6 ± 3.4% viable EGFP positive T-cells after 24h and 8.3 ± 0.6% after 48h when h4B1P+12 is expressed 5' of T2A. When h4B1P+12 is expressed 3' of T2A, 25.1 ± 1.8% EGFP positive T-cells were viable after 24h and 10.6 ± 0.6% after 48h. Cells transduced with a control vector only expressing EGFP and non-transduced cells displayed no toxicity towards 4-ipomeanol. In conclusion, the function of the suicide gene is independent whether it is expressed up- or downstream of the T2A site.

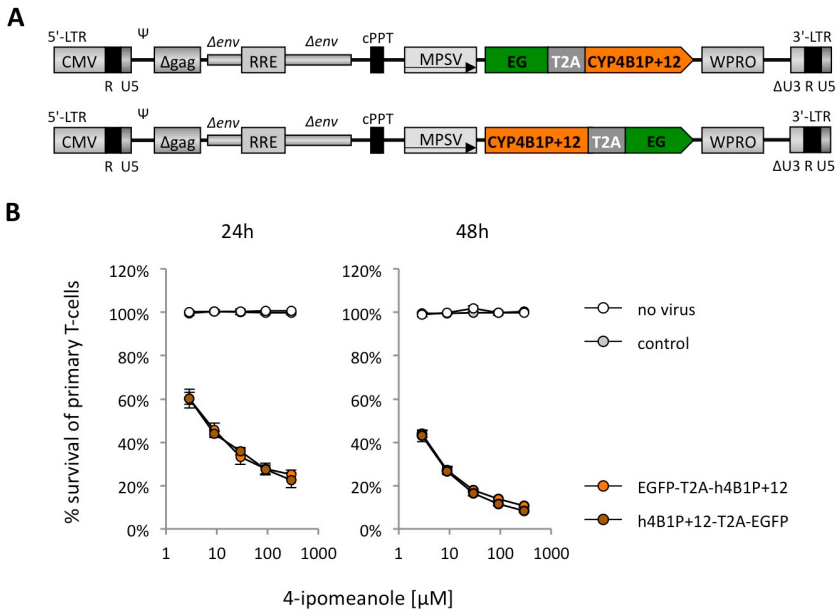

Figure 27. Position independency of h4B1P+12 and EGFP relative to T2A. A. Schematic outline of the lentiviral vectors. The transgene cassette consisting of EGFP and CYP4B1P+12 is driven by the MPSV promoter. **B. Survival of transduced primary T-cells after exposure to 4-IPO.** T-cells were incubated with increasing concentrations of 4-IPO, 2.9-290 μM, and analyzed by FACS after 24h and 48h. Control cells were transduced with EGFP only. For each construct, the mean ±S.E.M. from at least three different experiments is shown.

As a next step, the magnetic cell separation using two surface molecules was tested. The nerve growth factor receptor p75 [253, 254] and CD34 [255] were C-terminally truncated (ΔNGFR, ΔCD34), optimized for human codon-usage and cloned into the lentiviral vector described in Figure 26. Both surface markers are of human origin and not expressed on mature human T-cells. Importantly, GMP selection reagents are commercially available (Miltenyi Biotech). First, in order to evaluate the purity of the selection process, ΔNGFR and ΔCD34 were expressed together with EGFP. Also for these experiments, the surface markers were placed in both positions, before and after the T2A site. Three days after transduction, primary T-cells were selected using antibodies labeled with paramagnetic beads according to the protocol provided by Miltenyi Biotech. For quality control, cells prior and after selection as well as the flow through cells were analyzed by flow cytometry employing EGFP expression as a marker for positively selected cells (Figure 28). This analysis revealed that 48 ± 8.9% to 66 ± 4.0% EGFP positive T-cells can be enriched to 95 ± 0.6% to 97 ± 1.3% after two selection steps. In the flow through, there were almost no positive T-cells detected (Figure 28 B) demonstrating the high efficacy of this technology. Staining the final eluate with CD3, a mature T-cell marker, confirmed that the enriched cell populations are in fact >95% T-cells. Importantly for the intended usage, the position of ΔNGFR and ΔCD34 relative to the T2A site did not influence the efficacy of the selection process.

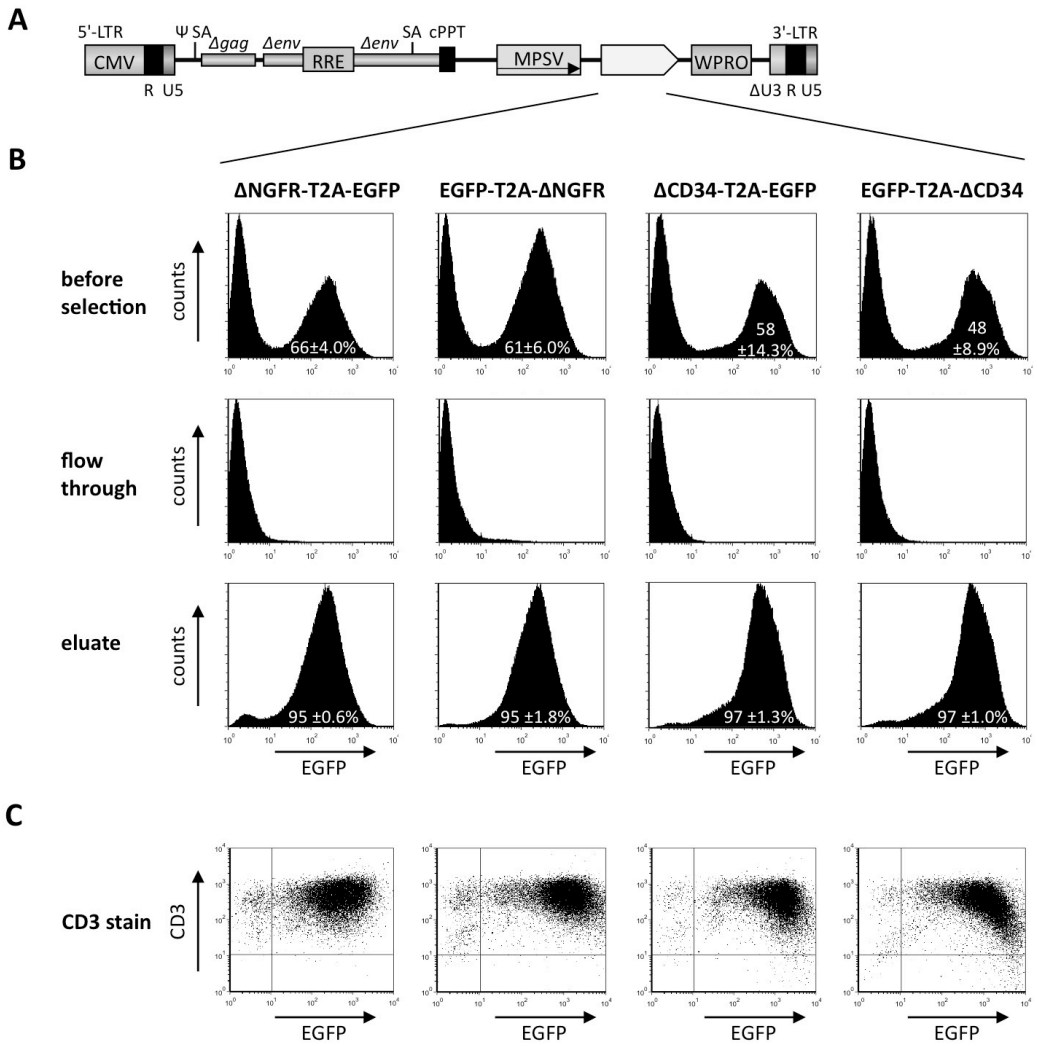

Figure 28: FACS analysis of primary T-cells using MACS selection. A. Schematic outline of the lentiviral vector backbone. B. MACS microbeads selection was performed on T-cells transduced with p2CL21ΔNGFRT2AEGwo, p2CL21EGT2AΔNGFR, p2CL21ΔCD34T2AEG and p2CL21EGT2AΔCD34, respectively. Representative histogram analyses before and after selection as well as the flow through are shown. **C.** Analysis of MACS microbeads selected T-cells after staining with CD3. Shown here are representative FACS analyses from at least three independent experiments are shown.

To perform Western Blots for analysis of the T2A-mediated cleavage efficiency, Jurkat cells were transduced with the same expression vectors used for MACS selection of primary T-cells (Figure 28). Staining with CD34 and NGFR antibodies showed that 96.9%-98.9% Jurkat cells were positive (Figure 29 A). Western Blot analysis was performed using a commercially available monoclonal antibody to detect EGFP and a β-Actin antibody as a control for demonstrating equal protein loading (Figure 29 B). EGFP was detected at around 27 kDa (second position) and at 29 kDa when EGFP was expressed upstream of T2A (with the remaining 17 amino acids fused to it). Also here due to 'uncleaved' protein, bands containing EGFP and ΔCD34 or ΔNGFR were detected around 64 kDa and 59 kDa, respectively. Surprisingly, T2A seemed to induce a better separation between ΔNGFR and EGFP than between ΔCD34 and EGFP (compare lanes 3 and 4 versus 1 and 2). Furthermore, when ΔNGFR was placed 5' of T2A the

separation worked better than ΔNGFR being placed 3' of T2A (compare lane 3 versus lane 4).

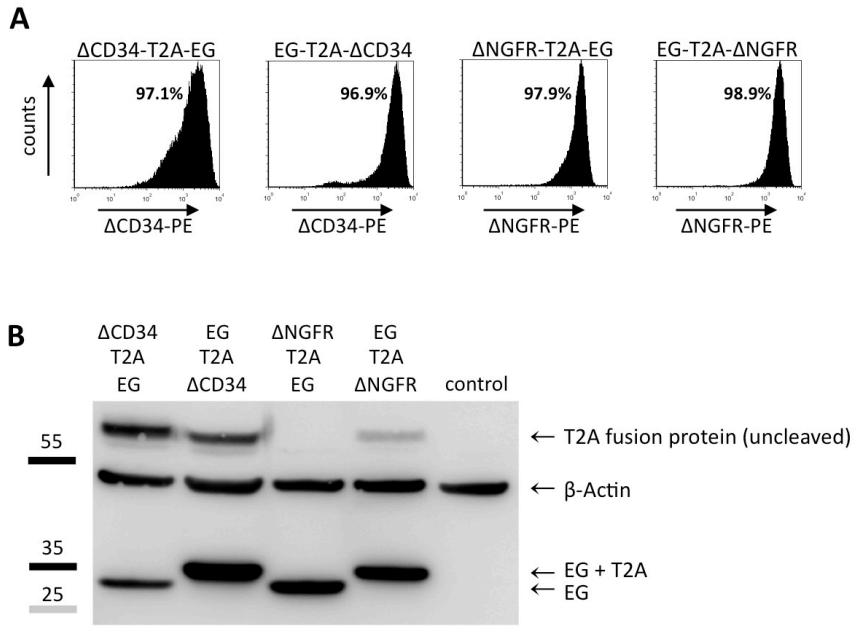

Figure 29: Analysis of Jurkat cells transduced with lentiviral vectors expressing EGFP and ΔNGFR or ΔCD34. A. FACS analysis of transduced Jurkat cells using CD34 and NGFR staining. **B.** Western Blot analysis of transduced Jurkat cells. An EGFP antibody was used to detect EGFP in whole cell lysates; β-Actin staining was used to visualize equal protein loading.

Finally, after confirming the position independency of all three cDNAs for h4B1P+12, ΔNGFR and ΔCD34 relative to the T2A site, the combination of the suicide gene and the ΔNGFR and ΔCD34 surface markers expressed in a single vector was tested. To this end, h4B1P+12 replaced EGFP in the vectors as shown in Figure 30. Transduced T-cells were selected using microbeads and 24h later challenged with increasing concentrations of 4-ipomeanol. At all times, T-cell medium contained IL-2 to ensure T-cell proliferation. Survival of T-cells was analyzed after 24h and 48h by flow cytometry after PI staining (Figure 30). T-cells transduced with h4B1P+12 showed a high toxicity towards 4-IPO independent of the position relative to T2A. Non-transduced or with a control vector transduced T-cells revealed no toxicity after 4-ipomeanol exposure. Notably, T-cells expressing ΔNGFR and h4B1P+12 displayed a stronger toxicity: after 48h only 6.85 ± 2.1% and 7.09 ± 1.8% cells were viable while ΔCD34 expressing T-cells showed 17.92 ± 2.6% and 18.37 ± 6.3% survival.

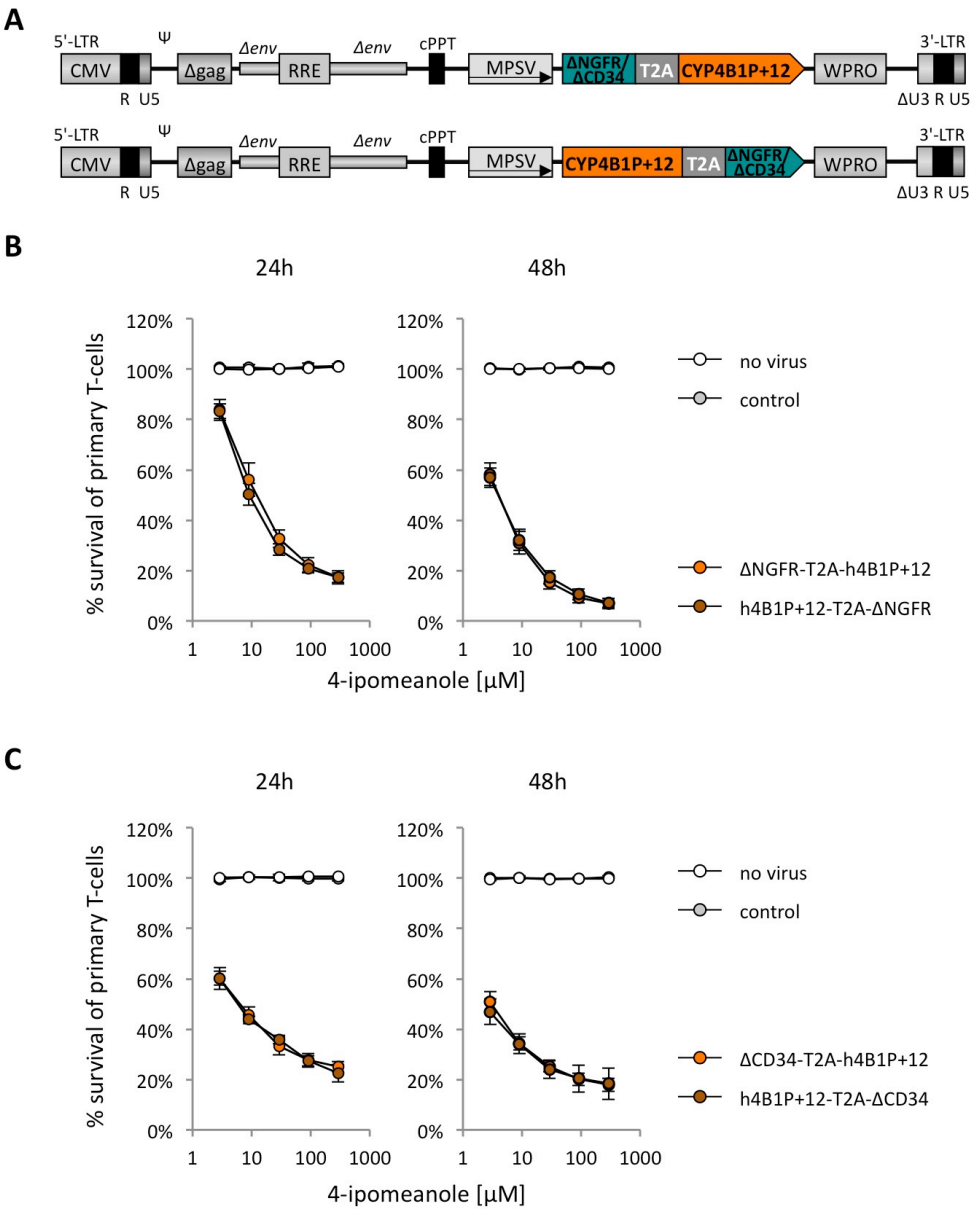

Figure 30: Cytotoxicity assay of primary T-cells. Primary T-cells were transduced with CYP4B1P+12 co-expressed with ΔNGFR (A) or ΔCD34 (B) using T2A, respectively. EGFP replaced CYP4B1P+12 in the control vector (A, B). Transduced T-cells were selected using MACS mircobeads. T-cells were challenged with increasing concentration of 4-IPO for 24h and 48h and then survival was assessed by flow cytometry after propidium iodide (PI) staining. For each construct, mean ± SEM is shown from at least three experiments.

Considering the different separation efficacy of ΔNGFR and ΔCD34 when co-expressed with EGFP, Western Blot analysis of Jurkat cells expressing h4B1P+12 with ΔNGFR and ΔCD34 was performed. First, the transduction of Jurkat cells was confirmed by antibody staining and subsequent FACS analysis (Figure 31 A). Co-expressed with h4B1P+12, 'uncleaved' fusion protein was only detected as a weak band of ΔCD34 expressing cells by a CYP4B1 antibody (Figure 31 B, lanes 1 and 2). In contrast, no fusion protein was detectable for the vector constructs expressing ΔNGFR and h4B1P+12 linked by T2A.

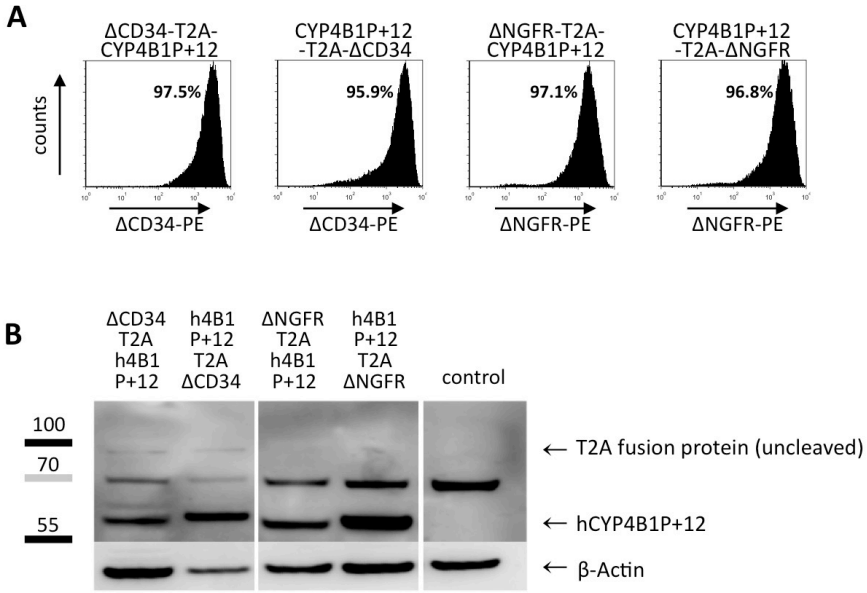

Figure 31: Analysis of Jurkat cells expressing with h4B1P+12 and ΔNGFR or ΔCD34. A. FACS analysis of transduced Jurkat cells using CD34 and NGFR staining. **B.** Western Blot analysis of transduced Jurkat cells. An CYP4B1 antibody was used to detect h4B1P+12 in whole cell lysates; β-Actin staining was used to visualize equal protein loading.

Based on the results within this chapter, especially on the improved cleavage and the higher level of cytotoxicity at lower 4-ipomeanol concentrations, p2CL21ΔNGFRT2Ah4B1P+12wo was chosen as a suitable vector for DLI application (Figure 32). The U3 region of the MPSV provides a strong expression of the transgene cassette and the T2A site facilitates an equal expression of both transgenes. Importantly for clinical usage, by not including an intron in the vector, high amounts of infectious particles can be easily produced under standard conditions.

Figure 32: Schematic representation of p2CL21ΔNGFRT2Ah4B1P+12wo. The MPSV promoter drives the transgenes ΔNGFR and h4B1P+12 connected by a T2A site.

3.2 Alternative prodrugs for the CYP4B1P+12 suicide gene

4-ipomeanol is a well-established prodrug for CYP4B1, although other substrates are processed by the highly active rabbit CYP4B1 such as arylamines, for example 2-aminoanthracene (2-AA) [256]. Heterologous expression of rabbit CYP4B1 in mammalian cells demonstrated the cytotoxic potential of 4-ipomeanol and 2-aminoanthracene [257] and different bystander activity [121, 258]. Therefore, this chapter focuses on determining the cytotoxic potential and bystander activity of different known und unknown substrates for the hCYP4B1P+12 suicide gene.

3.2.1 Comparison of different substrates

After the seminal identification of 4-ipomeanol as a major lung toxin for livestock, a quest started for other pneumotoxins that can cause outbreaks of atypical pulmonary emphysema in livestock [259]. Perilla ketone (PK) is a chemical substance present in the essential oil of *Perilla frutescens*, a food plant in Asia known in the US as 'beef-steak plant' or 'perilla mint'. Animal testing demonstrated a strong pulmonary toxicity for laboratory animals and livestock in the 1970s and 80s [134, 260-262]. The natural terpenoid consists of a furan ring with a six-carbon side chain like 4-ipomeanol. The only structural difference between the two substances is that perilla ketone has a methyl group in C-5, whereas 4-ipomenaol harbors a hydroxyl group at this position. These findings led to the question whether CYP4B1 can metabolize perilla ketone.

Baer *et* Rettie have summarized known substrates of CYP4B1 within their review [128]. The incubation of cells expressing CYP4B1 isoforms and analyzing by flow cytometry after PI staining allows the discrimination between dead and viable cells but not the detection of metabolites bioactivated by the enzyme. Therefore, I tested the substances with known cytotoxicity or potential cytotoxicity such as 2-aminoanthracene (2-AA), 2-aminofluorene (2-AF) and 3-methylindole (3-MI), which were mentioned in the review, and perilla ketone. Also, structurally related compounds of perilla ketone were examined. 2-heptylfuran (HepF), 2-hexylfuran (HexF) and 2-pentylfuran (PenF) contain a furan ring and a carbon side chain in different lengths. 2-furyl pentyl ketone additionally contains a ketone at C-2 like 4-ipomeanol and perilla ketone.

Since P450 cytochromes are predominantly expressed in the liver, a hepatic cell line offers an appropriate cellular environment Therefore, HepG2 cells were transduced with the native rabbit isoform (r4B1) and the native human (h4B1) as well as two modified isoforms: the human proteins with serin-to-proline exchange at position 427 (h4B1P427), which renders the enzyme capable of metabolizing 4-ipomeanol, and the engineered suicide gene isoform with twelve additional amino acid exchanges (h4B1P+12) [144]. These isoforms were expressed off the SFFV U3 promoter in a SIN lentivrial vector and co-expressed with EGFP using an IRES site to identify transduced cells (Figure 33).

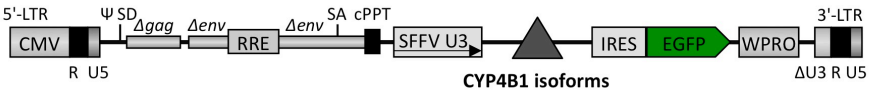

Figure 33: Schematic representation of the transgene cassette of the puc2CL6IEG vectors. The rabbit and human CYP4B1 as well as two human isoforms were placed before the IRES site.

Transduced HepG2 cells were seeded for the cytotoxicity assay as described before [144] and incubated with increasing concentration (2.9, 9, 29, 90, 290 and 900 µM) of the different substrates. After 24h and 48h cells were harvested, stained with PI for live/dead cell discrimination and then analyzed by flow cytometry (Figure 36).

Incubation with **4-ipomeanol** showed no toxicity to non-transduced or control vector transduced cells but led to a similar high toxicity in HepG2 cells expressing r4B1 and h4B1P+12 (Figure 34A). After 48h, at 290 µM 3.3 ± 0.3% r4B1 positive cells, 8.6 ± 1.1% h4B1P+12 positive cells and 32.1 ± 2.3% of h4B1P427 positive cells were still viable. In contrast, **perilla ketone** was much more toxic and showed similar toxicities as 4-ipomeanol in r4B1 and h4B1P+12 expressing cells at 290 µM, after 48h only 3.1 ± 0.5% and 3.6 ± 0.9% of the cells survived, but the toxicity in h4B1P427 positive HepG2 cells was dramatically increased (Figure 34B). After 48h incubation with 290 µM perilla ketone only 10.8 ± 1.9% cells were still alive. Another major difference could be observed after 48h incubation with 9 µM. 4-ipomeanol led to 61 ± 3.9% surviving cells expressing r4B1, whereas perilla ketone led to 36.2 ± 4.3% surviving cells. In summary, perilla ketone induced more cytotoxicity at low concentration in r4B1, h4B1P427 and h4B1P+12 expressing cells as 4-ipomeanol.

2-aminoantracene was already proposed as an alternative prodrug for the r4B1 suicide gene system [120, 121, 263]. Due to the strong autofluorescence of this substance, EGFP positive and EGFP negative cells could not be discriminated. 77.8 – 93.8 % of transduced HepG2 cells were positive for EGFP. 2-aminoanthracene showed no toxicity towards non-transduced, control-transduced and h4B1 transduced cells (Figure 34C). After 48h incubation with 290 µM, 8 ± 1.1% cells transduced with r4B1 were still viable, which is similar to cells incubated with 4-ipomeanol and perilla ketone, but 27 ± 1.8% of the cells transduced with h4B1P+12 survived. 24h incubation with 290 µM 2-aminoanthracene even led to 31.6 ± 4% surviving cells transduced with r4B1, whereas perilla ketone incubation led to 4.3 ± 0.8% surviving cells.

2-aminofluorene only showed unspecific toxicity at 290 µM after 24h and 48h (Figure 35 D), whereas 3-methylindole displayed unspecific toxicity at 90 µM but also specific toxicity at lower concentration (Figure 35 E).

A 24h incubation with **2-furyl pentyl ketone** led to toxicity in r4B1 expressing cells, at 290 µM only 45.6 ± 5.1% cells survived (Figure 35 F). After 48h, incubation with 290 µM decreased the amount of surviving cells to 33.1 ± 6.2%. Interestingly, 48h incubation led also to toxicity in h4B1P427 positive cells, even in a higher extent than in h4B1P+12 expressing cells, resulting in 69.6 ± 3.2% surviving h4B1P427 positive cells versus 81.5 ± 5.7% h4B1P+12 positive cells.

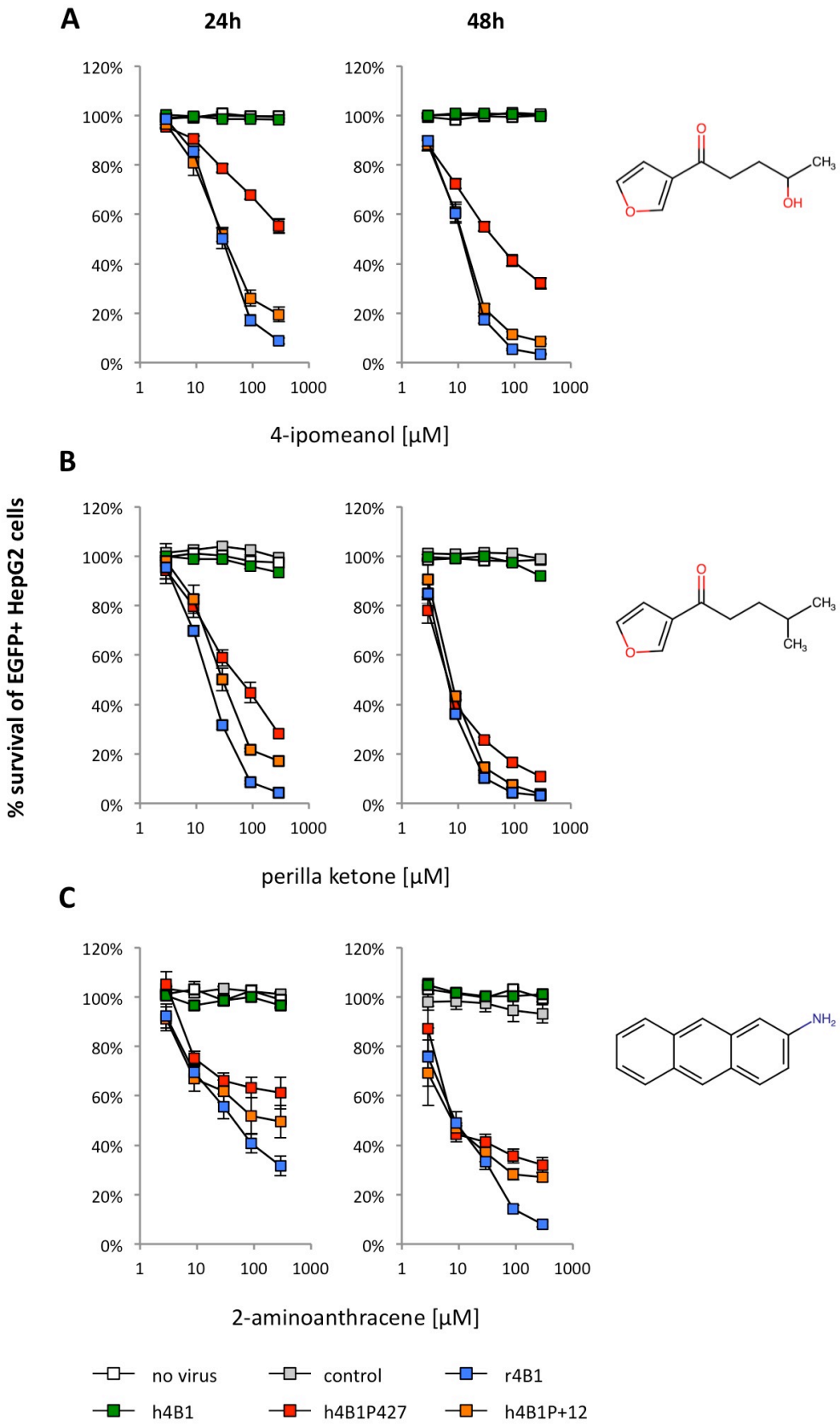

Figure 34: Survival assay of HepG2 cells expressing different isoforms of CYP4B1. Cells were challenged for 24h and 48h with increasing concentration of 4-IPO (**A**), PK (**B**), and 2AA (**C**). As control vector, puc2CL6IEGwo was used. Survival was assessed after 24h and 48h by PI staining and FACS analysis. The mean ±SEM of at least three different experiments is shown.

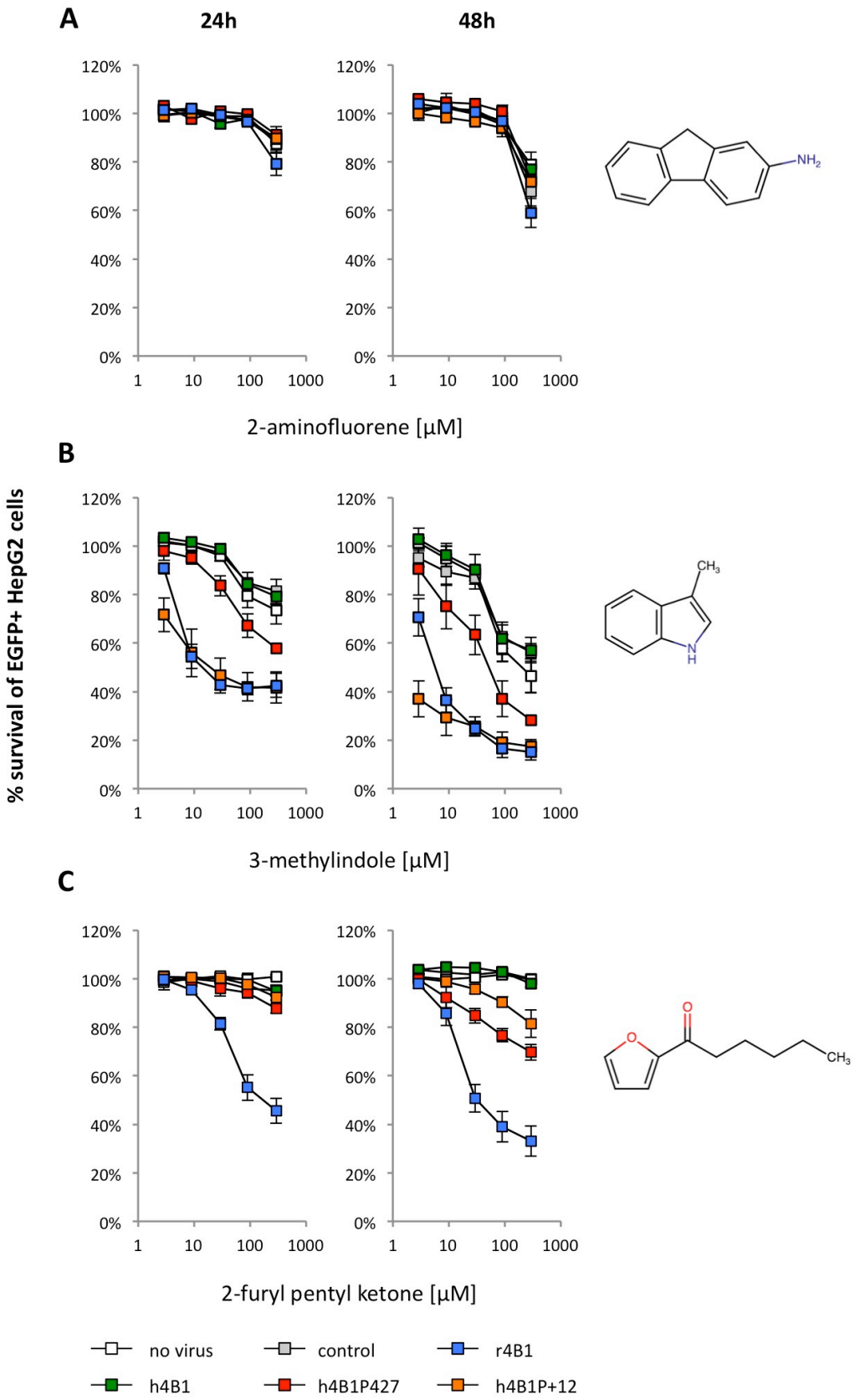

Figure 35: Survival assay of HepG2 cells expressing different isoforms of CYP4B1. Cells were challenged for 24h and 48h with increasing concentration of 2-AF (**A**), 3-MI (**B**), and FPK (**C**). As control vector, puc2CL6IEGwo was used. Survival was assessed after 24h and 48h by PI staining and FACS analysis. The mean ±SEM of at least three different experiments is shown.

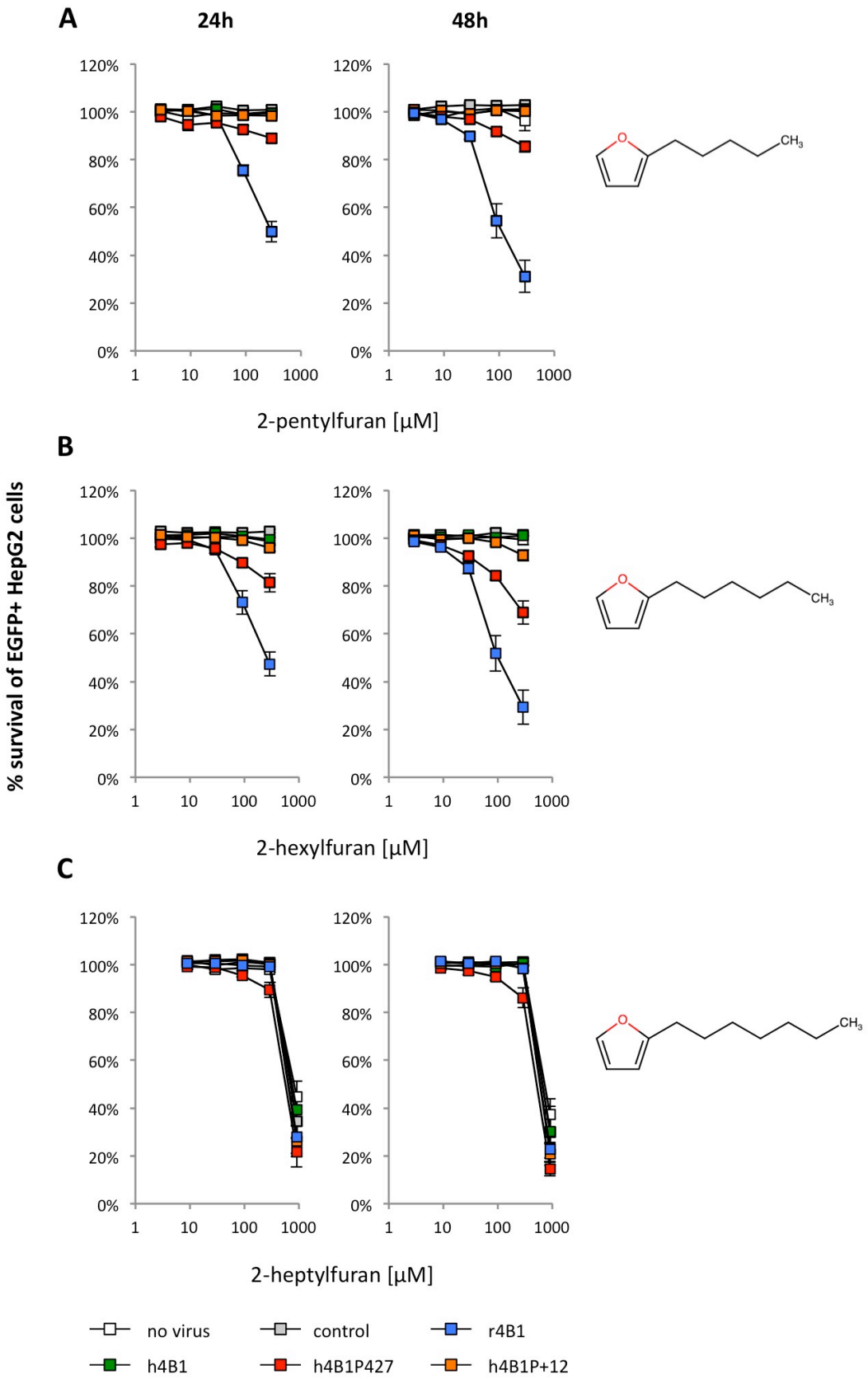

Figure 36: Survival assay of HepG2 cells expressing different isoforms of CYP4B1. Cells were challenged for 24h and 48h with increasing concentration of PenF (**A**), HexF (**B**), and HepF (**C**). As control vector, puc2CL6IEGwo was used. Survival was assessed after 24h and 48h by PI staining and FACS analysis. The mean ±SEM of at least three different experiments is shown.

This pattern continued with the furan ring harboring a carbon side chain in increasing length. **2-pentylfuran** has a five-carbon side chain. Toxicity was only induced in HepG2 cells expressing r4B1, 290 µM resulted in 49.9 ± 4.3% surviving cells after 24h and 31.1 ± 6.6% cells after 48h. After 48h, also h4B1P427 cells incubated with 290 µM displayed a weak toxicity, as much as 85.4 ± 2.2 cells were viable (Figure 36 G).

Adding another carbon to the side chain weakly increased the toxicity towards r4B1. Incubation with 290 µM **2-hexylfuran** resulted in 47.4 ± 5% viable cells after 24h and 29.3 ± 7.1% after 48. But it intensified the toxicity towards h4B1P427. Even after 24h, incubation with 290 µM resulted in 81.4 ± 3.8% viable cells and after 48h in 68.8 ± 4.8% (Figure 36 H). Both 2-pentylfuran and 2-hexylfuran did not affect non-transduced cells or cells transduced with a control vector, h4B1, or h4B1P+12.

Surprisingly, adding one more carbon to the side chain did not enhance the specific toxicity but rather diminished it almost at all. Unspecific toxicity could be observed upon incubation with 900 µM **2-heptylfuran** independently from the duration. Incubation with 290 µM led to a weak specific toxicity after 48h in cells expressing r4B1 resulting in 86 ± 2.9% viable cells.

3.2.2 Bystander activity

Of all tested substrates above, only perilla ketone and 2-aminoanthracene induced a strong and specific toxicity, which is a prerequisite for considerations of using it as an alternative prodrug. Bystander activity has been reported for 2-aminoanthracene but not 4-ipomeanol [121, 258]. For perilla ketone, it was unknown. Since the cells, which will be transduced with the suicide gene, are T-cells, a bystander activity and unspecific killing of cells in the bloodstream is not desirable. To evaluate the potential bystander activity of perilla ketone, the cDNAs for CYP4B1 isoforms r4B1, h4B1P427 and h4B1P+12, which displayed toxicity upon incubation with the substance, were cloned into the puc2CL6IPwo vector harboring a resistance gene against puromycin instead of EGFP behind the IRES site (Figure 37).

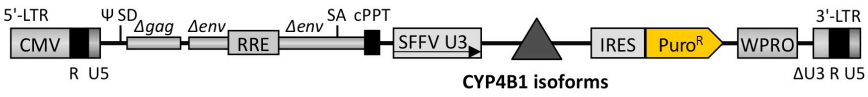

Figure 37: Schematic representation of the transgene cassette of the puc2CL6IPwo vector. The cDNA of r4B1, h4B1P427 and h4B1P+12 were cut out of the corresponding puc2CL6IEGwo vectors and cloned into puc2CL6Ipwo using the same restriction enzymes.

HepG2 cells were stably transduced with the different vectors and then selected using puromycin. Transduced and non-transduced cells were seeded in various ratios. After 24h, 90 µM perilla ketone, 4-ipomeanol and 2-aminoanthracene were applied. 24h and 48h later, cells were harvested, stained with PI and analyzed by flow cytometry (Figure 38).

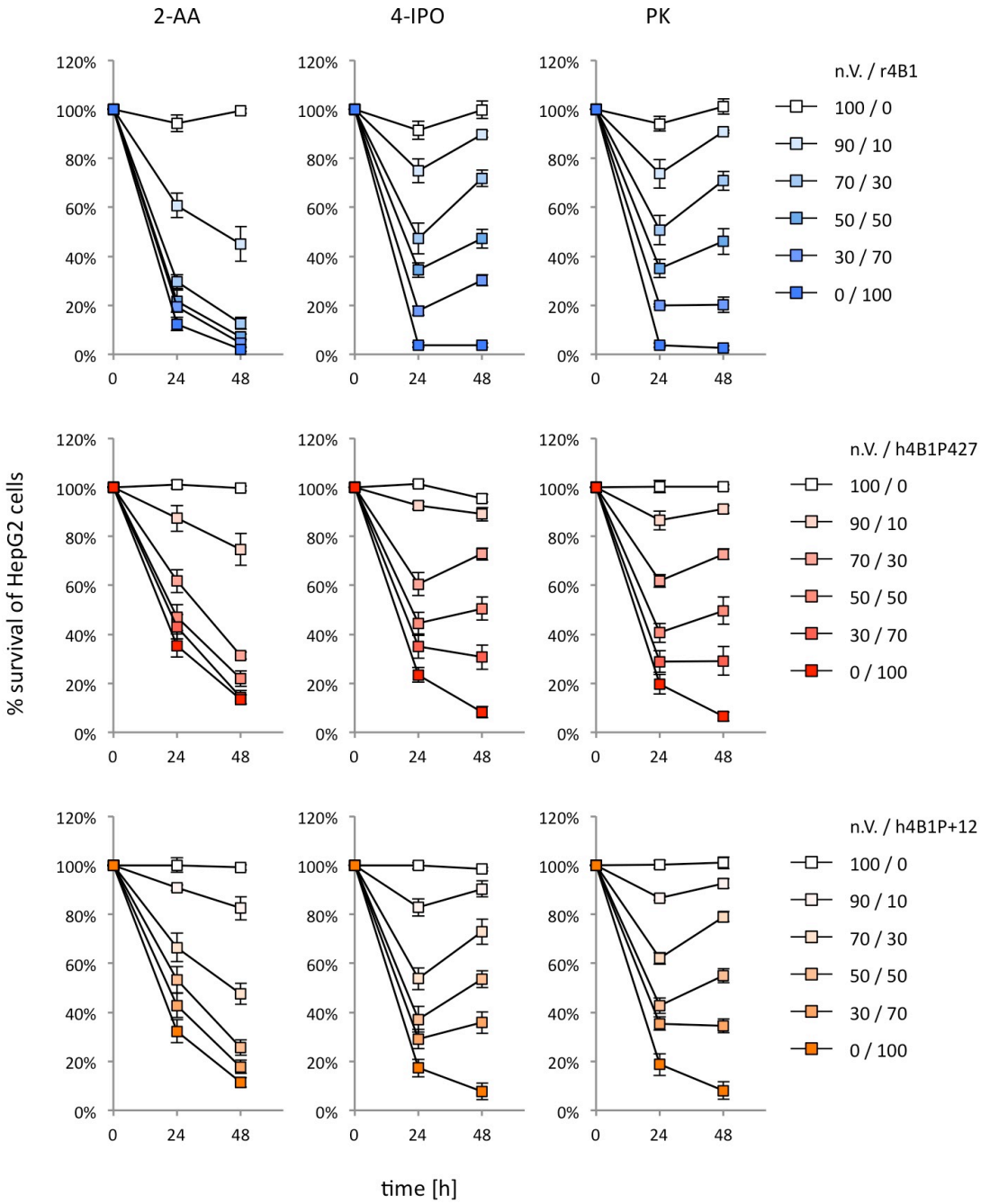

Figure 38: Bystander killing in HepG2 cells. HepG2 cells transduced with LV vectors carrying the cDNA for r4B1, h4B1P427 and h4B1P+12 were incubated with 90 µM 2-AA, 4-IPO and PK in different ratios with non-transduced (n.V.) cells. After 24h and 48h cells were harvested and analyzed by FACS using PI staining. The mean ±SEM of at least three different experiments is shown.

Incubation with 90 µM 2-aminoanthracene led to no toxicity in non-transduced cells. However, when 10% r4B1 expressing cells were added, only 60.8 ± 5% cells were viable after 24h. Increasing the number of r4B1 positive cells dramatically decreased the number of viable cells in the mixed cultures. When 30% r4B1 expressing cells were incubated for 24h, only 29.7 ± 2.9% of the cells survived, but when the number of r4B1 positive cells increased to 100%, the survival only decreased to 12.4 ± 2.8%. After 48h, the survival in all cell ratios decreased further. Similar observed was made when 2-aminoanthrace was incubated with cells that expressed h4B1P427 or h4B1P+12. Importantly, the survival data did not reflect the cell ratio set up condition for this assay, therefore non-transduced cells are killed by a bystander activity.

In contrast, incubation of cells expressing the three CYP4B1 isoforms with 4-ipomeanol did not induce any bystander activity. After 48h, non-transduced cells proliferated to such an extent, that the survival in these cultures actually increased in mixed populations containing ≥50% non-transduced cells. For example, when 10% r4B1 expressing cells are mixed with 90% non-transduced cells, the survival increased from 74.9 ± 4.8% after 24h to 89.8 ± 1.3% after 48h. A similar pattern is observed with h4B1P427 and h4B1P+12 expressing cells. Incubation of the transduced cells with perilla ketone led to the exact same effect.

In conclusion, 2-aminoanthracene leads to a strong bystander activity as reported [121, 258], whereas 4-ipomeanol and perilla ketone have none at all.In contrast to perilla ketone, 2-aminoanthracene not only shows bystander activity, but also is carcinogenic. These two features make this compound not favorable as prodrug.

3.2.3 Perilla ketone toxicity in primary T-cells

As shown in the previous chapters, perilla ketone has a toxicity profile similar to 4-ipomeanol and thus is a suitable alternative prodrug for the h4B1P+12 suicide gene. Since the target cells are primary T-cells, the effect of perilla ketone exposure in T-cells was evaluated. Primary T-cells were transduced with the expression vector p2CL21ΔNGFRT2Ah4B1P+12wo established in chapter 3.1. The MPSV promoter drives the cDNA of ΔNGFR and h4B1P+12 linked by T2A, ΔNGFR is placed first. The control vector contained the native, inactive h4B1 instead of h4B1P+12. T-cells were selected using the MACS selection system and incubated with increasing concentrations of 4-ipomeanol and perilla ketone. After 24h and 48h, cells were harvested, stained with PI and analyzed using flow cytometry (Figure 39).

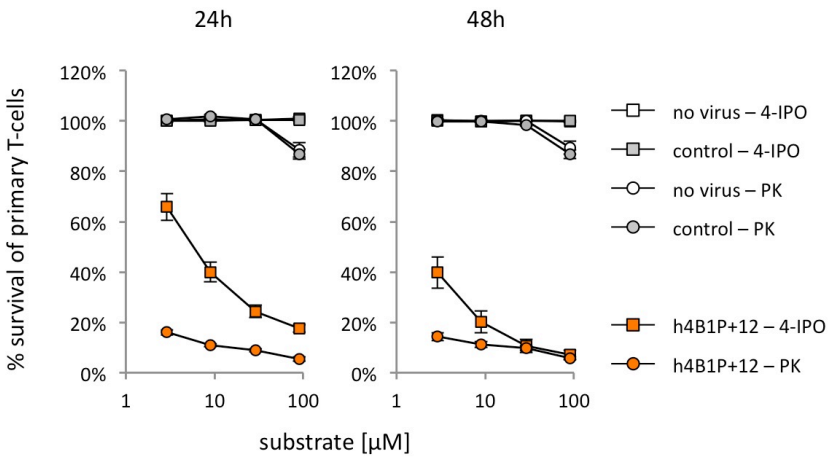

Figure 39: Cytotoxicity assay of primary T-cells. Primary T-cells expressed a transgene cassette containing h4B1P+12 and ΔNGFR. The inactive isoform h4B1 replaced h4B1P+12 in the control vector. Transduced T-cells were selected using MACS microbeads. Transduced T-cells were challenged with increasing concentration of 4-IPO and PK and survival was assessed by flow cytometry after PI staining. For each construct, mean ± SEM is shown from at least three experiments.

Non-transduced and control-transduced T-cells displayed no toxicity towards 4-ipomeanol regardless of the concentration or the incubation period. In contrast, perilla ketone induced a slight toxicity at 90 µM, leading to 89.5 ± 2.6% viable non-transduced cells after 24h and 88.6 ± 2.9% after 48h. The length of the incubation with perilla ketone did not change the toxicity observed in control-transduced cells, 86.9 ± 1.8% after 24h and 86.9 ± 2% after 48h. Incubation of h4B1P+12 with 2.9 µM 4-ipomeanol resulted in 65.8 ± 5.3% surviving cells after 24h and 39.8 ± 6.3% after 48h. Increasing the concentration to 90 µM decreased the survival rate to 17.5 ± 1.5% (24h) and 7.1 ± 0.13% (48h). Surprisingly, only 16 ± 1.1% h4B1P+12 expressing cells survived the incubation with 2.9 µM perilla ketone for 24h. 90 µM perilla ketone led to 5.4 ± 0.8% viable cells after 24h.

Thus, in order to find out at which concentration of perilla ketone toxicity begins, MACS selected T-cells were incubated with limiting concentrations of perilla ketone and again analyzed by flow cytometry for survival (Figure 40). Also here, incubation with 90 µM perilla ketone induced mild unspecific toxicity. At 0.09 µM perilla ketone a weak toxicity begun with 82 ± 1.2% viable cells after 24h, but the induced toxicity rapidly intensified as incubation with 0.9 µM resulted in only 13.2 ± 0.5% surviving cells after 24h. Increasing the concentration further only gradually increased the toxicity. The same pattern was observed after 48h.

In summary, perilla ketone induced a weak unspecific toxicity at high concentrations, but more importantly, it was clearly more toxic for T-cells than 4-ipomeanol.

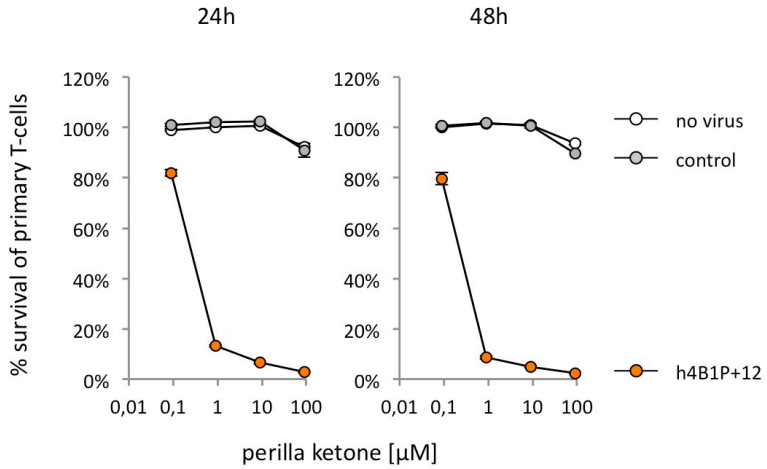

Figure 40: Cytotoxicity assay of primary T-cells. Primary T-cells expressing a transgene cassette with h4B1P+12 and ΔNGFR. The inactive isoform h4B1 replaced h4B1P+12 in the control vector. Transduced T-cells were selected using MACS mircobeads. T-cells were challenged with increasing concentration of PK, survival was assessed by flow cytometry and PI staining. For each construct, mean ± SEM is shown from at least three experiments.

3.2.4 Apoptosis in primary T-cells

After demonstrating the absence of bystander activity and the strong potency of perilla ketone, the kinetic in inducing apoptosis in primary T-cells was evaluated. Staining with PI effectively marks dead cells, but not cells undergoing apoptosis; thus apoptotic and dead cells were stained using an annexin V-Fitc antibody and PI combined. Primary T-cells were transduced with p2CL21ΔNGFRT2Ah4B1P+12wo. Three days later, T-cells were selected by MACS and then seeded for a cytotoxicity assay. The next day, 9 and 90 µM perilla ketone and 29, 90 and 290 µM 4-ipomeanol were added. After 1, 2, 4 and 10h cells incubated with perilla ketone were harvested and stained for apoptosis. Cells incubated with 4-ipomeanol were harvested after 2, 4, 10 and 24h. Analysis was performed using flow cytometry (Figure 41).

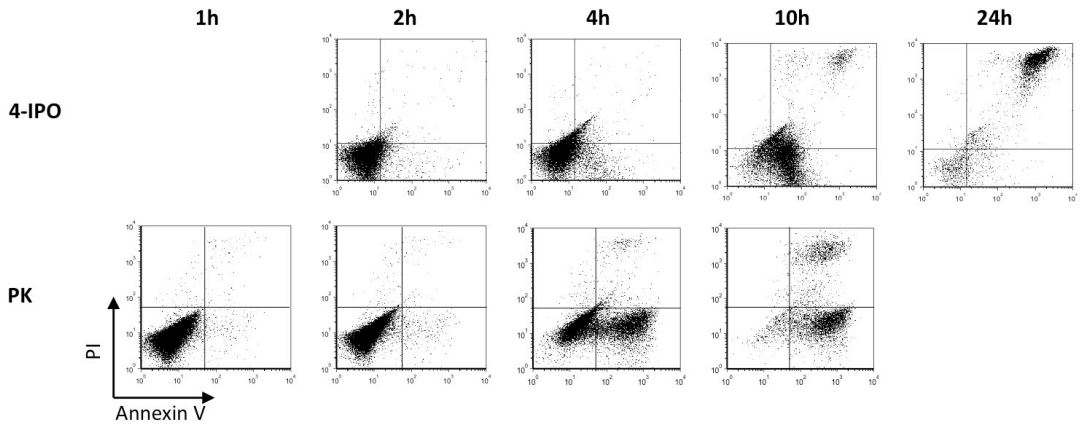

Figure 41: FACS analysis of apoptosis induced in primary T-cells transduced with p2CL21ΔNGFRT2Ah4B1P+12wo upon prodrug incubation. T-cells were harvested at the indicated time points after incubation with 290 µM 4-IPO or 90 µM PK and stained with Annexin V-Fitc and PI. Representative dot plots from at least three experiments are shown.

Neither 4-ipomeanol nor perilla ketone induced apoptosis in T-cells transduced with a control vector carrying the inactive h4B1 cDNA in these short time periods (Figure 42). Incubation of h4B1P+12 expressing T-cells with 29, 90 and 290 µM 4-ipomeanol led to 93 ± 2.7%, 87.4 ± 2.9% and 82.3 ± 1.8% viable cells after 4h; after 10h it decreased to 24.5 ± 4.3%, 20.4 ± 4.9% 18.3 ± 2.4% viable cells. 24h after the addition of 4-ipomeanol, only 9.4 ± 0.4%, 5.8 ± 0.9% and 4 ± 0.6% cells were still viable. In contrast, cellular toxicity was already detectable after incubation of h4B1P+12 positive T-cells with 9 and 90 µM perilla ketone after 2h resulting in 88.7 ± 2.1% and 89.7 ± 1% non-apoptotic cells. After 4h, non-apoptotic cells decreased to 60.2 ± 5.5% and 59.3 ± 7.7% and after 10h to 5.5 ± 1.9% and 4.5 ± 1.5%. The results revealed clearly the induction of apoptosis after 4h incubation with perilla ketone. In conclusion, perilla ketone induces apoptosis in T-cells almost twice as fast as 4-ipomeanol at the same concentration.

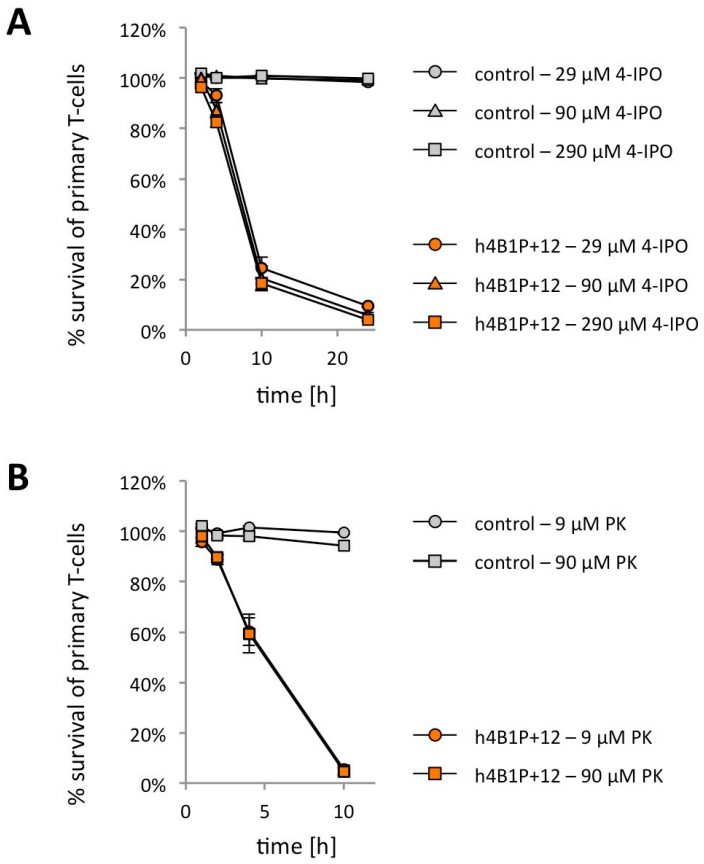

Figure 42: Apoptosis of primary T-cells transduced with p2CL21ΔNGFRT2Ah4B1P+12wo. (A) Transduced T-cells were incubated with 29, 90 and 290 µM 4-IPO and collected after 2, 4, 10 and 24h. **(B)** Transduced T-cells were challenged with 9 and 90 µM PK and collected after 1, 2, 4 and 10h. Cells were stained with annexin V and PI. Control cells express this transgene cassette with h4B1 instead of h4B1P+12. The mean ±SEM of at least three different experiments is shown.

3.3 T-cell stimulation

For T-cell experiments, primary peripheral blood lymphocytes were isolated using Ficoll and stimulated with immobilized CD3/CD28 antibodies and 100 U/ml IL-2 supplement in the medium before they were transduced. After transduction, IL-2 was always present in the medium except where otherwise indicated. Such a strong stimulation with the antibodies and retroviral transduction can inverse the CD4/CD8 ratio [264], enrich memory T-cells [265], change the TCR repertoire [266] and reduce alloreactivity [267, 268]. In this chapter, the influence of continuous stimulation on the 4-ipomeanol induced cytotoxicity is analyzed. Primary T-cells were isolated, stimulated as described and transduced with p2CL21ΔNGFRT2Ah4B1P+12wo. In control vectors, EGFP replaced h4B1P+12. After MACS selection, T-cells were divided in three groups: T-cells in group A were stimulated over night using CD3/CD28 coating, T-cells in group B were permanently stimulated by antibody coating after MACS selection and T-cells in group C were not stimulated by CD3/CD28 coating. All groups received medium supplemented with IL-2 and were incubated with increasing concentrations of 4-ipomeanol. After 8, 24 and 48h T-cells were harvested and analyzed by flow cytometry after PI staining (Figure 43).

4-ipomeanol displayed no toxicity towards non-transduced and control-transduced cells at all time points and concentrations. After 8h, 81.5 ± 4.3% of h4B1P+12 positive T-cells in group C were viable, whereas no toxicity was detectable in T-cells of group A and B. After 24h, the difference between the 3 stimulations was clearly visible. Incubation with 290 µM 4-ipomeanol led to 19.8 ± 0.6% viable T-cells of group C, in sharp contrast, 46,1 ± 4.1% T-cells of group A and 57.7 ± 3.1% T-cells of group B were viable. After 48h incubation, the differences between the three groups vanished. Somehow, the additional stimulation affects the cytotoxicity induced by 4-ipomeanol.

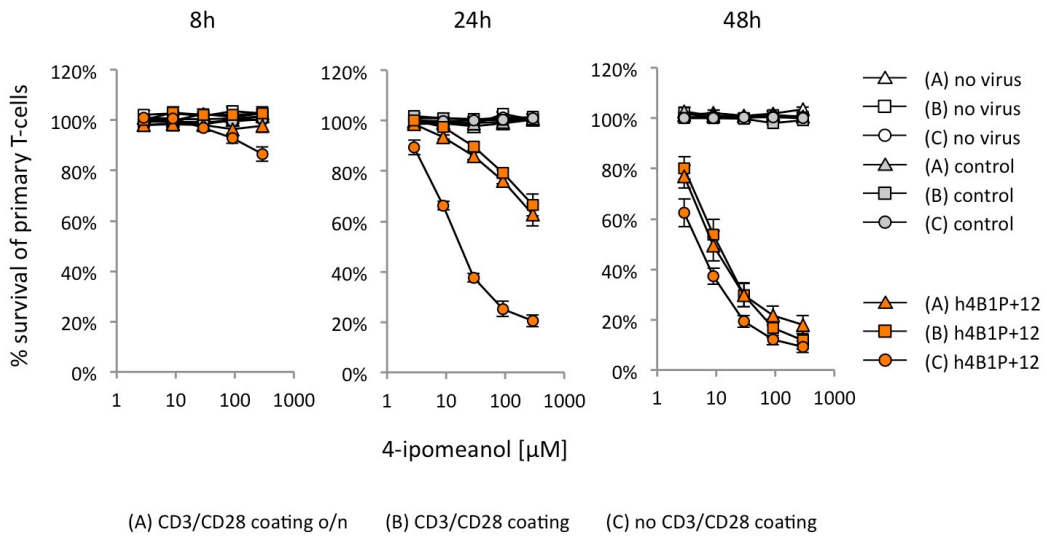

Figure 43: Cytotoxicity assay of primary T-cells. Primary T-cells were transduced with CYP4B1P+12 co-expressed with ΔNGFR using T2A. EGFP replaced CYP4B1P+12 in the control vector. Transduced T-cells were selected using MACS mircobeads. T-cells were challenged with increasing concentration of 4-IPO, survival was assessed by flow cytometry and PI staining. For each construct, mean ± SEM is shown from at least three experiments.

3.4 CAR mediated T-cell cytotoxicity

Another important concept of adoptive cell therapies are strategies using chimeric antigen receptors. To create a CAR, the variable light and heavy chain (scFv) of a monoclonal antibody is fused to a transmembrane domain and signaling domains of T-cell receptors. After binding to the specific antigen, signaling via the ζ-chain and the costimulatory domain (in second generation) takes place and cytolysis is induced. For this approach, candidate antigens expressed on ALL cells are CD19 and CD22 and on AML cells CD33. In order to establish that the h4B1P+12 suicide gene can be utilized to control CAR expressing T-cells, already established CARs against CD22, CD19 and CD33 were co-expressed with h4B1P+12 in a single vector as depicted in Figure 44. Previous experiments have shown that the functionality of a CAR is not influenced by its position relative to the T2A site (data not shown), the CAR is placed 5' of the T2A site like the selection marker in an equivalent constructed SIN lentiviral expression vector. Both transgenes are connected by a T2A site and driven by MPSV promoter.

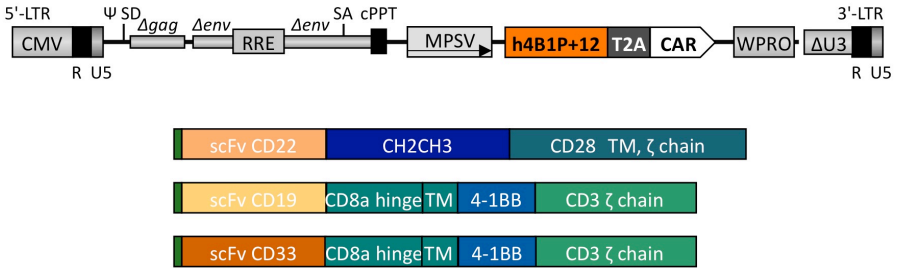

Figure 44: Schematic representation of the lentiviral vector for co-expressing h4B1P+12 and a CAR. The MPSV promoter drives the expression cassette containing h4B1P+12, T2A and a CAR. The different CARs are depicted below. CD19-CAR and CD33-CAR have the hinge region and transmembrane domain of CD8a, the costimulatory domain of 4-1BB and the signaling domain of the CD3 chain. The CD22-CAR has the Ch2CH3 region as hinge region and the transmembrane domain and chain of CD28.

Primary T-cells were transduced and MACS selected 3 days later using the $F(ab')_2$ fragment specific antibody conjugated with biotin-SP. By employing MACS anti-biotin microbeads, transduced cells were enriched from 39.4 to 98.8% (data not shown). The next day, T-cells were incubated with EGFP positive cells from the ALL cell line REH cells and the AML cell line Molm14 in different effector-to-target-ratios. Cell cultures were harvested the next day, stained with PI and analyzed by flow cytometry. T-cells transduced with CARs recognizing CD19 or CD22 effectively lysed target REH cells (Figure 45 A). At a 0.03 effector : 1 target cell ratio, CD19-CAR T-cells mediated lysis of 79.9 ± 2.2% REH-cells and at 0.01:1 even of 49.3 ± 5.3% whereas CD22-CAR T-cells lysed 57 ± 2.7% at a 0.03:1 ratio and 27.1 ± 4.2% at 0.01:1. Hence, CD19-CAR T-cells induced lysis at low effector to target cell ratios more efficient than CD22-CAR T-cells. T-cells transduced with a CD33-CAR served as control since REH cells do not express the myeloid lineage-specific CD33. High effector to target cell ratios like 3:1 and 1:1

resulted in cell lysis of REH cells independently from the expressed CAR (62.6 ± 3.5% and 35 ± 4.3%). At lower ratios this effect vanished rapidly.

The AML cell line Molm14 proved to be more sensitive against T-cells than REH cells (Figure 45 B). Molm14 cells were incubated with T-cells expressing a h4B1P+12 plus CD33-CAR cassette or co-expressing a CD19-CAR with h4B1P+12 as a control. Both T-cell populations induced cell lysis in 16.3 ± 2.3% to 30.9 ± 6.6% at the low cell ratios 0.03:1 and 0.01:1. Lysis mediated by the CD33-CAR T-cells rose rapidly with increasing effector to target cell ratios. At the ratio 0.1:1, 59.4 ± 3.8% of Molm14 cells were killed, at 0.3:1 it was 88.4 ± 0.6%. Control T-cells killed only 33.6 ± 3.5% Molm14 cells at 0.1:1 and 37.8 ± 2.8% at 0.3:1. At high effector to target cell ratio, both T-cell populations unspecifically induced efficiently cell lysis.

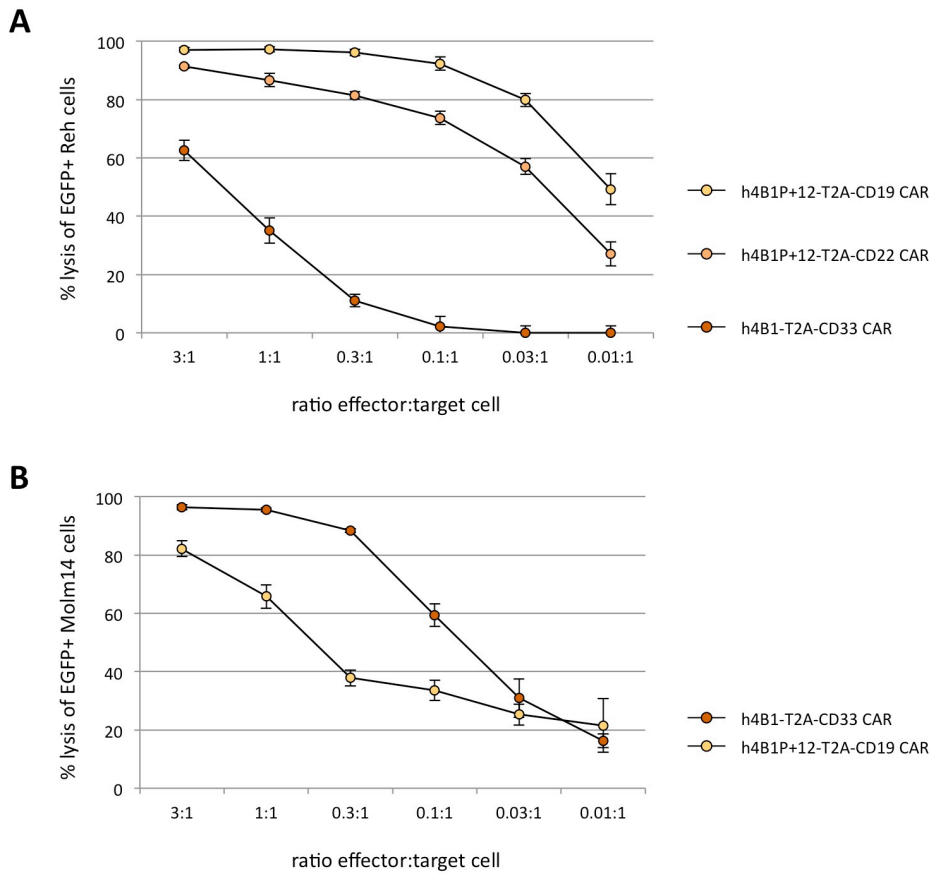

Figure 45: Specific cytotoxicity of CAR expressing T-cells. A. Cell lysis induced in REH cells. EGFP positive REH cells were incubated in different ratios with primary T-cells transduced with a transgene cassette containing either a CD19-CAR or a CD22 with h4B1P+12. As controls, REH cells were incubated with T-cells co-expressing a CD33-CAR. Survival of REH-cells was assessed by FACS analysis after PI staining. For each construct, mean ± SEM is shown from at least three experiments. **B. Cell lysis induced in Molm14 cells.** EGFP positive Molm14 cells were incubated in different ratios with primary T-cells transduced with a transgene cassette containing a CD33-CAR and h4B1P+12. As controls, Molm14 cells were incubated with T-cells co-expressing a CD19-CAR. Survival of Molm14 cells was assessed by FACS analysis after PI staining. For each construct, mean ± SEM is shown from at least three experiments.

In order to ensure that h4BP+12 effectively functions co-expressed with a CAR, part of the transduced T-cell culture, that were used for the CAR-mediated cytotoxicity assays, were challenged with increasing concentrations of perilla ketone for 24h (Figure 46). Control cells were transduced with a CD33-CAR vector construct that co-expressed the inactive h4B1 isoform. As observed before, 90 µM perilla ketone induced low toxicity in control cells. T-cells expressing h4B1P+12 demonstrated potent toxicity upon incubation with even low concentrations of perilla ketone. The different survival rate of T-cells challenged with 0.9 µM perilla ketone, from 8.6 ± 1.7% viable cells expressing CD19 and 46.2 ± 7.1% viable cells expressing CD33, may reflect the heterogeneous enrichment using the biotin conjugated $F(ab')_2$ antibody and anti-biotin microbeads. In conclusion, the h4B1P+12 suicide gene functions with all three different CAR constructs expressed in a single vector.

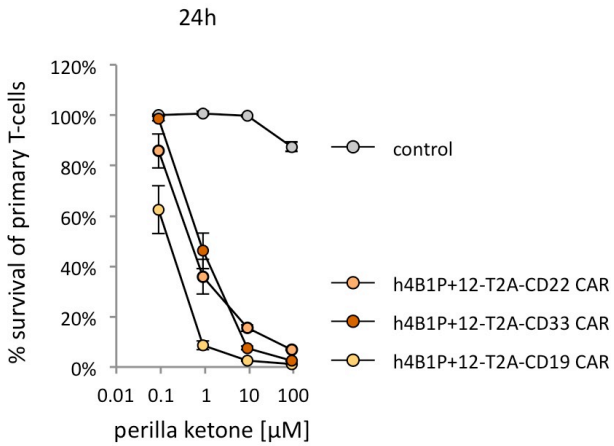

Figure 46: Cytotoxicity assay of primary T-cells. Primary T-cells were transduced with CARs co-expressed with h4B1P+12 using T2A. The inactive isoform h4B1 replaced CYP4B1P+12 in the control vector. Transduced T-cells were selected using MACS mircobeads. T-cells were challenged with increasing concentration of PK, survival was assessed by flow cytometry and PI staining. For each construct, mean ± SEM is shown from at least three experiments.

4. Discussion

In the context of hematological malignancies, the importance of infused donor lymphocytes along with allo-HSCT as adoptive immunotherapy has been clearly demonstrated [80]. Alloreactive donor-derived T-cells recognize HLA-antigens or minor histocompatibility molecules on the surface of cancer cells [269], which can result in a graft-versus-leukemia effect and thereby induce complete remission, on the other side the infused T-cells can also lead to a life-threatening graft-versus-host-disease with the alloreactive T-cells recognizing predominantly liver, intestine and skin cells [270]. Precautions to prevent a GvHD include less intense preconditioning regimens, administration of immunosuppressive drugs, and *ex vivo* T-cell depletion that also minimize the GvL effect. However, impairing the activity of the donor T-cells in the graft is also associated with higher relapse rate [97]. Here, another approach to control GvHD is to equip the T-cells prior to infusion with a suicide gene [99, 100]. A suicide gene allows the specific elimination of transduced T-cells after a non-toxic prodrug is applied. The suicide gene should fulfill following criteria:

1. The suicide gene should be of human origin to prevent immunogenicity.
2. For a fast elimination, the apoptosis-inducing mechanism should not depend on cell proliferation.
3. The prodrug should be non-toxic and induce a specific elimination.
4. Dependent on the product application the prodrug should not induce a bystander effect.

The suicide genes most frequently used in clinical studies are the HSVtk and iCasp9. Although the HSVtk induced immunogenicity in patients due to the fact that it is a viral protein [104, 105], has a bystander effect, a cell proliferation dependent elimination [271], and a prodrug that is used as antiviral drug after CMV infections, it is currently still used in a phase III clinical trial to treat patients with high risk acute leukemia with DLI (Gov. Trial: NCT00914628). Despite its disadvantages, it is the suicide gene with a broad experience [272] and clearly can achieve clinical benefit [273]. The suicide gene iCasp9 is currently also used in clinical I/II studies (e.g. NCT01822652), and meets most of the requirements stated above. The human cDNA of caspase 9, a proapoptotic protein, is fused to the CID-binding dimerizing domain of the FK506-binding protein 12, thus generating a protein that dimerizes upon the administration of the synthetic molecule AP1903 and thereby induces apoptosis. Hence, the elimination of suicide gene positive cells is independent of cell proliferation, fast by directly mediating apoptosis and specific for transduced cells since the molecule AP1903 is inert in human and not

therapeutically used. There are only a few disadvantages known so far: the prodrug is synthetic and can mediate efficient but incomplete elimination of cells [274].

The suicide gene generated in our lab is the P450 cytochrome CYP4B1 with 13 changed amino acid residues rendering the inactive endogenous protein capable of efficiently processing the furan prodrug to the highly active isoform h4B1P+12 [144]. By changing amino acid residues, which are conserved in other members of the same human P450 cytochrome family, the likelihood to cause an immune reaction appears to be low. The activated prodrug forms highly DNA alkylating metabolites and induces apoptosis independently from cell proliferation [128]. The corresponding prodrug 4-ipomeanol has been extensively tested in human clinical phase I/II trials more than 15 years ago, when 4-ipomenaol was thought to be an ideal drug against lung cancer. Although serum concentration of up to 100 µM was achieved with administrations of 1290 mg/m^2 and patients received 1032 mg/m^2 every three weeks, only weak hepatotoxicity occurred at the highest doses in some patients [140, 141]. Also the fact, that no bystander activity is reported [258], makes h4B1P+12 highly suitable for DLI application.

For the infusion of allogeneic T-cells, all cells must express the suicide gene in order to effectively control the GvHD in the patient. Therefore, transduced cells have to be enriched and the gene(s) facilitating the selection should be expressed with the suicide gene in a single vector. In addition, the clinical vector expressing the suicide gene and the selection gene should be safe and stable, and inflict minimal toxicity towards the cell and the genome. Due to the stable integration and cargo size, retroviral vectors are often used. In contrast to γ-retroviral vectors, lentiviral vectors can integrate into non-dividing cells and carry longer DNA sequences [168]. Although integration of retroviral vectors was thought to induce insertional mutagenesis [248, 275], long-term follow up of patients who received transduced T-cells reveal that lentiviral integration sites are only partial random and do not favor proto-oncogenes or tumor suppression genes [276, 277]. Also, less than five integrations per cell are considered safe and due to limited cell division, mature T-cells do not get transformed [278, 279]. Therefore, a lentiviral expression system was used here.

Establishing a clinical applicable lentiviral expression vector

The P450 cytochrome CYP4B1 is membrane bound within the endoplasmic reticulum and requires electrons transferred from the NADPH as reductase [128]. The selection markers ΔCD34 and ΔNGFR on the other hand are surface molecules. Therefore a suicide-sort gene as designed for CD34/HSVtk [280] or CD20/CD34 [124] is not possible for h4B1P+12. In this thesis, different lentiviral systems were tested to co-express two distinctly located transgenes in cells. For the initial testing, a dual promoter system consisting of the human PGK promoter and the viral SFFV U3 promoter were

used in combination with two fluorescent proteins. Even though the successful expression of two transgenes had been reported for γ-retroviral vectors [281] and lentiviral vectors [282], in this setting, I, similar to others, was not able to reproduce these results here [241, 242, 283]. Analyzing the MFI of dTOMATO and EGFP driven by hPGK and SFFV, strong promoter interferences were observed generating cell populations that are positive for only one fluorescent protein, although all transduced cells are genetically equipped with both EGFP and dTOMATO. Hence, the dual promoter system tested here is not suitable for co-expressing the selection marker and the suicide gene in T-cells.

IRES sites are internal ribosomal entry sites that initiate cap independent translation, especially when located in the middle of an mRNA [187]. Using the IRES site of encephelomyelocarditis virus, the most commonly used IRES site in hematopoietic cells, the translation of the downstream cDNA starts at the 11th ATG. In naturally infected cells, the IRES site initiates the translation at the beginning of the transcribed polyprotein. By employing the IRES site, both EGFP and dTOMATO are expressed in transduced cells, although the downstream cDNA always to a much lower degree. This effect has also been observed by others [284, 285].

A 2A site is a virus-derived CHYSEL acting element that mediates co-translational cleavage; 'uncleaved' protein is not subsequently cleaved [188]. The cleavage actually is not a proteolysis but a translational effect [286]. Donnelly *et al.* tested the cleavage efficacy of various 2A mutants derived from FMDV [189]. By adding 14 aa to the N-terminus, the cleavage activity was increased from 90% to 99% (construct #33). She also performed a database search for the consensus motif and found 2A-like sequences in other picornaviruses and 'picornavirus-like' insect viruses, one of them a 2A site from the insect virus *Thosea assigna* with a cleavage efficacy of 99%. In this thesis, a human codon usage optimized cDNA of FMDV construct #33, named 14+2A, and the 2A site of *Thosea assigna* (T2A) were tested in a lentiviral vector system. Both 2A sites facilitated a strong and comparable expression of dTOMATO and EGFP. Western Blot analysis revealed some 'uncleaved' EGFP and dTOMATO fusion proteins. Since there was no difference in performances between 14+2A and T2A, T2A was chosen as best co-expression strategy due to its smaller size and hence the lower likelihood to cause immunogenicity by adding virus-derived 18 aa to the first transgene. Western Blot analysis demonstrated also that the amount of 'uncleaved' protein with the T2A site can depend on the transgenes. Co-expression of the selection marker ΔNGFR and EGFP resulted in low levels of 'uncleaved' proteins, which was even lower when ΔNGFR was placed before the T2A site. In contrast, co-expression of ΔCD34 and EGFP led to a considerably high amount of 'uncleaved' protein. Replacing EGFP by h4B1P+12 enhanced the cleavage activity further. These results clearly demonstrated the influence of the transgenes co-expressed via T2A on its cleaving efficacy.

Selection markers are an important genetic tool to identify and enrich gene-modified cells within gene therapy settings [287, 288]. For clinical usage they should meet specific requirements [255]:

1) In order to prevent an immune reaction, the marker gene should be of human origin.
2) The expression should only occur on engineered cells and not interfere with the physiological functions of the target cell.
3) The selection process should not require a long-term *ex vivo* cultivation.
4) The selection process should be GMP compatible.

In my thesis, I used C-terminally truncated versions of the surface molecules CD34 and NGFR [255, 289] and optimized the mRNA for human codon usage. CD34 is glycosylated type I transmembrane protein with an extracellular domain involved in cell adhesion and an intracellular domain carrying signaling domains [290]. The recognition sites for the protein kinase C within the intracellular domain are required for the function of CD34 for differentiation and adhesion of hematopoietic progenitor cells [291-293]. CD34 is expressed on hematopoietic stem and progenitor cells, cells of B and myeloid lineage, endothelial cells, and some embryonic fibroblasts [290, 294, 295], but not on lymphocytes and antigen-presenting cells. A major advantage of using CD34 as selection marker for transduced cells is the fact that GMP compatible enrichment of CD34 positive cells are commercially available [296].

The low affinity nerve growth factor receptor is also a type I transmembrane protein. The extracellular domain is comprised of four cysteine-rich domains presenting a conserved motif and a hinge region [297], whereas the intracellular domain contains a chopper and a death domain [298, 299]. The endogenous function of NGFR is the regulation of a range of neuronal functions such as cell proliferation, migration, axon guidance, and survival [300, 301]. NGFR is not expressed on T-lymphocytes and, equally important, GMP compatible selection products are available. Co-expressed with EGFP, both selection markers, ΔCD34 and ΔNGFR, were introduced into T-cells with lentiviral vectors, selected with MACS microbeads and then evaluated by FACS analysis. Both selection markers facilitated a strong enrichment of transduced cells while loosing only few transgene positive cells in the through flow. Surprisingly, when EGFP was replaced by h4B1P+12, survival assays with 4-ipomeanol showed less activity in ΔCD34 positive cells than in ΔNGFR positive cells. This might be due to the relative high amount of ‘uncleaved’ fusion protein as demonstrated by Western Blot analysis. In summary, ΔNGFR seems to be a more suitable selection marker for the h4B1P+12 suicide gene, although I could not detect functional differenced between ΔCD34 or ΔCD271, when placed 5’ or 3’ off the T2A site.

An important part of the transfer vector is the internal promoter, which drives the transgene expression cassette. To compare the strength of different promoters in the SIN lentiviral vector, they were cloned into the same lentiviral vector backbone driving an EGFP-NeoR fusion protein. The MFI of transduced primary T-cells was compared by flow cytometry. Here, viral, human and rodent promoters were tested. Promoters of human house keeping genes, UbiC, PGK, and EF1α, only facilitated weak EGFP expression compared to the viral promoters SFFV and MPSV. HLA-DR is an activation dependent protein in T-cells, therefore either the activation status of the T-cell was not high enough to induce the expression or the activity of the core HLA-DR promoter is relatively weak. Asct2 expression was shown to be upregulated by glutamine availability [302], probably by initial activation of the farnesoid X receptor (FXR) promoter, because the FXR/RXR dimer binds to the Asct2 promoter [303]. Although glutamine was added to media, expression of EGFP-NeoR was weak, however, it is unknown if increasing the glutamine concentration would lead an increased activation of the Asct2 promoter in primary T-cells. Several factors that control the promoter activity of Glut1 in mammalian cells were identified, including oncogenic transformation [304] and mitogen stimulation [305]. In a hematopoietic cell line, increased glucose transport was increased by IL-3 as mitogenic stimulus [305]. Also here, independent from the promoter length or origin of Glut1, EGFP expression was low in activated T-cells. Whether the expression is in general relatively low or the proliferation conditions of the T-cells were not optimal remains to be determined. Both promoter, Asct2 and Glut1, were supposed to be upregulated in activated T-cells (personal communication), however, the activation level of T-cells might not have been high enough.

Interestingly, adding the natural intron to the hEF1α promoter dramatically increased the EGFP expression but simultaneously drastically reduced the infectious titer by 90%. Splice sites and an appropriate intron can be beneficial when they are located in the 5'UTR of transgenic RNA [244, 245] by promoting mRNA export [244, 246] and translation [245], but inefficient splicing can also impair the protein expression. The MPSV U3 promoter was tested in two variants, one without an intron and one with the addition of a modified untranslated leader derived from the murine embryonal stem cell virus. The modification involves the deletion of the *gag* sequence and potential start codons but integration of an additional splice donor and splice acceptor sites [306]. When using EGFP as a transgene, there was no significant difference between MPSV and MPSV+intron with respect to the protein expression or the production of infectious particle. Schambach *et al.* also compared viral promoters with different modifications within the LTR and RNA transport elements and reported that the introduction of splice sites within the 5'UTR of mRNA and WPRE within the 3'UTR lead to the highest enhancement of transgene expression [307]. Given that SFFV had been shown to be associated with strong insertional mutagenesis [248-250] and efficient production of

infectious particles is of economical importance, the MPSV promoter without an intron was chosen as the promoter to further work with.

Pseudotyping of lentiviral vectors allows redirecting the tropism to a specific cell type. For any target cells, an envelope protein that mediates a gentle transduction is favorable. Therefore, I tested several envelopes for transduction of T-cells. The glycoprotein of the *Vesicular stomatitis virus* (VSV-G) facilitates effective transduction of a broad range of cells but is also associated with cellular cytotoxicity [200, 201]. The receptor of the envelope protein of the *gibbon ape leukemia virus*, Galv, is the phosphate transporter protein PRT1, which is highly expressed on T-cells which in turn mediates optimal cell entry into lymphocytes [211, 212, 308, 309]. Because the native Galv protein cannot be incorporated with the lentiviral vector due to the long intracellular domain of the TM unit, this unit was exchanged with the TM of the *amphotrophic murine leukemia virus*. The resulting chimeric envelope protein GalvTM can now be incorporated into lentiviral particles [213] and has already been used in preclinical studies [310-312].

Spumaretrovirus, also known as foamy virus (FV), is a subfamily of retroviruses [214]. They are endemic to non-human primates, cats, horses and cattle [313, 314]; humans are not their natural hosts, however, infections occur through zoonosis [216, 217]. Foamy viruses exhibit a broad host and tissue tropism with a favorable integration profile [315]. In preclinical studies, FV vector-mediated has been used to treat genetic diseases such as CD18 deficiency and Fanconi anemia by gene transfer into hematopoietic stem cells in animal models [315, 316]. The envelopes VSV-G, GalvTM and PE01, the FV glycoprotein with point mutations within the leader regions, were compared here regarding the production of infectious particles and the transduction efficacy of human primary T-cells. GalvTM and PE01 were also tested after optimization to human codon usage, which dramatically increased the infectious titers and also the transduction efficacy of primary T-cells. While the envelope PE01 achieved a similar transduction rate to VSV-G, the efficacy of GalvTM was slightly less but the transduction was more gentle due to the low toxicity of the protein. The optimization of cDNA to human codon-usage can lead to increased mRNA stability and export as well as translation efficacy [183-186]. Therefore, the envelope protein of both coop PE01 and coop GalvTM were more efficiently generated from lower cDNA levels resulting in more envelope protein present in the cell membrane of the producer cell line and hence incorporated into the lentiviral particles. Importantly, the transduction efficiency of codon optimized GalvTM and PE01 in primary T-cells increased when the amount of envelope DNA decreased. This might be due to the overrepresentation of envelope proteins in the cell membrane of the producer cell line leading to an inefficient processed envelope precursor protein as competitor of the processed envelope protein and inhibiting the binding of the envelope SU unit to the cellular receptor.

In conclusion, both codon-optimized envelope proteins display a favorable transduction efficacy, are only slightly toxic and there are no problems for the usage of these envelope proteins. Thus, they are suitable of replacing VSV-G for transduction of T-cells.

Prodrug characterization of h4B1P+12

Several substances are known to induce pulmonary toxicity in livestock and lab animals, among these are 4-ipomeanol, perilla ketone and 3-methylindole [134, 260-262, 317]. Although 4-ipomeanol is a well-established and potent prodrug for the h4B1P+12 suicide gene, other substances might exhibit different cytotoxic characteristics, which can be beneficial for the use as a prodrug. Therefore, I tested several substances in respect of their cytotoxic potential in HepG2 and T-cells.

Perilla ketone is the main substance in the essential oil of the Asian food plant *Perilla frutescence*, known in the US as 'beef-steak plant', and caused outbreaks of atypical pulmonary emphysema in the 1970s [259]. Although it was not formally shown that perilla ketone is processed by CYP4B1, the striking structural similarity to 4-ipomeanol prompted me to hypothesize that CYP4B1 might be the enzyme which bioactivates perilla ketone. In contrast, northern blot analysis identified CYP4B enzymes as the main enzyme that processes 3-methylindole [318], which was confirmed after heterologous expression of CYP4B1 in HepG2 cells and subsequent analysis of binding assays [319]. 2-aminoanthracene and 2-aminofluorene were among the first substrates identified for rabbit CYP4B1 [256]. Historically, the rabbit CYP4B1, due to its stability and early availability, was used as the main CYP4B1 to further characterize substrates for mammalian CYP4B1s.

Beside these substrates, I have tested the other potential substrates, 2-pentylfuran, 2-heptylfuran, 2-hexylfuran and 2-furyl pentyl ketone, because of their structure similarity to 4-ipomeanol and the commercially availability. Stable transduced HepG2 cells and survival assays revealed that 2-pentylfuran and 2-hexylfuran induce toxicity in cells expressing the rabbit isoform r4B1 and to a much lesser extent in cells expressing h4B1P427. Astonishingly, no toxicity was induced through h4B1P+12 expression, which only differs in 12 amino acid positions from h4B1P427. Importantly, prolonging the fatty acid linked to the furan ring about one methyl group largely abolished toxicity, except for h4B1P427, where a slight toxicity at 290 µM 2-hetylfuran was observed after 24h and 48h. For all HepG2 cells transduced with the different isoforms, incubation at higher dose (900 µM) 2-heptylfuran led only to high unspecific cell killing. Within our cooperation with Dr. Marco Girhard of the Institute of Biochemistry at the Heinrich-Heine University Düsseldorf, r4B1 was N-terminally deleted to facilitate heterologous expression in *E. coli* for high-level protein production. CO difference spectra showed that the N-terminally modified r4B1 was able to bind 2-pentylfuran, 2-hexylfuran and

also 2-heptylfuran, but only GC/MS analysis demonstrated the conversion of 2-pentylfuran and 2-hexylfuran but not 2-heptylfuran (unpublished data). These data is consistent with the results I obtained with the survival assays. With the highest resemblance to 4-ipomeanol and perilla ketone, 2-furyl pentyl ketone induced toxicity in r4B1 positive HepG2 cells at lower concentrations than 2-pentylfuran and 2-hexylfuran, but did not exceed the toxicity observed at high concentrations. Interestingly, after 48h cytotoxicity was found in cells expressing h4B1P427 and h4B1P+12, although to a lesser degree. Apparently, adding a ketone to the backbone 2-hexylfuran seems to enhance the toxicity and specificity towards h4B1P+12. Further biochemical analyses are undergoing in collaboration with Dr. Girhard to prove this hypothesis.

3-methyindole, also established as a pneumotoxin, is produced by fermentation of tryptophan by ruminal and intestinal microorganisms. The induced toxicity of 2.9 µM 3-methylindole was higher in h4B1P+12 positive HepG2 cells than in r4B1 positive cells, albeit it equaled out at higher concentrations. However, even at the highest concentration, 290 µM, only 60% of h4B1P+12 or r4B1 expressing cells died. Importantly, concentrations higher than 29 µM lead to unspecific toxicity in control cells. That was surprising since Thornton-Manning *et al.* incubated microsomes of transduced HepG2 cells with 200 µM 3-methylindole, analyzed the covalent binding and metabolites and observed no unspecific metabolism [319]. But he found, that beside rabbit CYP4B1, human CYP1A2 and CYP2F1 can bind and metabolize 3-methylindole. Thus, considering the unspecific toxicity and the conversion through CYP1A2 and CYP2F1, 3-methylindole can be neglected as an alternative prodrug.

2-aminoanthracene and 2-aminofluorene are bioactivated by P450 cytochromes to DNA-binding agents by initial heteroatom oxidation to the N-hydroxy metabolites [320, 321]. Imaoka *et al.* reported a correlation between CYP4B1 mRNA and protein levels and 2-aminofluorene activation in human bladder tumor preparations [322]. The same authors also published that a fusion protein of a reductase, POR, and a human isoform carrying an additional serine residue at position 207 and still the serine at position 427 in the meander region were stable and able to metabolize 2-aminofluorene and lauric acid [323]. These observations were not reproducible in our lab [data no shown, 144, 252]. 2-aminofluorene only exhibited unspecific toxicities in HepG2 cells at 290 µM, in control cells and independently of the CYP4B1 isoform expressed. When rabbit CYP4B1 was heterologous expressed in a mammalian cell lines, Smith *et al.* also observed no cytotoxicity of 2-aminofluorene, which was hypothesized to be due to deficient sulfonylation and/or deacetylation activities [257]. In contrast, in my experiments 2-aminoanthracene induced a strong toxicity against cells expressing r4B1, h4B1P+12 and h4B1P427, though weaker as the one achieved with 4-ipomeanol and with no toxicity in control cells. Differences in the potencies between the three isoforms

with 2-aminoanthracene were marginal. However, the substance belongs to a class of aromatic amines that are known mutagens and carcinogens [128] and becomes a highly reactive unsaturated dialdehyde intermediate upon conversion [257]. Although it has been considered and was tested as a prodrug for the rabbit CYP4B1 suicide gene [121, 258, 263], due to its mutagenic properties it is not clinically usable. The demonstrated bystander activity upon 2-aminoanthracene conversion was reproducible in my experiments.

Therefore, except for perilla ketone, none of the substances mentioned above can be considered as an alternative prodrug. Importantly, a strong toxicity of perilla ketone similar to the one upon 4-ipomeanol incubation was demonstrated here. There was no unspecific toxicity in control HepG2 cells at higher dosis. Remarkably, though the cytotoxicity in r4B1 and h4B1P+12 expressing cells achieved by perilla ketone and 4-ipomeanol was very similar, perilla ketone efficiently induced cell death at a higher rate at lower concentration than 4-ipomeanol. Like 4-ipomeanol, perilla ketone does not induce bystander activity when using HepG2 cells transduced with different CYP4B1 isoforms. These results prompted me to further investigate perilla ketone as an alternative prodrug for h4B1P+12 in T-cells. Therefore, primary T-cells were transduced with the previously established lentiviral vector, p2CL21ΔNGFRT2Ah4B1P+12, and subsequently selected using microbead enrichment. Considering the very similar characteristics of perilla ketone and 4-ipomeanol in the kill kinetics HepG2 cells, two new striking insights were obtained. First, concentrations of perilla ketone of more than 90 µM induced unspecific toxicity in control T-cells. Secondly, h4B1P+12 transduced T-cells were much more sensitive to perilla ketone than to 4-ipomeanol. Survival assays with lower concentration showed, that already 0.9 µM perilla ketone induced cell death comparable to 90 µM 4-ipomeanol.

Up to here, the cellular survival assays were analyzed by staining with propidium iodide, which reliable discriminates between viable and dead cells. However, for better understanding of the kill kinetics of 4-ipomeanol and perilla ketone and thus increasing the sensitivity of the analysis, analysis of an apoptosis marker was also included. Staining with annexinV antibody detects phosphatidylserine, which is translocated from the inner side to the outer layer of the plasma membrane already during early induction of apoptosis. Therefore, in combination with propidium iodide, cells can be further differentiated between being viable, apoptotic and necrotic/dead [324, 325]. Using this ‘double’ staining with annexin V antibody and PI clearly demonstrated the much faster kinetics of perilla ketone to induce apoptosis, almost twice as fast as 4-ipomeanol at similar concentrations.

These characteristics of perilla ketone for induction of apoptosis in transduced T-cells appear to have advantages *in vivo* over 4-ipomeanol. It is more potent in T-cells and induces faster cell death. Therefore, perilla ketone is the only potential alternative for 4-

ipomeanol as a prodrug. Further investigations in *Cyp4b1* knock-out mice will have to prove if the use of perilla ketone also has these advantages *in vivo*. Importantly, although the oil is used for cooking, the concentrations that are used here cannot be reached during the preparation of the food. Also, recent publications suggest that perilla ketone activates rat TRPA1 [326, 327]. TRPA1 belongs to the transient receptor potential channel family, is expressed in a subset of peripheral sensory neurons and can respond to mustard oil, allicin, other chemical irritants and probably noxious cold sensation [328]. Also, TRPA1 is considered to be the transduction channel in auditory hair cells [329]. Until now, it is unknown if perilla ketone induces liver toxicity as it has been shown for 4-ipomeanol by being catalyzed by other cytochromes and if other, non-P450 cytochrome mediated toxic reactions develop, therefore the effect of perilla ketone has to be tested in the *Cyp4b1* null mouse. Both prodrugs, perilla ketone and 4-ipomeanol, have to be tested in their feasibility regarding specific T-cell killing in an *in vivo* model. Although perilla ketone is more potent than 4-ipomeanol it is also more lipophilic, which can change the distribution characteristics *in vivo*. Due to the generation of *Cyp4b1* null mouse [145], we can begin to answer these questions.

Stimulation of primary T-cells

T-cell activation achieved by CD3/CD28 cross-linking with immortalized monoclonal antibodies in combination with high doses of IL-2 have been used in this thesis [266, 330]. Such a strong stimulation can lead to a change in the TCR repertoire [266] and an inversion of the CD4/CD8 ratio [264]. Although it has been reported, that T-cells retain their normal functions after CD3/CD28 activation [331, 332], others showed that the 'standard' T-cell production leads to an enrichment of effector memory T-cells (T_{EM}) or effector type T-cells and to a loss of naïve T-cell subsets [333] and reduced alloreactivity [267, 268]. Here, recent studies suggested that less differentiated T-cell subsets possess superior *in vivo* engraftment and antitumor activity in murine model systems [334, 335].

Therefore, alternative cytokines were tested that also signal through the IL-2R γ-chain (CD130) [330-341]. IL-7 displays some favorable characteristics: it does not induce proliferation of long-term naïve T-cells *in vivo* [338, 339], no switch from a naïve to a memory phenotype [340, 341], and only a slight upregulation of activation markers *in vitro* [336]. Although IL-7 can transfer resting T-cells into a state permissive for HIV and HIV-based lentiviral vectors, the transduction efficiency is depending on the time point, the MOI, and the use of fibronectin among other parameters [337, 338, 342]. IL-15 enhances the enrichment of CD8 positive effector T-cells [343]. The combination of IL-15 and IL-21 promotes the enrichment of less differentiated T-cells [344, 345] and

the functionally more potent effector T-cells [346], thus enabling more intense interaction with tumor cells [346].

The complex cellular pathway regulation between T-cell stimulation, activation, transduction, *in vivo* engraftment, and anti-tumor activity makes the search for the ideal prestimulation and transduction protocol extremely challenging. Transduction efficiency relies also on the retroviral vector backbone and the envelope protein/pseudotype used for cell entry. Detailed analysis of T-cells prior to and after transduction as well as after infusion into the patients are necessary to start to understand the consequences of *in vitro* manipulations for the T-cell function *in vivo*.

In my thesis, I investigated the effect of constant high activation/stimulation on the survival of T-cells challenged with the prodrug. High activation was achieved by a combination of CD3/CD28 signaling and IL-2 supplementation. Interestingly, when analyzing the cell death of h4B1P+12 positive T-cells, there was no difference between constant high activation and high activation only over night prior to 4-ipomeanol incubation. Both strong stimulations led to an excessive decrease in prodrug-induced cytotoxicity with T-cells being more resistant to incubation with 4-ipomeanol compared to the more 'gentle' culture with IL-2 only. After 24h, more than 60% of the strongly stimulated T-cells survived, while only 20% T-cells stimulated by IL-2 were viable. In contrast, the killing curves after 48h were almost identical. Probably a high stimulation delays apoptosis induction. Although further studies are necessary to understand these observations, cell death induction after 48h is almost identical for all three conditions. Here, it will be important *in vivo* in murine models, which of the three *in vitro* stimulation protocols closely resemble the activation process and intensity present *in vivo*.

Chimeric antigen receptors to treat AML and ALL

An important approach of adoptive T-cell therapies is to educate of T-lymphocytes to recognize antigens that are present on cancer cells. This retargeting can be achieved by expressing either a cDNA for affinity enhanced TCRs α- or β-chains or a cDNA for a CAR. Most of the clinically utilized TCRs are directed against the melanoma-associated antigen recognized by T cells 1 (MART-1) [347, 348] or the melanoma-associated antigen 3 (MAGE-A3) [21, 22]. Both antigens are only recognized on the tumor cells when HLA-A2 is present. In contrast, CARs recognize antigens in a MHC-independent manner. Composed of stimulatory domains of a TCR and single chain variable fragment of a monoclonal antibody [23, 24], they can be directed against a broad range of antigens. CARs targeting CD19, a B-cell lineage restricted marker, which is expressed on malignant B-cells, was the first CAR to reach clinical success in several studies, which

was acknowledged by the FDA in 2014 by giving the CTL019 'breakthrough therapy' status with advanced market entry.

While 1st generation CARs only contained the CD3ζ-chain as signaling domain, 2nd generation CAR employed essential domains of other co-stimulatory molecules. Extensive research demonstrated that CD19 CARs harboring co-stimulatory domains of CD28 or 4-1BB in combination with CD3ζ domains have increased cytokine production as well as the T-cell survival and proliferation after target antigen recognition compared to CD19 CAR harboring only the CD3ζ chain [31, 349-352]. Up to date, patients with B-cell malignancies treated with CD19 CAR therapy have a response rate of relapse/refractory disease of 90% [53]. However, patients, who achieved remission upon CD19 CAR therapy, are constantly depleted of B-cells, which is clinically manageable by life-long regular immunoglobulin infusions. Another B-cell lineage restricted antigen is CD22; the expression starts at the pro-B-cell stage and is lost only in plasma cells [353]. The recombinant immunotoxin BL22, a fusion protein consisting of the scFv portion of a CD22 antibody and an exotoxin of *Pseudomonas* [354], has been successfully targeting B-cell leukemia and lymphomas [355]. To improve the therapeutic success, a mutant of the scFv portion with a higher affinity to CD22 (HA22) was generated. Therapy with HA22 resulted in complete remission in patients with drug-resistant hair cell leukemia [356]. Because of the anti-tumor activity of both CD22 immunotoxins, BL22 and HA22, shown in clinical trials [357, 358], the efficacy of a CAR incorporating the scFv CD22 portions BL22 and HA22 were tested [359, 360]. Although the increase in potency of HA22-therapy was reported, a direct comparison of HA22-scFv- and BL22scFv-CARs demonstrated no significant differences in the activity of CAR-transduced T-cells [359]. Since both HA22 and BL22 possess a high antigen affinity, it confirms that over a certain threshold, the affinity of the scFv is not enhancing the CAR activity further [26].

In my thesis, I tested both CD19 and CD22 (BL22) CARs expressed in combination with h4B1P+12 for their ability to lyse the ALL cell line REH cells. These results demonstrated that firstly, both CARs efficiently lysed REH cells, secondly, that the suicide gene h4B1P+12 functions expressed from both vector constructs and, thirdly, that the CD19 CAR appears to mediate a better cell killing, however, both CAR constructs had a different CAR design. The CD19 CAR incorporated the hinge domain of CD8 whereas the CD22 CAR incorporated the IgG1 CH2CH3 region. Although these differences should not matter *in vitro, in vivo* studies have demonstrated that IgG molecules enable the activation of CAR T-cells through Fcγ-bearing cells and cross-activation of cytokine production by innate immune cells thereby inducing the loss of anti-tumor activity due to activation induced cell death [43]. Hudecek *et al.* also showed by testing a CD19-CAR with different length of the hinge region that the activity *in vitro* is independent of the length but that *in vivo* a short hinge region lead to more cytokine production and a higher proliferation rate [40].

Conventionally treated, AML is a devastating disease in children and adults and when treated with a modern chemotherapy protocol leads to complete remission in 60-80% of AML patients but 50-70% of them experience relapses [361-364]. If resistant to the chemotherapy, the leukemic stem cells (LSC) induce a new manifestation of the disease [365]. Although CD19 is successfully used in clinical applications, there is no antigen specific for AML blasts. CD123, which is restricted to myeloid lineage, is present on AML blasts and on LSCs of 75-89% of AML patients, and is a promising candidate for targeting AML with CAR therapy [366-368]. It is thought to be absent on normal hematopoietic stem cells, however present on early myeloid cells [367]. CD33 is expressed on leukemic blasts of 90% of the AML patients and on AML stem cells [58, 369], but like CD123 absent on normal hematopoietic stem cells [370-373]. Therefore, targeting CD33 has been the goal of several research strategies in the last 20 years [374]. The usage of both antigens for treatment with CAR transduced T-cells has been tested [57, 65, 375, 376]. Recently, the folate receptor β (FR β) has been proposed as a novel target antigen for redirecting T-cells [377]. FRβ is expressed on AML blasts of 70% of patients [378, 379], but its expression has also been reported on normal myeloid-lineage cells and is inducible on macrophage after activation [380]. This will potentially lead to on-target off-tumor toxicity. One major benefit of the FRβ antigen is, that the treatment of the patient with all-*trans* retinoic acid (ATRA) enhances the expression of FRβ on AML blasts [381, 382].

In my thesis, I tested the CD33-CAR in combination with h4B1P+12 for its cytolytic activity against the AML cell line Molm14 and demonstrated that the function of both transgenes are not impaired by co-expression via the T2A site.

Therapy with CARs directed towards CD33 or CD123 can lead to killing of AML blasts but also to a reduction of normal myeloid progenitor cells and consecutively peripheral blood cytopenia [57]. Although patients can be treated with immunoglobulin infusions after constant B-cell depleting, patients cannot survive long-term without a functioning myelopoiesis. To address these safety concerns for CAR therapy for AML, to control off-target or on-target off-tumor toxicities and the ability to eliminate the CAR T-cells *in vivo*, when a complete remission is achieved, several strategies have been developed. One strategy is to include a suicide gene into the transgene cassette containing the CAR thus allowing to specifically eliminate CAR positive T-cells. Another strategy is a transient expression of CARs in T-cells. Kenderian *et al.* electroporated T-cells with CD33 CAR-encoding mRNA, thus achieving high-level expression however with little benefits in a preclinical model [57]. Although it is an alternative to a stable retroviral transduction and CAR expression quickly ceases due to mRNA degradation in proliferating cells, this strategy is not well controllable. As an alternative, tumor specificity can be enhanced by directing T-cells against towards two antigens at the same

time resulting in two CARs expressed in a single T-cell. Consecutively, the costimulatory and signaling domains should be split on both CARs.

The split CAR design has recently been taken a step further. Wu *et al.* reported the design of an 'ON-switch' for CARs [383]: here, the CD3ζ signaling domain is removed from the CAR and transferred to a new polypeptid. Both molecules, the CAR and the CD3ζ domain, are fused to heterodimerizing domains, which dimerize upon binding a small synthetic molecule, AP21967. A CAR mediated T-cell response only occurs upon antigen binding and AP21967 supplement. By this safety mechanism, CAR T-cells don't have to be removed from the patient to stop the therapy or off-tumor toxicities.

In turn, these approaches result in another transgene, which has to be incorporated into the retroviral vector. Since the suicide gene should not be abandoned, even with the 'ON-switch', T-cells could be transduced with the vector established for DLI therapy, ΔNGFR and h4B1P+12, selected for positive transduction and subsequently transduced with a second vector harboring 2 CARs. This would increase the *ex vivo* cultivation time as well as viral copies per cells. Alternatively, depending on the size, a third transgene in addition to the suicide gene and the CAR could be expressed via a second 2A site. But further investigations will have to prove if all three transgenes would be equally expressed in transduced cells.

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6. Appendix

6.1 List of abbreviations

°C	Celsius
2-AA	2-aminoanthracene
2-AF	2-aminofluorene
3-MI	3-methyindole
4-IPO	4-ipomeanol
ALL	Acute lymphatic leukemia
Allo-HSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute myeloid leukemia
Asct2	Amino acid transporter 2
bp	Base pairs
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
CD	cluster of differentiation
cDNA	complementary DNA
CLL	Chronic lymphatic leukemia
CR	E2 Crimson
CTL	Cytotoxic T-lymphocyte
CXCR	C-X-C chemokine receptor
Da	Dalton
DLI	Donor lymphocyte infusion
DNA	Desoxyribonucleinacid
dNTP	Desoxyribose nucleoside triphosphate
EF1α	Elongation factor-1 alpha
EGFP	Enhanced green fluorescent protein
FACS	Fluorescent activated cell sorting
FCS	Fetal calf serum
Fitc	Fluorescein isothiocyanate
FPK	2-furyl pentyl ketone
FRβ	Folate receptor β
FXR	Farnesoid X receptor
g	gramm
GC/MS	Gas chromatography/mass spectroscopy
Glut1	Glucose Transporter 1
GvHD	Graft-versus-Host-Disease

GvL	Graft-versus-Leukemia
h	hour
H_2O	Water
HepF	2-n-heptylfuren
HexF	2-n-hexylfuren
HLA	Human leucocyte antigene
Ig	Immunoglobulin
IL	Interleukin
IRES	Internal ribosomal entry site
k	kilo
K_d	Dissociation constant
LSC	Leukemic stem cell
LV	Lentivirus
m	milli
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
min	minutes
MPSV	Myeloproliferative sarcoma virus
mRNA	messenger RNA
NFkB	nuclear factor kappa-light-chain-enhancer
NGFR	Nerve growth factor receptor
PE	R-Phycoerythrin
PenF	2-n-pentylfuren
PGK	Phosphoglycerate kinase 1
PI	Propidium Iodide
PK	Perilla ketone
RNA	Ribonucleic acid
RT	Room temperature
RXR	Retinoid X receptor
s	seconds
ScFv	Single-chain variable fragment
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
TCR	T-cell receptor
TIL	Tumor infiltrating lymphocyte
TO	dTOMATO
UbiC	Ubiquitin C
VSV	Vesicular stomatitis virus
µ	micro

6.2 List of figures

6.3 List of tables

6.4 Publications

"**Optimized human CYP4B1 in combination with the natural alkylator prodrug 4-ipomeanol serves as a novel suicide gene system for adoptive T-cell therapies**"

Katharina Roellecke[1], Elizabeth L. Virts[2], Ralf Einholz[3], Bianca Altvater[4], Claudia Rossig[4], Dorothea von Laer[5], Kathrin Scheckenbach[1], Martin Wagenmann[1], Dirk Reinhardt[6], Christof M. Kramm[7], Katheryne Z. Edson[8], Allan E. Rettie[8], Constanze Wiek[1¶], Helmut Hanenberg[1,6¶*]

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submitted in Molecular Therapies, in review

"**Ligand characterization of CYP4B1 isoforms modified for high-level expression in E.coli and HepG2 cells**"

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in preparation

"Characterization of an Additional Splice Acceptor Site Introduced into CYP4B1 in Hominoidae during Evolution"

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"Identification of amino acid determinants in CYP4B1 for optimal catalytic processing of 4-ipomeanol"

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"Amiloride derivatives are effective blockers of insect odorant receptors"

Röllecke K[1], Werner M, Ziemba PM, Neuhaus EM, Hatt H, Gisselmann G.

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Chem Senses. 2013 Mar;38(3):231-6. doi: 10.1093/chemse/bjs140

Conferences

"Human suicide gene systems for adoptive cellular therapies"

K Röllecke

Talk at the 5th Pediatric AML symposium

14-17 April 2014, Essen, Germany

"High-level co-expression of a novel human suicide gene, CYP4B1, with CD19 or CD22 CARs in SIN lentiviral vectors"

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Poster presentation at European Society of Gene and Cell Therapy

23-26 October 2014, The Hague, The Netherlands

"Re-engineering of human CYP4B1 for optimal catalytic processing of 4-ipomeanol and use as a suicide gene in adoptive cell therapy"

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6.5 Curriculum vitae

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Universitäre Ausbildung

02/2012 – 11/2015	Dissertation an der Heinrich-Heine Universität Düsseldorf in der Hals-Nasen-Ohren Klinik des Universitätsklinikums. Titel: „A Modified P450 Cytochrome as Safety Mechanism in Adoptive T-cell Therapy“, Betreuer: Prof. Dr. Hanenberg
10/2010 – 09/2011	Masterarbeit an der Ruhr-Universität Bochum am Lehrstuhl für Zellphysiologie. Titel: „Pharmakologische Untersuchung von chemorezeptiven Ionenkanälen in Drosophila melanogaster“, Betreuer: Prof. Dr. Dr. Dr. Hatt
03/2009 – 09/2009	Bachelorarbeit an der Ruhr-Universität Bochum am Lehrstuhl für Zellphysiologie. Titel: „Analyse zur potentiellen Multimerisierung von Ionenkanälen, die zur TMEM16-Familie gehören“, Betreuer: Prof. Dr. Dr. Dr. Hatt
06/2006 – 09/2011	Studium der Biologie an der Fakultät für Biologie und Biotechnologie der Ruhr-Universität Bochum mit dem Abschluss Master of Science

Schulische Ausbildung

08/1997 – 06/2006	Stadtgymnasium Dortmund mit dem Abschluss Allgemeine Hochschulreife

Berufserfahrung

11/2009 – 06/2010	Studentische Hilfskraftstelle in der Ruhr-Universität Bochum am Lehrstuhl für Zellphysiologie

6.6 Acknowledgment

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Meiner Familie danke ich, dass sie mir immer den Rücken stärkt, für mich da ist und ich mich auf sie verlassen kann. Und mir gegebenenfalls einen Tritt verpasst um mich wieder auf die richtige Spur zu bringen.

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6.7 Declaration

I declare that I have compiled my dissertation independently and without any undue assistance by third parties under consideration of the 'Principles for the Safeguarding of Good Scientific Practice at Heinrich Heine University Düsseldorf'.

This dissertation has not been submitted in its present or a similar form in any other institution. I have not made any successful or unsuccessful attempt to obtain a doctorate before.

Düsseldorf, 4th November 2015