Aus der Klinik für Pädiatrische Hämatologie und Onkologie der Medizinischen Hochschule Hannover

Generation of genetically engineered precursor T cells from human umbilical cord blood using an optimized alpharetroviral vector platform

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I Background

I.1 T cell based therapies for cancer

Cancer is one of the most widespread lethal illnesses in the world, with 14 million new patients every year, as reported in the World Cancer Report 2014 by the World Health Organization. It is not by chance that writer and oncologist Siddharta Mukherjee called cancer the "emperor of all maladies" (Mukherjee, 2010). New therapeutic approaches are required and many are based on the fact that the immune system plays a crucial role in tumorigenesis. Therefore, diverse seminal works in the field of immunology allow the translation of immunologic principles into cancer immunotherapies. This development began with William Coley in 1893 who was the first one to explore the immune system to target cancer. He treated sarcoma patients with topic injections of streptococcus and achieved durable cancer regression (Coley, 1991).

Later, Paul Ehrlich was the first one to hypothesize that the immune system can prevent emergence of neoplasia (Ehrlich, 1909). More than 50 years later, this was denominated concept of immunological surveillance (Burnet, 1970). The main thought was that the immune system fights malignant tumor cells owing to the expression of tumor-associated antigens that can be specifically targeted by immune cells. Several studies have supported this hypothesis. For instance, transplanting tumor cells from immunodeficient mice to wild type mice led to rejection of tumor cells (Shankaran *et al.*, 2001). Nowadays, it is established that a complex interplay of several immune cells, of both innate and adaptive immunity, recognizes and destroys transformed cells (Smyth *et al.*, 2001). Of all immune cells, T cells are being assigned a major role for tumor destruction.

However, up to the middle of the 20th century immunological research focused on antibodies and the roots of cellular immunology only date back to recent times. The function of lymphocytes was first explored in the 1950s, when lymphocytes were found to play an important role in delayed hypersensitivity reactions (Landsteiner *et al.*, 1941) and to mediate allograft rejection in animals (Mitchison, 1955). Subsequently, researchers made use of syngeneic lymphocytes from rodents that were immunized against the tumor and slight growth reduction of small tumors was seen in cancer-bearing hosts (Delorme *et al.*, 1964; Fefer, 1969). The capability of T lymphocytes for tumor control and for specific interaction with antigens is now being exploited for adoptive cell immunotherapy (ACT), which is a highly individualized cancer therapy approach consisting of isolation, *ex vivo* handling and administration of immune cells with anti-cancer properties to a cancer-bearing recipient (Kolb *et al.*, 1995). This was first explored in the setting of allogeneic hematopoietic stem cell transplantation (HSCT). Today, ACT has become an effective treatment option enabling total and ongoing regression of refractory cancers, where most extensive experience has been gained for metastatic melanoma. T cell therapies such as anti-CD19 chimeric antigen receptor (CAR)-transduced T cells are fostered by receiving breakthrough designation status from the United States Food and Drug Administration (FDA) allowing efficient design of clinical trials to ensure future approval (Grupp *et al.*, 2013). Research is still ongoing and new technologies in gene transfer open up new possibilities to target various tumor antigens as long as a T cell receptor (TCR) or antibody sequence is known.

I.1.1 Adoptive immunotherapy after allogeneic hematopoietic stem cell therapy

Development of allogeneic HSCT dates back to the Cold War where it was meant to be a salvage therapy for United States soldiers following a nuclear weapon incident (Welniak *et al.*, 2007). The underlying principle was first discovered by Lorenz and colleagues because they reported that bone marrow transfer saves lethally irradiated mice from irradiation sickness (Congdon *et al.*, 1952; Lorenz *et al.*, 1952). This theoretical background was then applied to cancer treatment after mouse leukemia studies revealed that irradiation alone could not eliminate tumor cells unless combined with allogeneic bone marrow transfer (Burchenal *et al.*, 1960; Barnes *et al.*, 2001).

The anti-tumor effect of allogeneic HSCT is mediated by bystander T cells found in the donor stem cell graft. Due to their genetic disparity, especially regarding minor histocompatibility antigens, to the recipient cells, allogeneic T cells bind allo-antigens on patient tumor cells. Thereby, they exert an immune reaction and play an important role in cancer control, leading to an increased chance of tumor remission as seen in improved survival of allogeneic compared to syngeneic transplant recipients (Weiden *et al.*, 1979; Fefer *et al.*, 1987). This mechanism is called graft versus tumor effect or in the case of hematological malignancies graft versus leukemia effect (GVL). However, the allo-reaction is also the reason for the development of graft versus host disease (GVHD) as an undesired side effect after allogeneic HSCT due to alloreactive graft T cells binding normal tissue cells. Therefore, with less GVHD there is also less GVL and hence a greater risk of leukemia relapse as seen after T cell depletion of allogeneic MHC-matched transplants (Marmont *et al.*, 1991). Later, it was noted that natural killer cells also contribute to the GVL effect (Ruggeri *et al.*, 2002).

In addition, the T cell allo-reaction is not only important for allogeneic HSCT, but also being exploited in donor lymphocyte infusions (DLI) in case of disease progression after allogeneic HSCT. DLI is especially successful for chronic myeloid leukemia (CML) where 73% of patients underwent complete remission following DLI infusion. This result was long-lasting with a chance of 87% for remission three years after treatment (Kolb *et al.*, 1995). Tumor responses for other malignant diseases such as acute lymphocytic leukemia and multiple myeloma have been disappointing (Collins *et al.*, 2000; Salama *et al.*, 2000). Nevertheless, adoptive T cell transfer through DLI does not only benefit malignancy treatment but also improves general immunity which is important because one of the major risks after HSCT is slowed reconstitution of the immune system and following risk for infection and malignancies (Lum, 1987; Storek *et al.*, 1997). Examples for major infections are varicella zoster virus, streptococcus pneumonia and cytomegalovirus (Hoyle *et al.*, 1994; Ketterer *et al.*, 1999). Despite these major benefits of DLI, severe adverse events also occur. As for allo-HSCT, GVHD is an important risk. This needs to be taken into consideration, because it is a main reason for non-relapse related mortality after HSCT (Horowitz *et al.*, 1990). Reduced intensity, non-myeloablative pre-transplant conditioning regimens appear to cause less serious, but later occurring GVHD. Nonetheless, the frequency of chronic GVHD does not seem to change with the modified regimen (Mielcarek *et al.*, 2003).

In spite of this serious side effect, allogeneic HSCT for human hematological malignant diseases such as leukemia, lymphoma und multiple myeloma is nowadays an efficient therapeutic approach and the first and most developed type of ACT.

I.1.2 T cell based therapies using autologous T cells

The application of immunotherapy to treat tumors is becoming more important in the field of cancer therapy. As seen for DLI in the allogeneic HSCT setting, the cellular part of the immune system plays a substantial role in controlling malignant cells. Several studies investigated how the cytotoxic function of T cells can be enhanced, for example by immunizations. Nonetheless, it is feared that these T cells might be anergic *in vivo* in the cancer patient. In contrast, active anti-cancer T cells were reported to be found in tumor tissue. Initial research focused on these autologous tumor-specific and tumor-infiltrating lymphocytes (TILs) and now has more turned towards peripheral blood TCR- and CAR-transduced T cells. All of these three have distinct characteristics: Whereas TCR and CAR techniques have only a single antigen specificity, the advantage of TILs is the wide range of T cell binding to defined and unknown tumor antigens. Yet, not only antigen specificity is of interest, but also other methods, for example to improve trafficking of T cells to tumor cells and to decrease suppression of T cells in the tumor environment. It appears that the combination of different immunotherapeutic approaches and conventional treatment strategies could potentially become the major therapy approach for cancer in the future. The speed of research has especially increased over the last decade with development of new tumor target antigens and clinical trials, which will further be presented here.

I.1.2.1 *In vivo* stimulation for enhanced T cell function

In 1976, the T cell growth factor interleukin-2 (IL-2) was described and facilitated ACT. Directly administrating IL-2 showed reduced tumor growth in mice (Rosenberg *et al.*, 1985) and combining IL-2 and adoptive cell administration resulted in improved therapeutic potency of the transferred T cells (Donohue *et al.*, 1984). IL-2 was then first used for metastatic melanoma and renal cell carcinoma. The response rates only reached up to 17%, but 4 to 9% of the patients underwent total tumor regression (Rosenberg *et al.*, 1998; Klapper *et al.*, 2008). Importantly, these responses were long-lasting in 24 of 33 complete regressions up to 25 years after treatment (Smith *et al.*, 2008; Rosenberg, 2012). Another class of cytokines, interferons, was shown to improve disease-free survival time if administered in an adjuvant manner for melanoma (Kirkwood *et al.*, 1996).

Not only cytokines, but also immune checkpoints influence T cell activity. These checkpoints restrict T cell response and are necessary to avoid autoimmunity. Nonetheless, they also impede the magnitude of desirable anti-cancer responses. Key molecules include cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1), both expressed on T cells. If T cells within the tumor express these markers, it leads to hyporesponsiveness and immune exhaustion (Callahan *et al.*, 2010). Blocking these checkpoints can enhance anti-tumor response and promising results have been observed with ipilimumab, an anti-CTLA-4 monoclonal antibody. When patients with metastatic melanoma received ipilimumab, they profited from enhanced overall

survival (Hodi *et al.*, 2010). However, autoimmune adverse events such as endocrinopathies and enteropathies occurred (Mitchell *et al.*, 2013). Nevertheless, after a successful phase III trial ipilimumab was approved by the FDA for immunotherapy of metastatic melanoma (McDermott *et al.*, 2013). Another option to circumvent an immune checkpoint is to block PD-1 or its ligand PD-L1. In a murine allo-transplant model, PD-L1 blocking could restore the GVL effect without emergence of GVHD (Koestner *et al.*, 2011). In humans, durable responses after PD-1/PD-L1 interruption were seen for disparate types of solid tumors, such as non-small-cell lung cancer, renal cell cancer or melanoma (Topalian *et al.*, 2012). More recent approaches target T cell immunoglobulin and mucin domain-containing protein 3 (Tim-3) and lymphocyte activation gene-3 (LAG-3) (Pardoll, 2012). Instead of blocking negative pathways, stimulating positive ones is also an option to augment T cell function. For instance, CD137 and CD134 can be stimulated *in vivo* with ligand fusion proteins or agonistic antibodies (Hernandez-Chacon *et al.*, 2011).

Another approach to booster the host's immune system *in vivo* is the use of vaccines. However, they are often ineffective because the cancer patient's antigen-presenting cells, such as dendritic cells (DCs) are dysfunctional (Enk *et al.*, 1997) and the T cells are exhausted after long-time exposure to the cancer antigens (Fourcade *et al.*, 2010). As an example, an irradiated, whole-cell melanoma vaccine, called canavaxin, seemed promising at first, but then displayed no benefit in a phase III trial (Morton *et al.*, 1992; Morton *et al.*, 2002). An option to improve immunogenicity of vaccines is transfection with genes coding for proinflammatory cytokines or costimulatory molecules (Jaffee *et al.*, 2001). Later, discovery of tumor antigens paved the ground for peptide-based vaccines. Increasing immunogenicity remains a concern for this type of vaccines as well and injection of costimulatory factors or cytokines are possible solutions (Schaed *et al.*, 2002). A final approach is usage of DC vaccines. These consist of antigens bound to anti-DC antibodies, DCs that either capture the peptide or nucleic acid *in vivo* or are loaded *in vitro* with antigens (Palucka *et al.*, 2013), DCs that were transfected with tumor cell mRNA (Specht *et al.*, 1997) or DCs bound to whole tumor cells (Gong *et al.*, 1997). In addition, it was recently reported that lentivirally transduced DCs are more potent than conventional DCs and can even be produced under good manufacturing practice conditions (Sundarasetty *et al.*, 2015). However, precautions have to be taken because DC immunization can cause autoimmune reactions and destruction of self-antigen expressing cells (Roskrow *et al.*, 1999).

I.1.2.2 Use of tumor-infiltrating T lymphocytes

Several patients with metastatic melanoma underwent complete long-lasting tumor regressions by administration of the T cell cytokine IL-2, which led to further interest in the underlying T cell involving mechanism and discovering of TILs. TILs are a mixture of CD4+ and CD8+ T cells which were shown to be a subpopulation of T cells infiltrating the stroma of tumors and found to be able to recognize tumor cells *ex vivo* (**Figure i**).

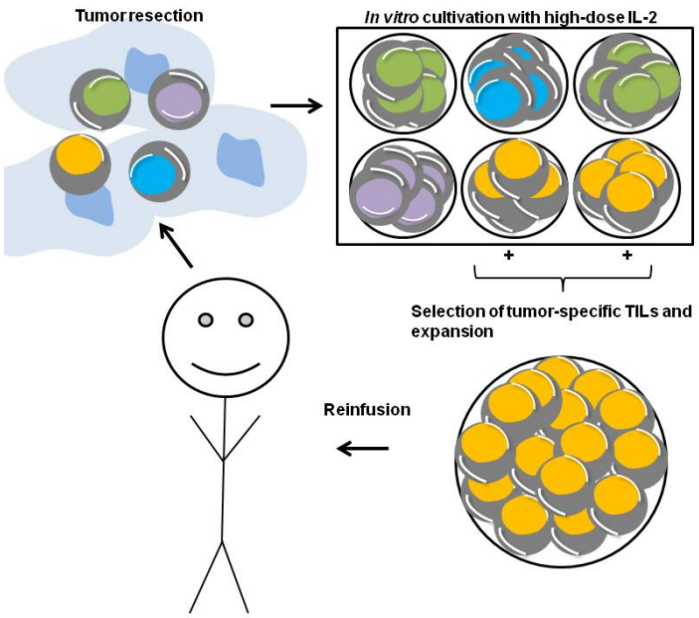

Figure i: Tumor-infiltrating T lymphocytes. TILs derived from tumor specimens are expanded *ex vivo*, screened for anti-cancer efficacy and injected back to the patient.

Adoptive transfer of TILs emerged from the group of Steven A. Rosenberg, which could show that syngeneic murine TILs expanded *in vitro* with IL-2 treated mice with liver and lung tumors (Rosenberg *et al.*, 1986). TILs were also found in human melanoma tissue (Muul *et al.*, 1987) and two years later, for the first time, it was shown that autologous human TILs could lead to reduction of tumor burden in metastatic melanoma patients (Rosenberg *et al.*, 1988). At the beginning of human ACT with TILs, a significant drawback was the fact that injected cells quickly disappeared from the circulation even days after administration and that anti-cancer activity only lasted for a short period of time (Rosenberg *et al.*, 1990). A huge advancement was made in 2002, when it was reported that nonmyeloablative chemotherapy for lymphodepletion led to improved cancer regression and ongoing host repopulation with the administered anti-cancer lymphocytes (Dudley *et al.*, 2002). Lymphodepletion kills regulatory T cells and lymphocytes that could possibly compete with transferred TILs for important cytokines, like IL-7 and IL-15 (Dudley *et al.*, 2005). The procedure of lymphodepletion is currently still being investigated, for example combination with total-body irradiation is explored in order to reduce T cell recovery after lymphodepletion that could interfere with the activity of TILs (Dudley *et al.*, 2002; Rosenberg *et al.*, 2011). Nonetheless, autologous TILs nowadays represent a potent therapy for metastatic melanoma with objective responses of around 50% of which 95% of complete remissions are ongoing for at least five years (Rosenberg *et al.*, 2011). Durable responses could also be reproduced by other treatment centers in the US (Radvanyi *et al.*, 2012), Israel (Itzhaki *et al.*, 2011) and Denmark (Ellebaek *et al.*, 2012). Despite positive treatment results, research to further optimize TIL strategies is continuing and focuses for instance on reducing negative regulatory cells (Yao *et al.*, 2012) or predictive biomarkers, such as telomere length (Zhou *et al.*, 2005), to select patients most susceptible to the treatment.

Application of the TIL approach to other tumors would be desirable, but melanoma turned out to be the only type of cancer that successfully generated TIL cultures with the ability to specifically recognize tumor cells. For some time, it has been hypothesized that the structures targeted by the immune system are caused by the mutations of tumor cells (Wolfel *et al.*, 1995) and melanoma has been found to possess a high mutation rate making it more immunogenic (Prickett *et al.*, 2009; Gartner *et al.*, 2012). Thus, T lymphocytes recognizing the mutated proteins are responsible for tumor responses in TILs-receiving patients. Other potential target tumors are lung carcinomas

(Lee *et al.*, 2010) and head and neck cancer (Agrawal *et al.*, 2011), especially in smokers, due to frequent somatic mutations.

I.1.2.3 *In vitro* expansion of T cells for therapeutic use

In vitro culture systems have been developed to be able to generate sufficient numbers of T cells for ACT. The response to ACT is largely correlated to the number of transferred cells as seen in mice with melanoma that got treated with CD8+ T cells (Klebanoff *et al.*, 2011). Two approaches exist: isolation of antigen-specific T cells from peripheral blood or tumor tissue and subsequent *in vitro* activation, or polyclonal *in vitro* activation. The latter approach is based on the hypothesis that tumor-specific T cells have already been primed in the patient and suffer from hampered *in vivo* function. Whereas the first approach ensures antigen specificity, only the second one has been used in randomized clinical trials due to cost-effectiveness (Takayama *et al.*, 2000; Rapoport *et al.*, 2005).

In the aforementioned second approach, generating large numbers of T cells *in vitro* could circumvent the necessity for antigen presentation and immune cell proliferation *in vivo*. For this reason, the T cell cytokine IL-2 was not only used for *in vivo* stimulation, but also *in vitro* expansion of T cells. Lymphocytes cultured *ex vivo* with IL-2 and subsequently injected intravenously efficiently treated subcutaneous lymphomas (Eberlein *et al.*, 1982). IL-2 is also being used for *in vitro* expansion of TILs from tumor fragments in combination with irradiated feeder lymphocytes and an anti-CD3 antibody binding the epsilon subunit. After six weeks of this so called rapid expansion protocol, about 10^{11} lymphocytes can be collected for infusion into the patient (Dudley *et al.*, 2002). It is important to note that prolonged culture time of adoptively transferred T cells has been associated with decreased efficacy *in vivo* due to reduced replication potential (Merrouche *et al.*, 1995; Economou *et al.*, 1996). In addition, phenotypic development of T cells towards effector cells during longer *in vitro* culture influences the homing and migration properties *in vivo* and especially IL-2 was found to be non-redundant for T cell effector differentiation. For example, L-selectin showed to be essential for rapid appearance of adoptively infused T cells to lymphoid organs in the periphery and was more prominently expressed on T cells of short-term culture (Sauer *et al.*, 2004). Additionally, not only duration, but also culture conditions themselves influence the differentiation pattern of T cells, which is relevant for their *in vivo* persistence (Huang *et al.*, 2005). As an example, the development of anti-TCR complex molecule CD3 and anti-costimulatory antigen CD28 antibodies was a major factor allowing proliferation of all T cell subgroups whilst avoiding final differentiation. These antibodies can either be used in a soluble form (Riddell *et al.*, 1990) or bound to plates, microspheres or cells in order to achieve an optimal crosslink of target structures on the T cells. This method has now even been developed to GMP-conform conditions, in which the antibodies are fused to paramagnetic beads (Bondanza *et al.*, 2006; Kaneko *et al.*, 2009).

I.1.2.4 *In vitro* priming of T cells to augment antigen specificity

Due to the low prevalence of naturally existing tumor-specific T cells, other options to prime T cells against tumor antigens and to expand them have been investigated. T cells can be stimulated with several methods: for example with autologous tumor cells, irradiated allogeneic peripheral blood mononuclear cells (Mannering *et al.*, 1998), CD40-stimulated autologous B cells (Schultze *et al.*, 1997), peptides, autologous (Ghosh *et al.*, 2008) or allogeneic (Montagna *et al.*, 2001) DCs.

DCs are a subpopulation of immune cells belonging to the professional antigen-presenting cells. Their characteristic function is to stimulate T cells to give an antigen-specific immune response. The main strategy consists of expanding autologous T cells from peripheral blood by several rounds of antigen stimulation by autologous DCs. These DCs can either be directly isolated from blood or be generated from autologous monocytes by incubation with specific cytokines, like IL-4 and granulocyte macrophage colony-stimulating factor. After maturation and loading with the desired peptide, they are able to specifically stimulate T cell expansion (Oelke *et al.*, 2000). Yet, in clinical trials for melanoma, only about 10% of patients responded modestly to these infused T cells and few patients underwent durable disease regression (Yee *et al.*, 2002; Mackensen *et al.*, 2006). This is potentially due to low affinity and little percentage of antigen-specific T cells. Additionally, the generation of autologous DCs has some negative aspects, such as labor- and cost-intensive procedures and difficulties owing to restricted replicative potential (Oelke *et al.*, 2005). Also, a large amount of blood is needed for their generation, which is impractical in case of severely ill patients. Moreover, in those patients, number and quality of *in vitro* generated DCs is often hampered due to previous chemotherapy and immunosuppression and it was reported that cancer itself impedes DC function (Ormandy *et al.*, 2006).

Due to the mentioned drawbacks of autologous DCs, artificial antigen-presenting cells, where cell lines and beads are engineered, have been developed. Either beads can be coated with CD3-specific antibodies or peptide-MHC complexes, or cells are made to express an MHC-molecule, costimulatory molecules and surface-anchored cytokines (Suhoski *et al.*, 2007; Forget *et al.*, 2014). Anti-MART-1 T cells, against melanoma antigen recognized by T cells 1, generated with this method were able to persist for four months but only generated a clinical response after ipilimumab treatment (Butler *et al.*, 2011).

Priming of T cells is not only investigated for solid, but also hematological malignancies. Normal donor T cells can be co-cultured with unmanipulated acute myeloid leukemia (AML) cells. Since these constitute poor antigen-presenting cells, addition of the cytokine interferon-γ (IFN-γ) (Brouwer *et al.*, 2002), generation of DCs from these tumor cells (Choudhury *et al.*, 1999) or CD34+-derived DCs (Fujii *et al.*, 1999) have been explored. As an alternative, in case there are enough leukemia cells expressing immunogenic proteins, they can be lysed and used to load DCs (Sauer *et al.*, 2004). With this method, DCs were harvested from an AML patient in remission, lysate-pulsed and used for generation of autologous AML-reactive T cells (Galea-Lauri, 2002). DCs can also be loaded with apoptotic leukemia cells or fused to leukemia cells. All of these three attempts can induce specific anti-leukemic activity of T cells with the hybrid vaccine being the most effective one (Galea-Lauri *et al.*, 2002). Another option, shown in a murine model, consists of using allogeneic recipient's DCs for priming of donor

lymphocytes which bears the unique potential for a strong GVL effect and reduced GVHD risk (Ghosh *et al.*, 2009).

I.1.2.5 The role of *in vivo* trafficking, persistence and expansion

T cell function after adoptive transfer is not only defined by antigen specificity, but also various other factors, such as migration and persistence.

Migration of adoptively transferred T cells to tumor sites was shown to be heterogeneous. In a murine tumor model, infused T cells could not be found in the central nervous system, ultimately leading to treatment failure (Sauer *et al.*, 2004). This was an unexpected finding because the blood-brain barrier is known to enable T cell trafficking. Approaches to improve T cell homing to tumors are transduction with chemokine receptor genes depending on chemokine expression by tumor cells (Peng *et al.*, 2010) and antiangiogenic substances such as anti-vascular endothelial growth factor (VEGF) antibody to normalize the blood vessels (Shrimali *et al.*, 2010). After trafficking, persistence of adoptively transferred T cells is the next critical step. For this, the T cell phenotype, which follows a precise development, is one of the influencing factors (**Figure ii**).

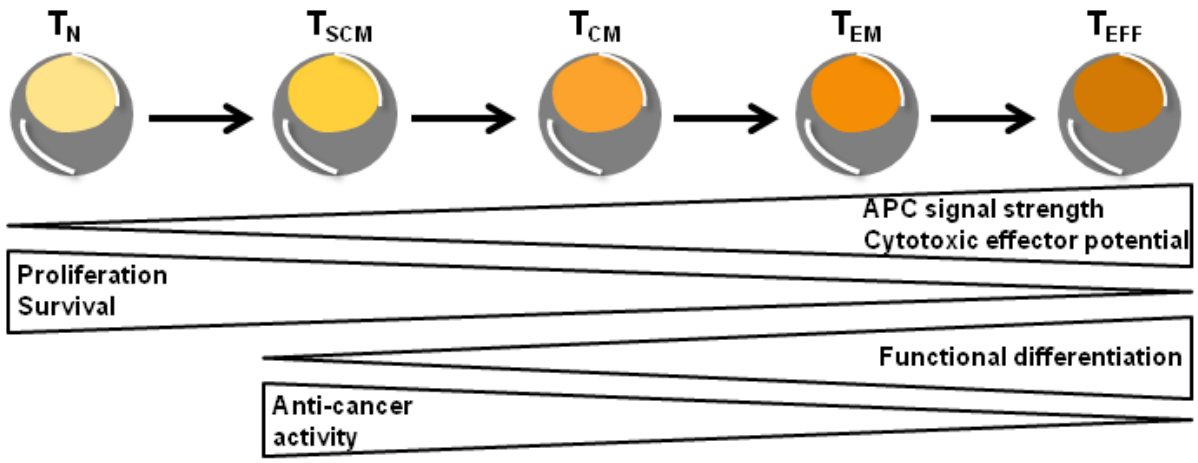

Figure ii: T cell subsets. Antigen-presenting cell (APC), naïve (T_N), memory stem (T_{SCM}), central memory (T_{CM}), effector memory (T_{EM}) and effector (T_{EFF}) T cell are indicated.

Naïve T cells (T_N) leave the thymus and upon antigen binding, they become memory, either effector memory (T_{EM}) or central memory (T_{CM}), and effector (T_{EFF}) T cells. Another new subtype has recently been described in mice (Zhang *et al.*, 2005) and humans (Gattinoni *et al.*, 2011), called memory stem T cells (T_{SCM}). At the beginning, it was thought that T_{EFF} would be most potent against tumors as seen by strong *in vitro* tumor cytotoxicity. Yet, it became clear that in comparison to a heterogeneous T cell population they exert a poor anti-cancer activity (Yee, 2010). In contrast, the aforementioned T_{SCM} possess great persistence and expansion capabilities in part due to long telomeres, which enables improved clinical responses compared to late differentiated adoptively transferred T cells (Huang *et al.*, 2005). The finding that less differentiated T cells show superior anti-tumor characteristics was supported by animal studies in mice and primates where T_{CM} proved superior to T_{EM} regarding *in vivo* tumor

eradication by improved persistence and function (Gattinoni *et al.*, 2005; Berger *et al.*, 2008). Extending these data, T_N compared to T_{CM} cells show greater expansion, cytokine secretion and anti-tumor response in mouse studies (Hinrichs *et al.*, 2009) This is also supported by human data where T_N-derived T cells rather than memory T cell-derived ones express more CD27 and have longer telomeres, which is associated with better tumor responses (Hinrichs *et al.*, 2011). Methods to limit T cell differentiation are T cell reprogramming to pluripotency (Nishimura *et al.*, 2013) or induced pluripotent stem cells (Vizcardo *et al.*, 2013), using IL-21 (Li *et al.*, 2005) or AKT kinase inhibitors (Crompton *et al.*, 2015). Another method to produce more efficient T cells for ACT is induction of specific differentiation by cytokines. For example, Type 17 T cells, which are distinguished by production of IL-17A and IL-17F, have shown promising tumor regression in mice (Muranski *et al.*, 2008; Hinrichs *et al.*, 2009).

In summary, because clonal expansion and effector differentiation are biologically coupled, shorter culture time is desirable in order to obtain a minimally differentiated phenotype. This favors certain phenotypic T cell populations, displaying rapid cell proliferation and longer telomeres, which have been associated with improved clinical results (Itzhaki *et al.*, 2011; Rosenberg *et al.*, 2011). Nevertheless, selection of one single T cell population for ACT might not be sufficient, because immune cells engage in complex interactions. As an example, mixing CD4 and CD8 T cells proved to be more successful than transferring CD8 T cells alone (Kalos *et al.*, 2011; Porter *et al.*, 2011; Grupp *et al.*, 2013).

I.1.3 Enhanced T cell specificity by T cell receptor gene transfer

The immune system often fails to respond to cancer. The decreased affinity of up to 1.5 logs against tumor self-antigens in contrast to foreign, for example viral, antigens represents one mechanism (Aleksic *et al.*, 2012). Additionally, there is data suggesting that the amplitude of T cell response to neo-antigens is higher than to tumor- or self-antigens (Castle *et al.*, 2012; Matsushita *et al.*, 2012). The reduced affinity is due to negative selection in the thymus where precursor T cells (preTs) expressing TCRs against self-antigens are deleted (Klein *et al.*, 2014). One possibility to overcome this hampered immune function against tumor antigens is the transfer of TCR-transduced T cells. This also allows applying the concept of ACT to a broader range of human cancers by genetic modification of T lymphocytes with various anti-tumor receptors. First proof to transmit functional specificity from one T cell to another was achieved by transferring α- and β-chain genes from one T cell clone to a different one (Dembic *et al.*, 1986). TCR-transduced T cells were then applied in mouse models (Kessels *et al.*, 2001) and afterwards, it was demonstrated in 2006 that autologous lymphocytes retrovirally transduced with a TCR encoding the MART-1 receptor led to reduced tumor burden in two out of 15 human patients (Morgan *et al.*, 2006). During this study it became evident that the TCR needs to have a high avidity against its target. One method to achieve this is to immunize HLA-transgenic mice with human tumor antigens to generate HLA-restricted T cells against the desired antigens. This approach has been applied to several cancer antigens, such as gp100 (Johnson *et al.*, 2009), MAGE-A3 (Chinnasamy *et al.*, 2011) and carcinoembryonic antigen (CEA) (Parkhurst *et al.*, 2011). Another antigen, the cancer/testis antigen NY-ESO-1 for melanoma and synovial cell

sarcoma, provides the best clinical example for TCR-transduced T cells. Nine of 17 patients underwent objective responses and two out of eleven melanoma patients had complete remission for more than twelve months (Robbins *et al.*, 2011). Yet, careful attention has to be paid to unintended cross-reactivity since these TCRs have not undergone thymic selection against the full repertory of physiologic human proteins.

Other boundaries to TCR gene transfer include the risk of mispaired transgene and native TCR chains, which can lead to dangerous neo-reactivity including autoreactivity as observed *in vitro* for human T cells (van Loenen *et al.*, 2010), but not during *in vivo* clinical trials. In murine studies a fatal GVHD-like syndrome occurred due to TCR mispairing (Bendle *et al.*, 2010). To decrease mispairing, the transgene α and β chains can be modified with different methods: adding a disulfide bond between the TCR constant domains (van Loenen *et al.*, 2010), including cysteines in the constant region (Kuball *et al.*, 2007) or codon modification (Scholten *et al.*, 2006). Similarly, knocking down the endogenous TCR β chain with a zinc finger nuclease or siRNA can reduce mispairing (Okamoto *et al.*, 2009; Ochi *et al.*, 2011; Provasi *et al.*, 2012) and additional knock down of endogenous MHC might even make allogeneic T cell banks imaginable (Torikai *et al.*, 2013).

I.1.4 Improvement of T cell specificity by chimeric antigen receptor gene transfer

One of the characteristic properties of TCRs is the fact that they specifically bind to antigens presented by the patient's MHC molecules, which uniquely allows them to detect intracellular proteins such as Wilms' Tumor-1 (Xue *et al.*, 2005). However, this is associated with two drawbacks: tumor cells escape the immune system by down-regulation of MHC molecules (Ryu *et al.*, 2001) or antigen processing defects, and generation of TCRs restricted to every MHC haplotype impedes clinical applicability as an "off-the-shelf" product. To overcome these negative aspects, CARs have been developed in which the characteristics of TCRs and antibodies are combined (Eshhar *et al.*, 1993). Antibodies bind epitopes in an MHC-independent manner and are specific not only for proteins but also carbohydrates or lipids that are potentially overexpressed by tumor tissue (Mezzanzanica *et al.*, 1998; Westwood *et al.*, 2005). In a CAR, which is a synthetic receptor, the antibody variable regions of the heavy and light chain are connected to intracellular TCR signaling domains, such as CD3ζ, CD28 (Maher *et al.*, 2002) or 4-1BB/CD137 (Imai *et al.*, 2004; Song *et al.*, 2011). With the T cell signaling moieties CAR T cells can respond to their respective antigen without costimulatory ligands on the tumor.

One of the most prominent examples for CAR development is the CD19 antigen, which is a surface epitope on about 90% of malignant B cell diseases and on B cells of different development stages. In 2010, an anti-CD19 CAR was first clinically applied and it was reported that a patient with B cell lymphoma underwent cancer regression for now four years after infusion of autologous genetically modified lymphocytes expressing an anti-CD19 CAR (Kochenderfer *et al.*, 2010) (**Figure iii**).

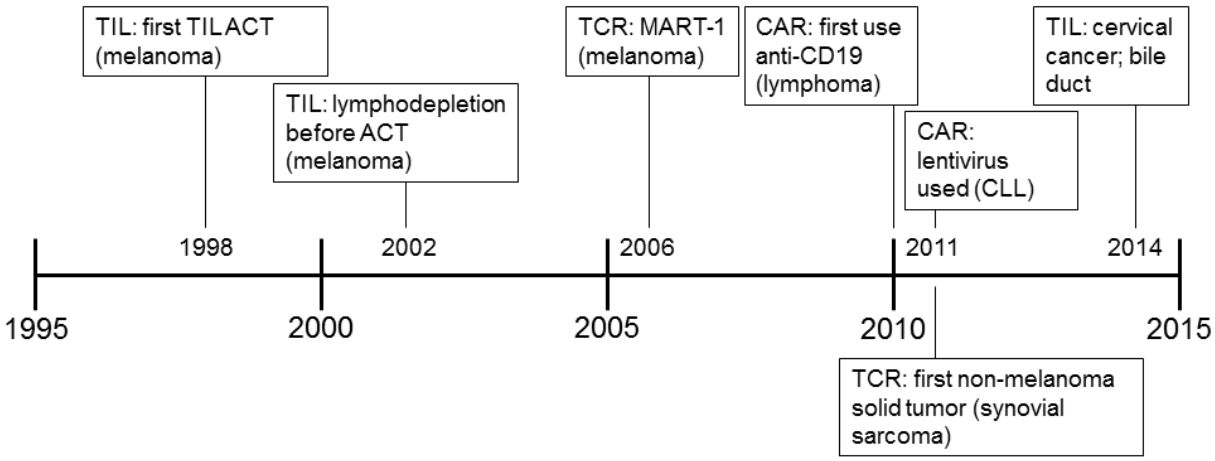

Figure iii: Selected clinical ACT trials for human cancer treatment.

This CAR has now also been applied for other types of lymphoma including follicular lymphoma and large-cell lymphomas, as well as chronic and acute lymphocytic leukemia, adding up to a total of about 200 treated patients worldwide (Kalos *et al.*, 2011; Porter *et al.*, 2011; Brentjens *et al.*, 2013; Grupp *et al.*, 2013; Kochenderfer *et al.*, 2015). Other target antigens currently being investigated include CD33 or CD123 for AML (Gill *et al.*, 2014; Pizzitola *et al.*, 2014) and CD30 expressed on Reed-Sternberg cells for Hodgkin lymphoma (Savoldo *et al.*, 2007). It is more difficult to generate CARs for solid compared to hematological malignancies because their antigens are often shared with essential normal cells. One exception is GD2 expressed on neuroblastomas (Louis *et al.*, 2011). Cancer/testis antigens were thought to be a suitable target because 80% of cancers upregulate it. However, only 10% express protein levels exceeding the threshold for sufficient recognition by anti-cancer T cells (Scanlan *et al.*, 2002).

As with other ACT approaches, persistence of T cells is a crucial area of interest. Possible solutions include transduction with cytokine encoding genes (Hoyos *et al.*, 2010) and depots of cytokines in cell surface-bound nanoparticles (Stephan *et al.*, 2010). These and other advances, such as different types of CAR generations or dual targeting strategies, will further foster application of CAR treatment for cancer.

I.1.5 On- and off-target toxicity

Improving the potency of adoptively transferred T cells bears the risk of increasing toxicity as well. On-target off-tumor toxicity occurs when the targeted antigen is not only overexpressed on tumor cells but also found on normal cells. Therefore, a suitable target antigen is only expressed by the tumor, or on normal tissue not indispensable for survival. As an example, in the previously mentioned anti-MART-1 study (Morgan *et al.*, 2006), which was subsequently expanded to 36 patients receiving anti-MART-1 or anti-gp100 melanoma-melanocyte antigen TCR transduced T cells (Johnson *et al.*, 2009), on-target toxicity developed in skin (vitiligo), eyes (uveitis) and inner ears because of physiologic expression of melanocytes in these sites. In another study investigating an anti-CEA TCR against metastatic colorectal cancer, critical colitis and colon bleeding occurred (Parkhurst *et al.*, 2011). Not only TCR-, but also CAR-transduced lymphocytes bear the risk of on-target toxicity: autologous anti-

CD19 CAR T cells led to B cell depletion in peripheral blood and bone marrow, which can be treated with intermittent infusions of immunoglobulin, and they also destroyed huge chronic lymphocytic leukemia tumor masses and led to tumor lysis syndrome, which is an example for on-target on-tumor toxicity (Kalos *et al.*, 2011; Porter *et al.*, 2011). Moreover, an anti-carbonic anhydrase 9 CAR against renal cell carcinoma caused serious liver toxicity and cholangitis because this antigen is also present on biliary duct epithelium (Lamers *et al.*, 2013) and a lethal pulmonary untoward event was noticed with CAR T cells recognizing the receptor tyrosine-protein kinase ERBB2 in a patient with metastatic colon cancer (Morgan *et al.*, 2010). In this case, CAR T cells were activated after binding the antigen target in the lungs. This in turn produced cytokine release syndrome that usually occurs in case of high tumor burden. Days after ACT the patient presented with fever, hypotension and increased levels of IFNγ, tumor necrosis factor-α, IL-6 and IL-10 which ultimately led to complications and intensive care unit admission. Corticosteroids and an anti-IL-6 antibody, tocilizumab, can be used as supportive treatment (Grupp *et al.*, 2013). Consequently, attentive selection of cell dosing and preparative lymphodepletion are a prerequisite.

Off-target toxicity is caused by four mechanisms. First, it occurs due to binding of an unknown antigen on normal cells. This happened when an affinity-improved anti-MAGE-A3 TCR, where site-specific mutations were generated by exchanging amino acids, was used. The antigen targeted was an extraneous cardiac muscle protein, titin, and this led to cardiogenic shock, myocardial necrosis and lethal outcome in one myeloma and one melanoma patient (Linette *et al.*, 2013). These adverse events could occur because physiologic negative selection of highly reactive TCRs against self-antigens in the thymus is not possible in case of ACT with mature T cells. Second, undiscovered cross-reactivity can cause toxicity: An anti-MAGE-A3 TCR, targeting a cancer/testis antigen, led to serious toxicity in the gray matter of the brain resulting in two fatal cases. The TCR also targeted MAGE-A12, which is closely related to MAGE-A3 and is expressed in brain tissue (Morgan *et al.*, 2013). Third, if allogeneic T cells are transferred there is the risk of GVHD as known for allogeneic HSCT and DLI. Last, off-target toxicity separate of target antigen binding can occur and includes fever, chills, myalgias and hypoxia transiently after infusion. Seldom, severe toxicities have occurred, culminating in one death due to a blood culture-negative sepsis-like syndrome (Brentjens *et al.*, 2010; Brentjens *et al.*, 2011).

I.1.6 Adoptive cell therapy with precursor T cells

Transferring mature T cells is associated with several drawbacks that for instance are related to the phenotype. Murine preclinical investigations pointed out that transducing T cells in earlier phenotypic differentiation leads to ameliorated anti-cancer responses (Klebanoff *et al.*, 2005). This finding was supported by monkey experiments where injected central memory cells proved to persist longer than effector memory cells (Berger *et al.*, 2008). Furthermore, the phenotypic differentiation stage of CD8+ T cells inversely correlated to *in vivo* expansion and persistence (Gattinoni *et al.*, 2005; Buchholz *et al.*, 2013; Gerlach *et al.*, 2013). This is important in clinical application and trials, where early differentiation T cells are also positively associated with enhanced effectiveness (Rosenberg *et al.*, 2011). Moreover, mature T cell transfer can cause GVHD and requires an MHC-

matched donor. Therefore, it is often reprimanded to be impractical for wider application. "Off-the-shelf" products, in the form of preTs, could be more extensively distributed. However, their *in vitro* generation has only recently been made possible on a larger scale.

Formerly, T cell development was thought to require a three-dimensional thymus environment (Hare *et al.*, 1999). Therefore, fetal thymus organ cultures (Jenkinson *et al.*, 1982) and reaggregate thymic organ cultures (Anderson *et al.*, 1993) were developed. However, little cell expansion and technical difficulties with thymic explants represent some limitations. Next, bone marrow stromal cell lines, such as S17 and MS5, were established to research hematopoiesis, but no T cell development was observed (Collins *et al.*, 1987; Suzuki *et al.*, 1992). Then, in 2002, another non-three-dimensional system was developed based on the finding that the Notch pathway is crucial for T cell development (Ohishi *et al.*, 2002; Schmitt *et al.*, 2002; Schmitt *et al.*, 2004). For this, the macrophage colony stimulation factor-deficient bone marrow stromal cell line OP9 was transduced to express Delta-like 1, a Notch ligand, that was shown to successfully generate murine preTs and mature CD8 T cells (Zakrzewski *et al.*, 2006; Zakrzewski *et al.*, 2008; Dervovic *et al.*, 2012). Zakrzewski and colleagues were the first to preclinically test murine hematopoietic stem cell-derived preTs. They coinjected preTs with T cell depleted allogeneic bone marrow into mice. ACT with preTs enhanced T cell reconstitution and general immunity as seen by improved resistance to pathogens after infection and higher cellularity in the thymus. Also, there was no GVHD observed and tumor-bearing mice showed improved survival. When they characterized the engrafting preT population, it showed a thymic double negative 2 phenotype ($CD44^+CD25^-$), which was achieved by high dose IL-7 leading to blockage of T cell development (Zakrzewski *et al.*, 2006). After these promising results, they could also show that allogeneic preTs can be infused regardless of MHC-mismatch and develop to host-MHC restricted T cells. When the preTs were then equipped with an anti-human CD19 CAR, lymphoma cell line bearing mice survived longer (Zakrzewski *et al.*, 2008).

Not only murine, but also human HSCs from fetal liver, fetal thymus (Weerkamp *et al.*, 2006), umbilical cord blood (UCB) (La Motte-Mohs *et al.*, 2005) and bone marrow (De Smedt *et al.*, 2004) undergo T cell development during OP9-DL1 co-culture.

I.1.7 Safety switches for the use of engineered T cells

It would be desirable to eliminate transferred T cells in case of toxicity. In order to achieve this, conditional safety switches which can be triggered to induce cell death can be introduced to T cells. They can be categorized depending on their mechanism: metabolic, dimerization induced and triggering by monoclonal antibodies.

The first clinically relevant safety switch was herpes simplex viral thymidine kinase (HSVtk). After infusion of ganciclovir, HSVtk metabolizes it to ganciclovir triphosphate which incorporates into DNA and leads to chain termination and cell death. Additionally, cell death is also achieved by CD95 aggregation and subsequent formation of Fas-associated death domain protein and caspase-8 complex (Beltinger *et al.*, 1999). Until now, 148 patients after HLA-identical and haploidentical HSCT have been treated with HSVtk-modified T cells and all cases of GVHD were successfully controlled (Ciceri *et al.*, 2009). Yet, HSVtk was found to have several

weaknesses: elimination of GVHD-causing DLI T cells after allo-HSCT was incomplete due to non-targeting of quiescent cells, activity onset is slow (Bonini *et al.*, 1997; Tiberghien *et al.*, 2001), using ganciclovir for CMV infection is excluded, and immunogenicity of the transgene (Berger *et al.*, 2006). Immunocompetent patients are capable of mounting an immune response against HSVtk antigens, which leads to undesired T cell elimination (Traversari *et al.*, 2007). However, the advantages outweigh, therefore HSVtk is being evaluated in a randomized phase III clinical trial. Another clinically used suicide gene is E. coli-derived cytosine deaminase gene (Freytag *et al.*, 2002), but because it is a xenoantigen it is also probable to cause host immune reactions.

One of the other promising approaches is human iCasp9 (Straathof *et al.*, 2005). It was successfully tested in four patients after MHC-matched HSCT and allodepleted iCasp9-transduced DLI. Induction of the suicide gene reversed cutaneous and hepatic GVHD within minutes, but similar to HSVtk the elimination of transduced T cells was incomplete (Di Stasi *et al.*, 2011). In addition, iCasp9 has also shown promising results in combination with anti-CD19/-CD20 CARs (Hoyos *et al.*, 2010; Budde *et al.*, 2013). iCasp9 has several advantages over HSVtk: less immunogenicity, rapid induction of apoptosis and functional activity against non-dividing cells.

Other strategies, that so far have only been tested in pre-clinical settings, include a truncated human EGF receptor (EGFRt), CD20, a c-myc protein tag consisting of ten amino acids (Kieback *et al.*, 2008), and RQR8 which combines CD34 and CD20 epitopes (Philip *et al.*, 2014). Cytotoxicity can be induced by a monoclonal antibody (mAb) in these cases, like cetuximab for EGFRt and rituximab for CD20 (Griffioen *et al.*, 2009; Wang *et al.*, 2011). As an advantage, EGFRt and CD20 not only allow *in vivo* elimination but also previous selection of transduced cells *ex vivo*. However, biodistribution of mAbs compared to small molecules like ganciclovir or the dimerizer agent for iCasp9 is smaller and in the case of CD20, activation of the safety switch also leads to undesired elimination of normal B cells therefore limiting clinical compatibility.

I.1.8 Genetic engineering of T cells and hematopoietic stem cells

Until now, mainly retroviral vectors, especially gamma- and lentiviral, were used for transduction of lymphocytes (**Table i**). The benefit is that the transgene becomes permanently integrated into the recipient cell genome. Nonviral attempts include transposon-transposase systems (Singh *et al.*, 2014), mRNA (Kenderian *et al.*, 2015) or CRISPR-cas (Doudna *et al.*, 2014). In case transient expression for a few days is desired, adenoviral vectors or mRNA transfection can be used (**Figure iv**).

Genus	Example
Alpharetroviruses	Rous sarcoma virus
Betaretroviruses	mouse mammary tumor virus
Gammaretroviruses	Moloney murine leukemia virus
Deltaretroviruses	human T lymphotropic virus
Epsilonretroviruses	Walleye dermal sarcoma virus
Lentiviruses	human immunodeficiency virus 1
Spumaviruses	human foamy virus

Table i: Retroviral genera.

Nowadays, most retroviral vectors are either murine or avian retrovirus derivates. The most extensively studied one is the Moloney murine leukemia gammaretrovirus (Uchida *et al.*, 1986).

In 1990, gammaretroviruses were first used for human trials of immunotherapy with genetically engineered T cells. Two patients with adenosine deaminase severe combined immunodeficiency (SCID) received gene-corrected T cells (Blaese *et al.*, 1995). Not only T cells, but also HSCs can be retrovirally modified. At first, HSCs were thought to be the most suitable population for retroviral gene modification, but in a murine model leukemia was caused by retroviral insertion (Li *et al.*, 2002). Moreover, side effects such as leukemia generation also occurred in human SCID-X1 and X-CGD gene therapy trials because of vector integrations near proto-oncogenes (Hacein-Bey-Abina *et al.*, 2008). It has been shown that progenitor cells are more likely to undergo transformation after retroviral insertion compared to more mature cell types (Kustikova *et al.*, 2009). Likewise, T cells are more resistant to transformation than HSCs (Newrzela *et al.*, 2008). Nevertheless, insertional transformation can occur *in vitro* in transduced mature T cells, as seen after activation of a proto-oncogene, like LMO2, and a synergistic effect via T cell signaling cytokines, like IL-2 or IL-15 (Newrzela *et al.*, 2011). Fortunately, until now malignant transformation has not occurred in trials of retrovirally engineered mature T cells (Scholler *et al.*, 2012). Apart from gammaretroviruses, also lentiviral vectors have been developed, mostly derived from human immunodeficiency virus. Their advantages are the possibility to transduce quiescent cells (Naldini *et al.*, 1996) and a more neutral integration site spectrum (Biffi *et al.*, 2011). So far, no oncogenic events have been reported from clinical trials (Levine *et al.*, 2006), but dominance of hematopoietic progenitors was observed in murine long-term hematopoiesis (Kustikova *et al.*, 2005) and when HSCs were engineered for thalassemia treatment (Cavazzana-Calvo *et al.*, 2010). Hence, insertional mutagenesis remains a safety concern also for lentiviral vectors. A new generation of retroviral vectors derived from alpharetroviruses has been reported to possess an even more neutral integration pattern and was constructed to not contain splice sites that could potentially interfere with mRNA processing (Suerth *et al.*, 2010; Suerth *et al.*, 2012). These safety issues do not have to be considered for transient transgene expression as with adenovirus vectors or mRNA transfection. They are often used in clinical trials, especially for gene silencing strategies such as zinc-finger nucleases (Perez *et al.*, 2008). A

decreased risk for insertional mutagenesis is also achievable with non-viral delivery methods where integration is more random. Plasmid DNA transfection using electroporation has been tested clinically in neuroblastoma, but T cell persistence was short (Park *et al.*, 2007). In contrast, transposons are more efficient than plasmids which do not harbor an integrating element (Dupuy *et al.*, 2005). A transposon is a mobile genetic component capable of insertion into the genome, even without sequence homology. The transposon system has been successfully applied for transfer of T cell receptors and for an anti-CD19 CAR (Kebriaei *et al.*, 2012; Field *et al.*, 2013). Apart from reduced gene toxicity, non-viral methods are also time and money saving and less immunogenic (Hackett *et al.*, 2010).

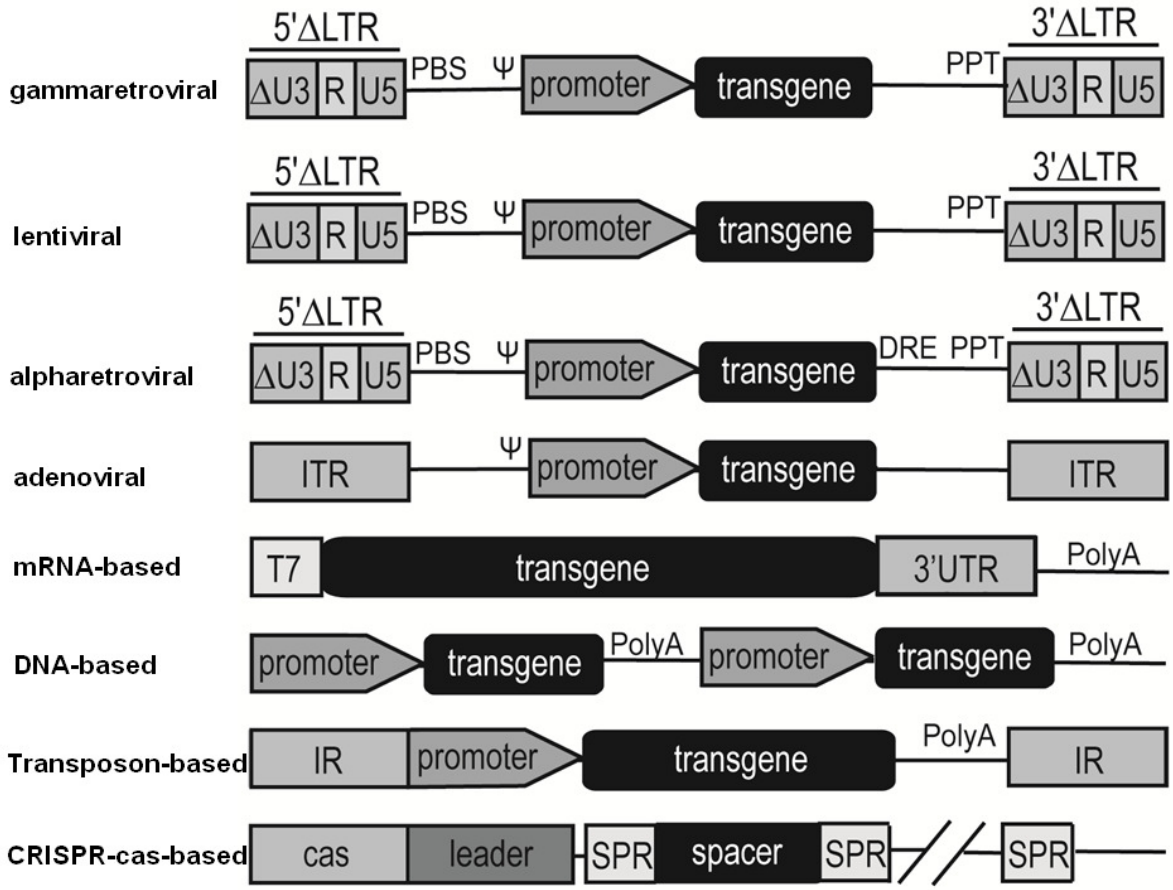

Figure iv: Vector systems for adoptive therapy. Indicated are unique 5 (U5), repeat (R), and self-inactivating unique 3 (ΔU3) regions, long terminal repeat (LTR), primer binding site (PBS), packaging signal (ψ), polypurine tract (PPT), direct repeat element (DRE, typical part of alpharetroviruses involved in RNA processing), inverted terminal repeat (ITR), T7 RNA polymerase (T7), untranslated region (UTR), poly(A) tail (PolyA), internal repeat (IR), and short palindromic repeats (SPR).

II Hypotheses and Aims of the Study

Adoptive transfer of TCR gene-engineered mature T cells is limited by some disadvantages:

a) necessity for strong *in vitro* prestimulation of T cells hampering their *in vivo* function and persistence
b) need for an HLA-matched donor
c) adverse effects of genetically engineered T cells such as on-target or off-target effects, and insertional mutagenesis

Hence we hypothesized that:

a) human precursor T cells can be effectively generated from frozen cord blood-derived CD34+ cells
b) engineering of cord blood-derived CD34+ cells allows generation of transgene positive precursor T cells
c) alpharetroviral platforms can be used to generate engineered precursor T cells expressing clinically relevant genes

III Methods

Primary samples and cell lines

Human UCB samples (approximately 50 mL/sample), that were not eligible for banking, were obtained after written, informed consent by the child's mother. Procedures for the use of UCB for this study were reviewed and approved by the medical ethics committee of Hannover Medical School. UCB mononuclear cells were isolated using Ficoll density centrifugation and CD34 selection was performed using a CD34 microbead kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions (**Figure v**). The purity of CD34+ cells was higher than 95% as determined by post-enrichment flow cytometric analysis. CD34+ cells were cryopreserved in 70% Roswell Park Memorial Institute (RPMI) medium, 20% human AB serum and 10% dimethylsulfoxide.

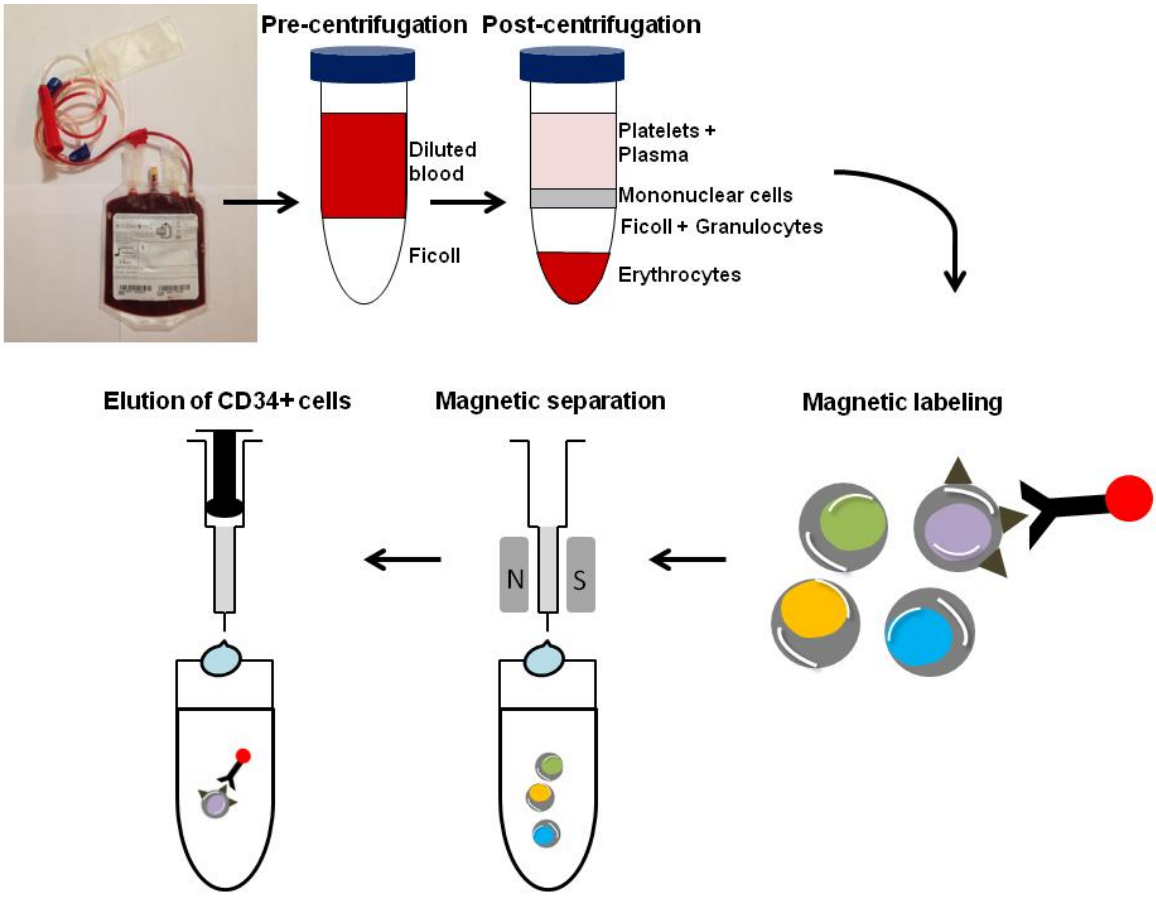

Figure v: CD34+ separation from UCB.

OP9-DL1 cells were cultured in minimum essential medium alpha containing 20% heat-inactivated fetal calf serum (FCS). CD34+ cells were transferred on 90% confluent OP9-DL1 cell monolayers containing 20% FCS, stem cell factor (SCF) (20ng/mL), thrombopoietin (TPO) (20ng/mL; until day 24), FMS-like tyrosine kinase 3 ligand (Flt3L) (10ng/mL) and IL-7 (10ng/mL, all cytokines from PeproTech, Rocky Hill, NJ, USA). Every four days, preTs were harvested, passed through a 70µm filter and transferred to new OP9-DL1 cell monolayers.

Human embryonic kidney 293T cells and the fibrosarcoma cell line HT1080 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. TdTomato.CD123-expressing 293T cells were generated by transduction with a gammaretroviral vector encoding TdTomato and CD123 linked by an internal ribosomal entry site (IRES) sequence. Acute T cell leukemia Jurkat cells were cultured in RPMI medium containing 10% FCS.

Vector construction and cloning

We utilized self-inactivating (SIN) alpharetroviral vectors, where transcriptional control elements are removed from the long terminal repeats (LTRs) to enhance genosafety, containing a myeloproliferative sarcoma virus variant (MPSV) or elongation factor 1 short-form (EFS) promoter and a woodchuck posttranscriptional regulatory element (PRE) (Gerull *et al.*, 2007). Enhanced green fluorescent protein (EGFP) was cloned into the constructs and expressed via an IRES sequence.

Inducible caspase 9 was kindly provided by Malcolm K. Brenner, Baylor College of Medicine, Houston, TX, USA. It is comprised of a human FK506 binding protein (FKBP12) containing an F36V mutation fused via an SGGGS linker to human caspase 9, and was linked by a 2A sequence to truncated human CD19 (ΔCD19). The F36V mutation enhances the binding capacity of FKBP12 to the dimerizer agent B/B Homodimerizer (Clontech, Palo Alto, CA, USA), which is a synthetic nontoxic FK506 analog that upon administration leads to aggregation and activation of iCasp9 monomers and eventually induction of apoptosis (**Figure vi**).

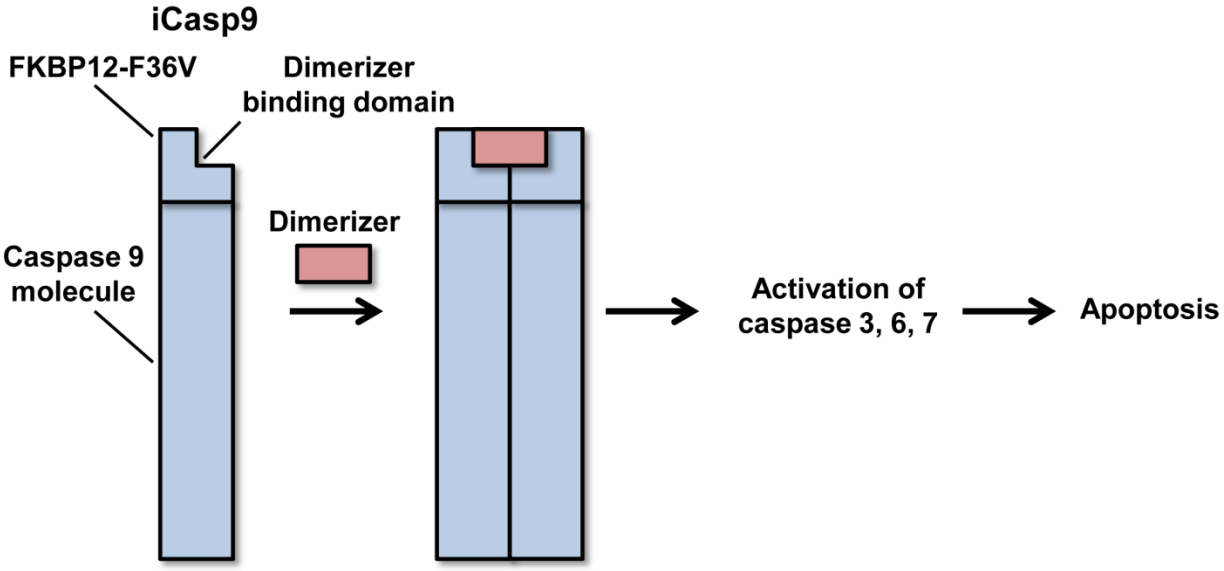

Figure vi: iCasp9-mediated apoptosis.

A third generation CD123-specific CAR containing the codon-optimized sequences for a CD123-specific single chain variable fragment (scFv), the transmembrane region of the human CD28 molecule, the co-stimulatory signaling endodomains of CD28 and 4-1BB, and the CD3ζ signaling domain, was cloned into a SIN alpharetroviral backbone driven by the MPSV promoter. The amino acid sequence is as follows:

MLLLVTSLLLCELPHPAFLLIPDIVMTQSHKFMSTSVGDRVNITCKASQNVDSAVAWYQQKPGQSPKALIYSASYRYSGVPDRF
TGRGSGTDFTLTISSVQAEDLAVYYCQQYYSTPWTFGGGTKLEIKRGGGGSGGGGSGGGGSGGGGSEVKLVESGGGLVQPG

GSLSLSCAASGFTFTDYYMSWVRQPPGKALEWLALIRSKADGYTTEYSASVKGRFTLSRDDSQSILYLQMNALRPEDSATYYC
ARDAAYYSYYSPEGAMDYWGQGTSVTVSSAAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGGVL
ACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRFSVVKRGRKKLLYIFKQPFMRPVQTT
QEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNE
LQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR.

EGFP or iCasp9 linked by an IRES sequence was cloned downstream of the CAR cassette.

Several generations of CARs now exist. The first one connected the variable heavy and light chain of an antibody to the transmembrane und cytoplasmic part of the CD3ζ signaling moiety. This generation resulted in poor T cell expansion and activation (Brocker *et al.*, 1995) likely because of missing costimulation leading to anergy. Therefore, the CD28 costimulatory endodomain or 4-1BB (CD137) was added to recapitulate the two-signal model postulated for activation of T cells (Hombach *et al.*, 2001; Maher *et al.*, 2002). Adding a third domain such as CD27, OX40 (CD134) or 4-1BB further enhanced T cell activation (Finney *et al.*, 2004) (**Figure vii**). Fourth generation CARs are additionally engineered to produce cytokines, for instance IL-12 (Pegram *et al.*, 2012).

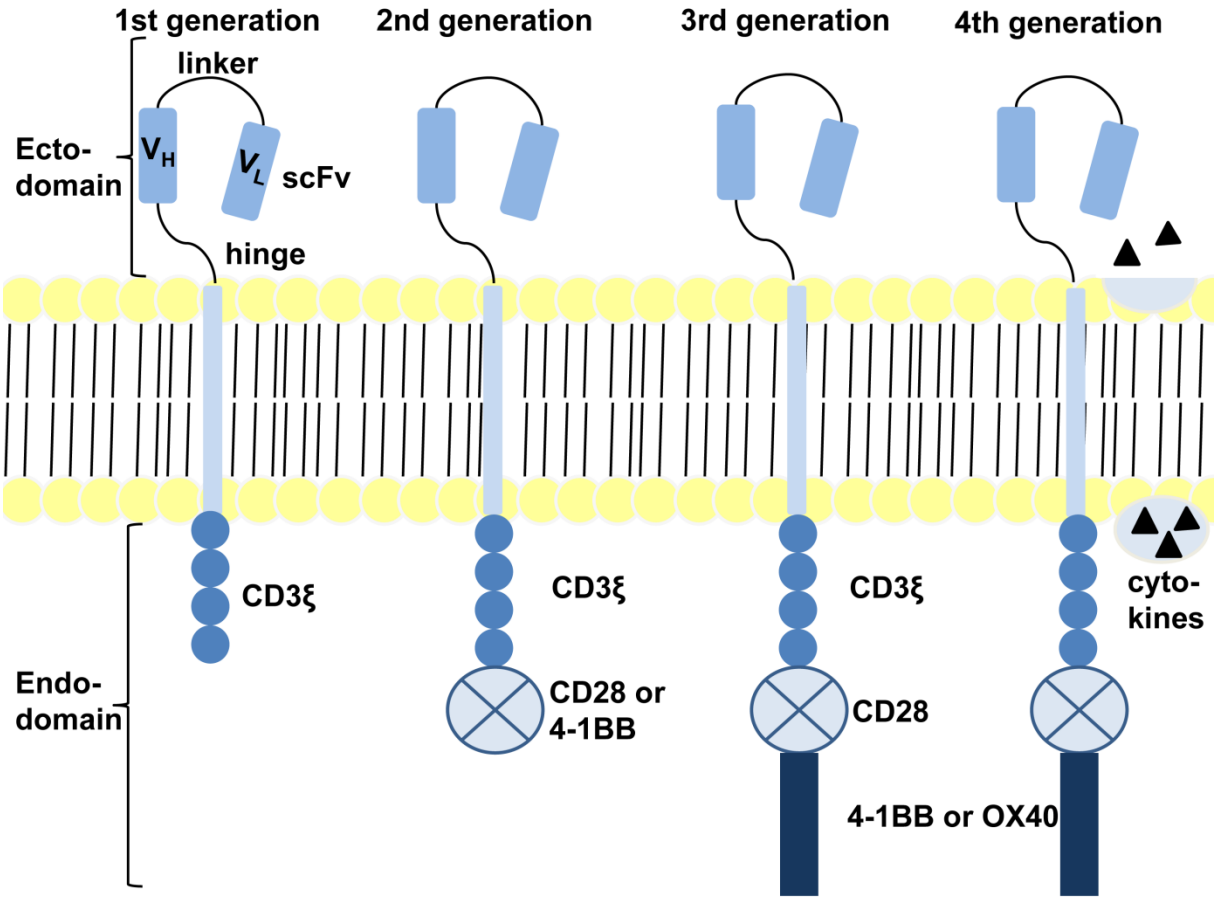

Figure vii: CAR generations. Indicated are variable heavy chain (V_H), variable light chain (V_L) and single chain variable fragment (scFv).

Cell transduction

For transient viral production, 293T cells were transfected using a calcium phosphate transfection kit (Sigma Aldrich, Steinheim, Germany) with MPSV or EFS constructs. They were combined with plasmids encoding retroviral structural and enzymatic proteins gag/pol and either a modified cat retrovirus glycoprotein (RD114/TR) (kindly provided by Els Verhoeyen, Lyon, France) or the vesicular stomatitis virus glycoprotein (VSVG) envelope (Sandrin *et al.*, 2002). Retroviral supernatant was harvested 36-48 hours after transfection, filtered through Millex-GP 0.22 µm filters (Millipore, Schwalbach, Germany) and either freshly used or concentrated by ultra-centrifugation, immediately frozen in dry ice and stored at -80°C for further usage. HT1080 cells were used for determining the viral titer.

Before transduction, CD34+ cells were pre-stimulated for 36 hours in X-VIVO 10 serum free medium (Lonza, Basel, Switzerland) at a maximal density of 0.6×10^6/mL per well of a 6-well plate in the presence of SCF, TPO and Flt3L (all 100 ng/mL). For the transduction of Jurkat cells and UCB-derived CD34+ cells, 24-well plates (ThermoScientific, Rockford, IL, USA) were coated with Retronectin (Takara, Otsu, Japan), a recombinant fibronectin fragment, overnight at 4°C. Retroviral supernatant was added and plates were spin-oculated (490xg, 1 hour, 4°C) to facilitate retrovirus binding to Retronectin. Subsequently, retroviral supernatant was removed and up to 15×10^4cells were added per well.

Flow cytometry

Cell suspensions were washed and incubated with 5% normal rat serum for 15 minutes at 4°C to block unspecific Fc-binding of fluorochromes. Transduction efficiency, cell-surface phenotype, and viability were assessed using the following fluorochrome-conjugated antibodies purchased from BioLegend (San Diego, CA, USA): CD3 (PerCPCy5.5), CD4 (Brilliant Violet 570™), CD5 (Brilliant Violet 421™), CD8 (PE), CD34 (PECy7), CD45RA (APCCy7), CD123 (APC), Annexin V (PE), or BD Biosciences (San Jose, CA, USA): CD7 (APC), CD19 (PE), and Annexin V (APC). To stain the CD123 CAR, human IL-3 receptor α/CD123 protein with a His Tag (Sino Biological, Beijing, China) and an anti-His Tag antibody (APC) (R&D Systems, Minneapolis, MN, USA) were used. Data were acquired using a FACSCanto or LSRII (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (TreeStar, Ashland, OR, USA). In all analyses, the population of interest was gated based on forward versus side scatter plot followed by doublet exclusion. GFP-expressing large OP9-DL1 cells were gated out through GFP expression and forward and side scatter characteristics. Numbers in quadrant corners represent percent of gated cells. Untransduced cells were used as control to set the negative gate.

Quantitative reverse transcription polymerase chain reaction (qRT PCR) for determination of vector copy numbers

Genomic DNA was isolated from transduced CD34+ cells and vector copy numbers determined by using the TaqMan system (Qiagen, Hilden, Germany). Quantitative PCR was performed on an Applied Biosystems Step One Plus real-time PCR (Darmstadt, Germany) (Suerth *et al.*, 2010). The primers are specific for the vector PRE and

the PTBP2 intron (Rahman *et al.*, 2004). Vector copy numbers of CD34+ cell samples were analyzed based on the mathematical model proposed by Pfaffl (Pfaffl, 2001).

Induction of apoptosis

Different cell populations were transduced with alpharetroviral vectors containing iCasp9. Jurkat cells transduced with vectors containing the iCasp9 and the ΔCD19 gene were sorted for CD19 using a FACSAria cell sorter (BD Biosciences, San Jose, CA, USA). Alternatively, a construct encoding CD123 CAR and iCasp9 was used for transduction of human peripheral blood mononuclear cells (PBMCs) or preTs. B/B Homodimerizer was added at increasing concentrations to transduced Jurkat cells. After 48-72 hours, cells were harvested, washed with Annexin V binding buffer (BD Biosciences, San Jose, CA, USA), and stained with Annexin V and CD19 according to the manufacturer's instructions. Analysis was performed within one hour by flow cytometry for apoptotic cells.

Generation of CAR T cells and cytotoxicity assay

For the assessment of the CAR functionality *in vitro*, PBMCs were isolated from blood samples of healthy volunteers using Ficoll-Paque PLUS reagent (GE Healthcare, Uppsala, Sweden) and were activated for two days with anti-CD3 antibody (50 ng/mL), anti-CD28 antibody (500 ng/mL), and IL-2 (25 U/mL). Cells were transduced on two consecutive days with alpharetroviral supernatant containing the CD123 CAR vector. After further expansion for four days, effector and target cells (293T cells expressing CD123 and tdTomato linked via an IRES sequence) were co-cultured at indicated ratios for two days. Cytotoxicity was assessed by fluorescence microscopy or flow cytometry for CD3.

Enzyme-linked immunosorbent assay (ELISA)

T cells ($2x10^5$) and target cells ($2x10^4$) were incubated (effector:target ratio of 10:1) in V-bottom 96 well plates in the presence of IL-2 (25 U/mL) and IL-7 (5 ng/mL). After 24 hours, the culture supernatant was harvested and used in duplicates for an IFN-γ ELISA (BioLegend, San Diego, CA, USA).

Mice

Animals in the experiments were used under protocols approved by the State Government of Lower Saxony, Germany. NOD.cg-Prkdc^{scid}IL2rg$^{tm/Wjl}$/Sz (NSG) mice were purchased from Charles River, housed and bred in a pathogen free facility.

Hematopoietic stem cell transplantation

PreTs together with UCB-derived $CD34^+$ hematopoietic stem cells (HSCs) were intrahepatically injected into four day-old irradiated NSG mice (Awong *et al.*, 2013). Transgene-positive preTs were sorted on day 11 of OP9-DL1 co-culture. $2x10^5$ preTs together with $2x10^4$ HSCs were resuspended in 30 µl PBS containing IL-7 (2,5 µg) and the IL-7 antibody M25 (0.5 µg) and subsequently injected intrahepatically into irradiated (1 Gy) newborn mice.

Control mice only received injections of HSCs alone. Mice were boosted with the IL-7/M25 mixture every 5 days. Six weeks after injection, thymi were harvested and cells analyzed by flow cytometry.

Statistical analysis

Unless specified in the text, data were presented as mean ± standard error of the mean. The Student's t-test was used to determine the statistical significance of differences between samples. P values <0.05 were considered to be statistically significant.

IV Results

In vitro generation of preTs derived from human UCB CD34+ cells

For generation of human preTs, UCB was used as a source of CD34+ hematopoietic stem cells. CB samples were obtained from consenting mothers and CD34+ cells were selected via two rounds of magnetic-activated cell sorting (MACS). Before selection, 23.5±1.1% (n=2) of the cord blood mononuclear cells were CD34+. After realizing that one selection round only yielded about up to 73.1±4% (n=2) purity, a second round was added for subsequent experiments. The CD34+ purity was consistently higher (93.6±3.1%) when two purification steps were performed (**Figure 1**).

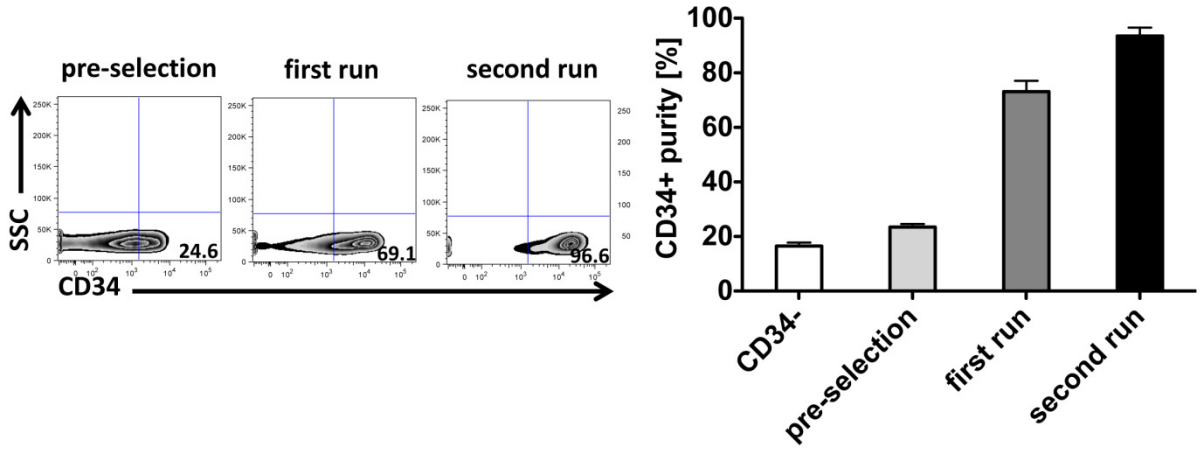

Figure 1: CD34+ cells were selected from UCB. CD34+ cells were isolated via positive selection by magnetic-activated cell sorting. Purity was determined by flow cytometry after one or two rounds of cell sorting (n=2).

One of our main aims in this project was to compare fresh and frozen CD34+ cells side by side, because frozen ones could potentially be used as an "off-the-shelf" product. Freshly isolated or thawed CD34+ cells were co-cultured with the Notch ligand-expressing OP9-DL1 stromal cells having previously been shown to support *in vitro* generation of human preTs. The kinetics of preT expansion, which were comparable for both, fresh and frozen, CD34+ cells, showed initially slower cell proliferation up to day 12 and a more rapid cell growth until day 28 yielding 10.15±4.46x10^6 (n=3) preTs in the fresh group (337.9±148.47 fold of the original cell input) and 14.41±4.45x10^6 (n=3) preTs (480±148.17 fold of the original cell input) in the frozen group. This was then followed by a plateau phase until day 40. On day 40, the final preT number derived from fresh CD34+ cells (28.4±23.4x10^6; 946.67±779.8 fold expansion, n=3) was comparable to the one from thawed CD34+ cells (44.36±18.84x10^6, 1479.67±629 fold expansion, n=3) (**Figure 2**).

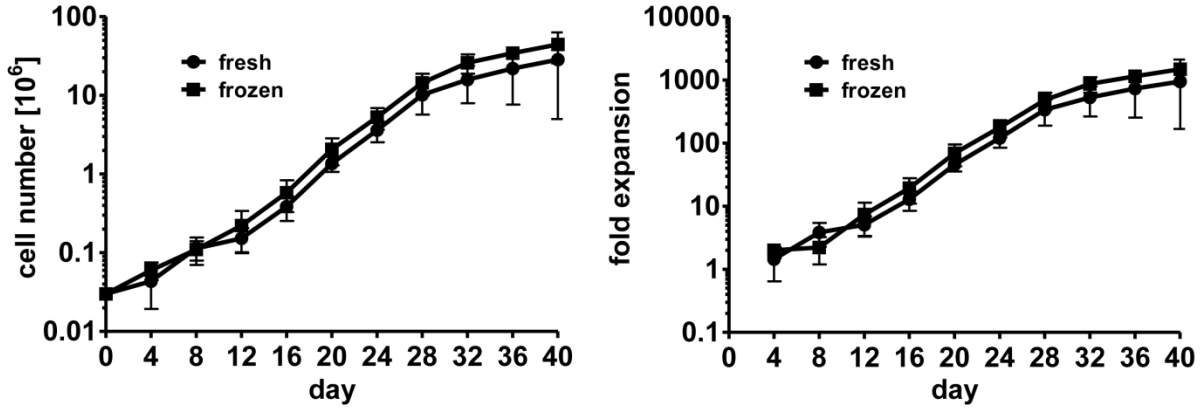

Figure 2: CD34+ cells expand on OP9-DL1 cells. Fresh or thawed CD34+ cells were co-cultured on OP9-DL1 stromal cells in the presence of SCF, TPO, Flt3L and IL-7 for preT differentiation. The proliferation rate of the cells was assessed every four days by Trypan blue staining (n=3).

To assess *in vitro* phenotype development, preTs were assessed by flow cytometry for the expression of CD34, CD45RA, CD5, CD7, CD4, CD8 and CD3 (**Figure 3**) and we used CD7 surface expression as a common marker for T cell development over time. The expression of CD34 decreased over time and disappeared by day 24 (36.23% on day 8, 11.13% on day 16 and 1.1% on day 24), which coincides with the increasing expression of T cell development markers CD45RA (81.8% on day 8, peaking 98.3% on day 16 and decreasing to 56.42% on day 40), CD7 (62.67% on day 8, stable expression from 88.15% on day 16 to 88.53% on day 40) and CD5 (35.05% on day 8, increasing to 79.81% on day 16 and staying stable from 89.4% on day 24 to 93.1% on day 40) during the culture period. A small population of cells only started to express CD3 by day 40. We did not observe the appearance of CD4 and CD8 positive cells over the whole culturing course. Of note, CD34+ CD7+ progenitor T cells that represent the thymus-engrafting population were most prominent on day 8 and had disappeared by day 24.

To conclude, after two-step purification of UCB, fresh and thawed CD34+ cells were expanded into substantial numbers and phenotypically differentiated into precursor T cells.

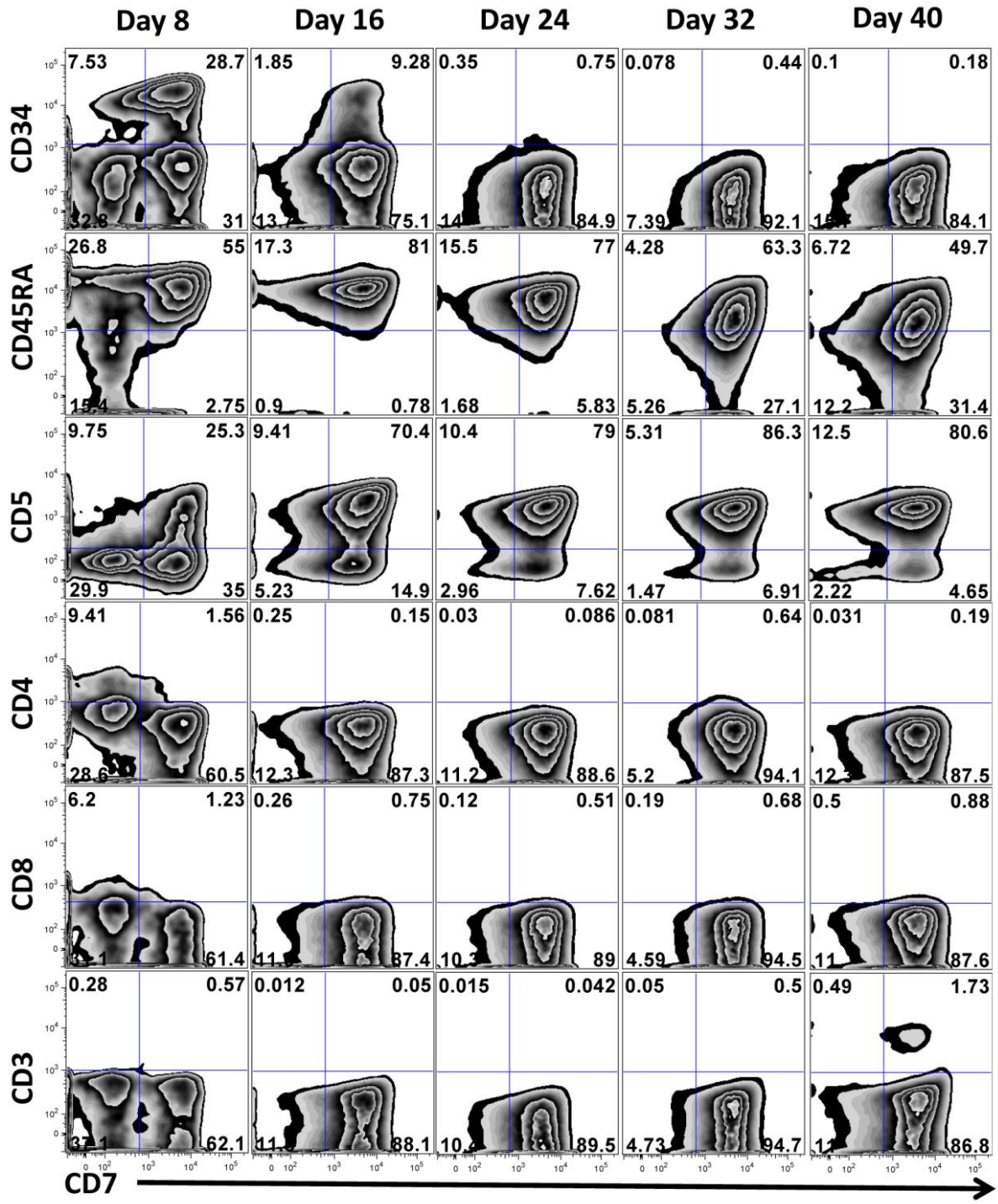

Figure 3: CD34+ cells phenotypically differentiate into preTs. The lymphoid phenotype of the cells was determined by multicolor flow cytometry serially every eight days for a period of 40 days. Results of a representative experiment are shown. Numbers in quadrants indicate percentage of cells.

Generation of alpharetrovirally engineered preTs derived from UCB CD34+ HSCs

To decrease the risk of insertional mutagenesis after transduction of CD34+ cells, we used SIN alpharetroviral vectors with a relatively neutral integration spectrum. To improve gene transfer into CD34+ cells, we compared alpharetroviral vectors containing two different promoters, either using the EFS or the MPSV promoter (**Figure**

4a). Respective retroviral particles were pseudotyped with either VSVG or RD114/TR envelope glycoprotein. EGFP was used as a reporter gene (**Figure 4b**).

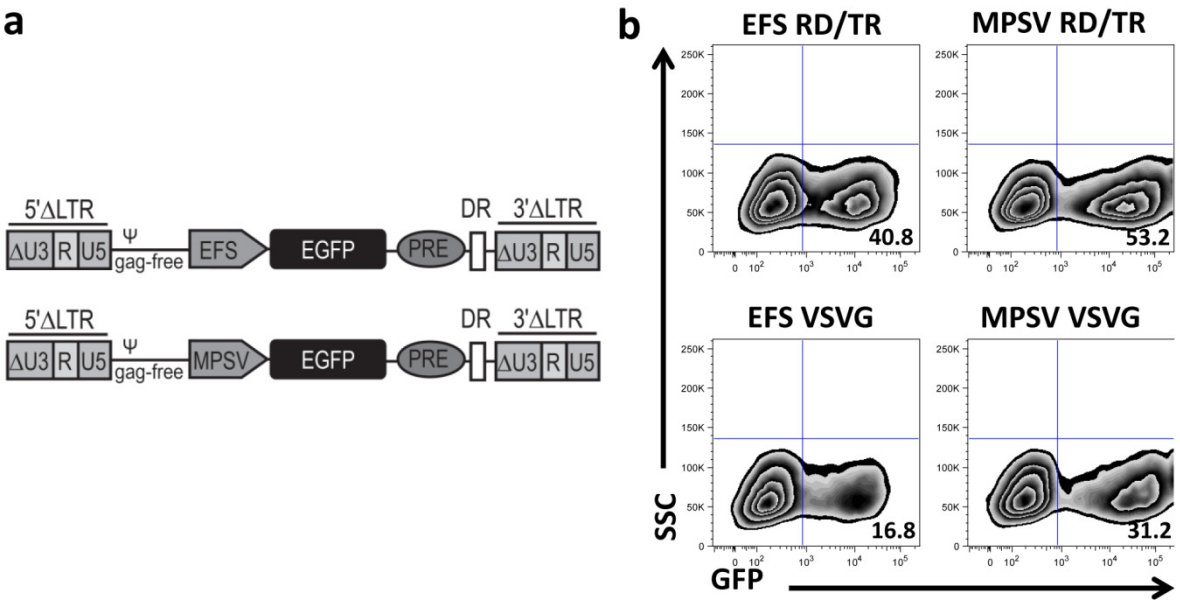

Figure 4: Alpharetroviral vectors efficiently transduce CD34+ cells. (a) SIN alpharetroviral vectors containing either an EFS or MPSV promoter combined with an EGFP reporter gene were generated. (b) EFS- and MPSV-driven alpharetroviral vectors were pseudotyped with either VSVG or RD114/TR and used to transduce CD34+ cells using an equal multiplicity of infection of 100. Indicated are unique 5 (U5), repeat (R), and self-inactivating unique 3 (ΔU3) regions, long terminal repeat (LTR), packaging signal (ψ), woodchuck posttranscriptional regulatory element (PRE) and direct repeat element (DR).

When comparing the four different vectors using the same viral multiplicity of infection (MOI), we observed similar viability of the respective groups six days after transduction as assessed by the relative percentage of live CD34+ cells in the forward scatter (FSC) vs. side scatter gate (SSC), and flowcytometric analysis with Annexin V and propidium iodide (PI) of the whole population in the FSC vs. SSC gate (**Figure 5**).

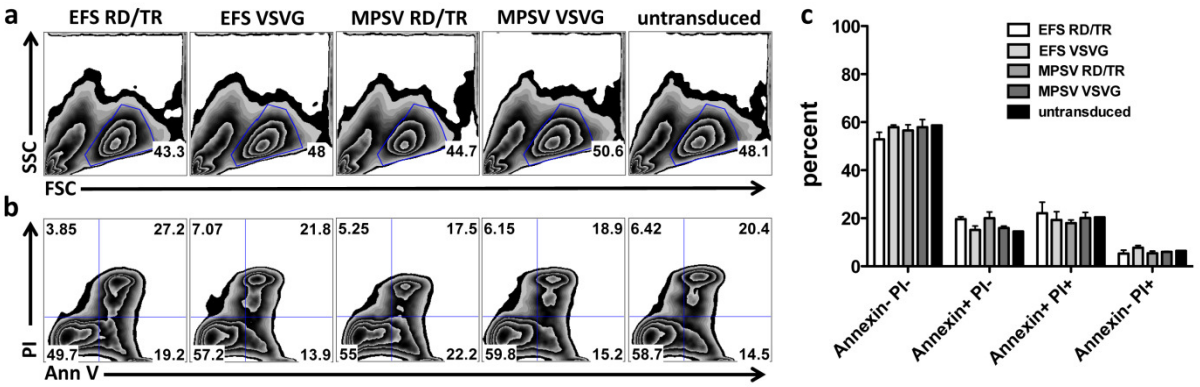

Figure 5: Transduction with alpharetroviral vectors does not influence the viability of CD34+ cell populations. Six days after transduction, CD34+ cells were stained with Annexin V (Ann V) and propidium iodide (PI) and analyzed by flow cytometry (n=3).

After assessing the total population, we next gated on viable CD34+ cells in the FSC vs. SSC gate to examine viability of GFP- and GFP+ CD34+ cells. CD34+ cells were mostly alive with about 90% of them being Ann V- PI- and viability staining between different groups was similar (**Figure 6**).

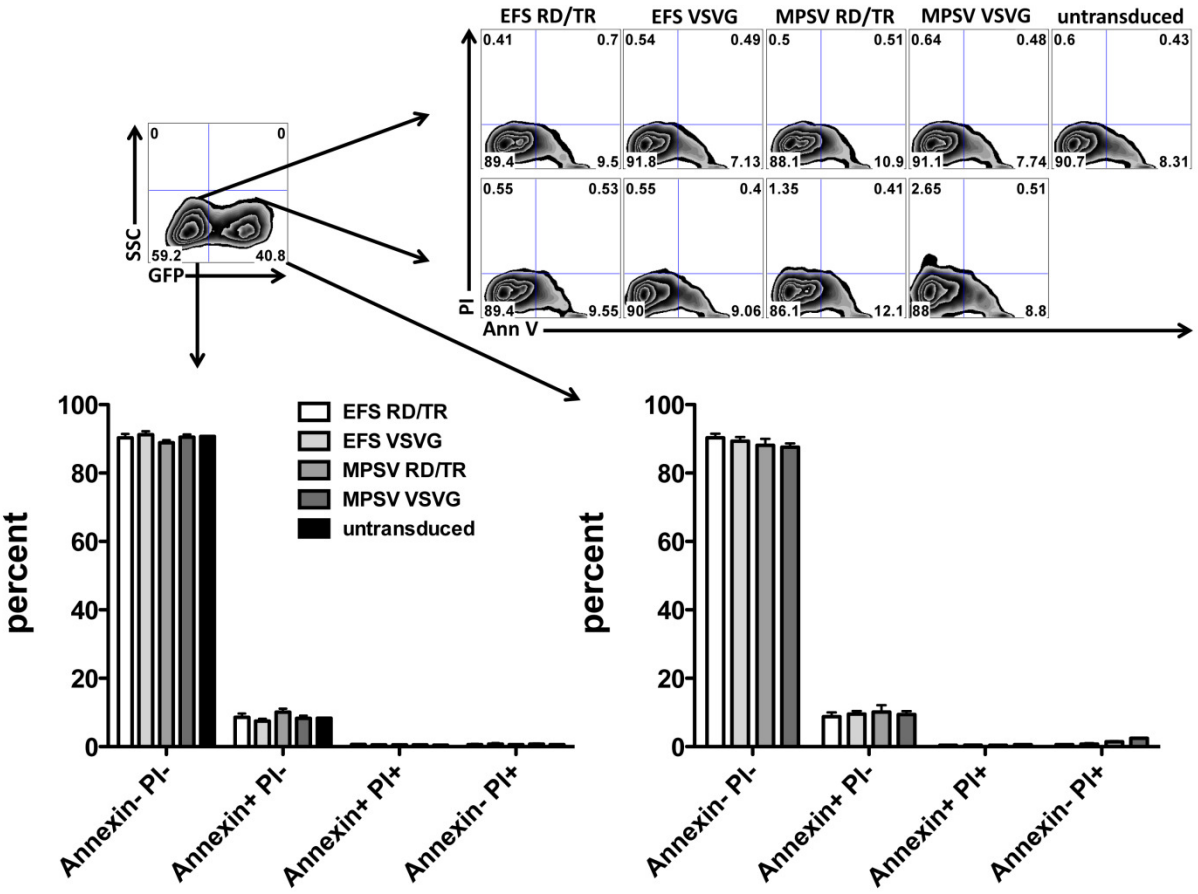

Figure 6: After transduction viability of GFP- and GFP+ CD34+ cells is similar. Annexin V (Ann V) and propidium iodide (PI) were used to stain CD34+ cells for viability six days after transduction (n=3).

Transduction efficiency of CD34+ cells with RD114/TR- versus VSVG-pseudotyped vectors was up to two fold higher (percentage of EGFP+ cells: 55.8±2.3 vs. 31.1±1.3 for the MPSV vectors and 37±0.3 vs. 16.9±0.2 for the EFS vectors, respectively) (**Figure 7a**). As compared to EFS, MPSV-driven vectors resulted in up to four fold increased transgene expression (EGFP mean fluorescent intensity: 45484±1428 vs. 13720±345 for the RD114/TR-pseudotyped and 55035±943 vs. 13274±431 for the VSVG-pseudotyped vectors, respectively) (**Figure 7b**).

As the number of viral vectors integrating into the genome can increase the risk of insertional mutagenesis, we assessed the impact of vector MOI on transduction efficiency and mean vector copy number (VCN). As shown in **Figure 7c**, increasing MOIs do enhance transduction efficiency of transduced human CD34+ cells. This increase is more prominent at lower MOIs and reaches a plateau level at MOIs of more than 100. Assuming that Poisson statistics applies to the transduction, increasing the MOI did not only augment the percentage of transduced CD34+ cells but also the likelihood of transferring more than one copy per cell and hence introduced an increasing number of vector copies per cell, as determined by real-time PCR detecting the PRE sequence. We observed a linear correlation between transduction efficiencies of less than 25% and vector copy number. For higher transduction efficiencies this correlation becomes exponential. Collectively, these data suggest that at lower MOIs, an increase in transduction efficiency is associated with a proportional enhancement of VCN.

Nevertheless, whereas higher MOIs are associated with modest increase in transduction efficiencies only, they result in a steep increase in VCN.

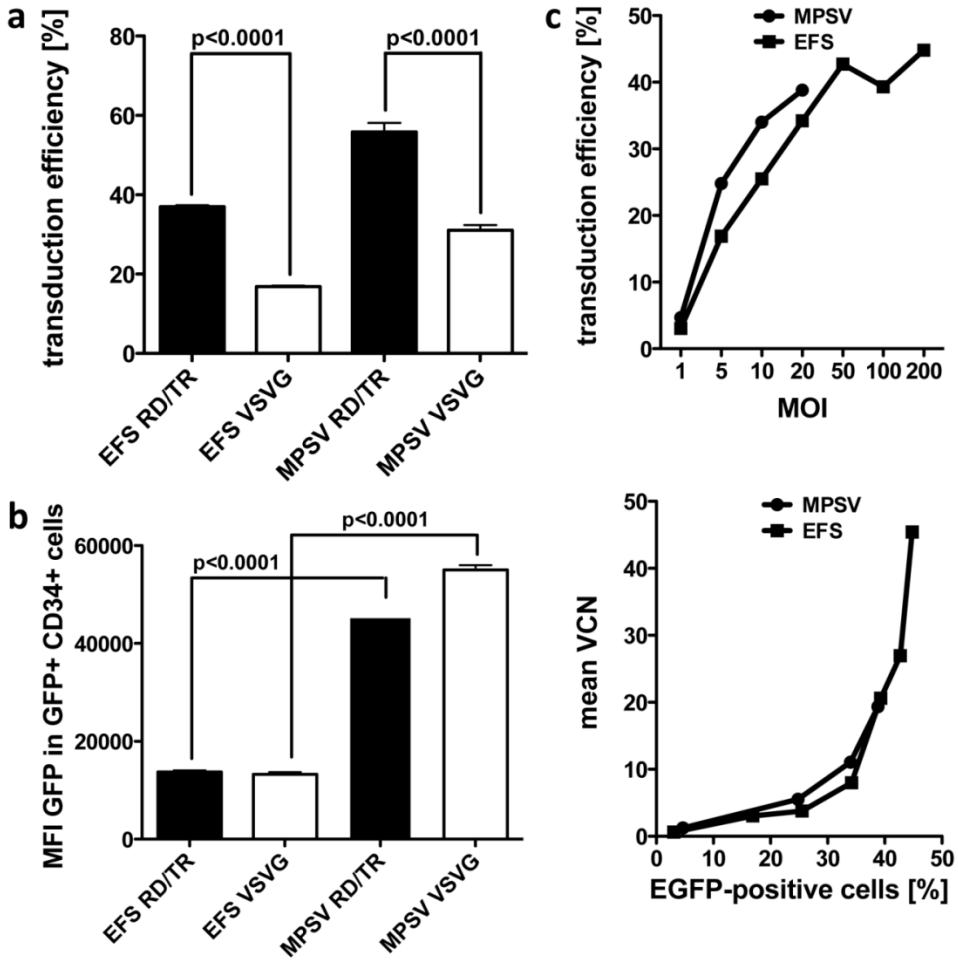

Figure 7: Alpharetroviral vectors containing an MPSV promoter and an RD114/TR envelope deliver enhanced gene transfer into UCB-derived CD34+ hematopoietic stem cells. (a, b) Transduction efficiency and mean fluorescence intensity (MFI) of EGFP were determined six days after transduction. Representative results of two independent experiments are shown (n=3). (c) UCB CD34+ cells were transduced with an increasing multiplicity of infection (MOI) using alpharetroviral MPSV- or EFS-driven vectors with the EGFP gene. After six days, transduction efficiency was determined by flow cytometry. Mean vector copy number (VCN) was assessed using quantitative real-time PCR for detection of woodchuck posttranscriptional regulatory element within the vector.

In order to keep the number of integrated vector copies low, but still yield a relatively high transduction efficiency, we aimed to compare an MOI of 10 and 20. There was a significant difference in transduction efficiency (**Figure 8a**), but not in viability as assessed by Trypan blue and Ann V/PI staining (**Figure 8b**). There was also no statistically significant difference between the number of viable GFP+ CD34+ cells, therefore an MOI of 10 seems adequate for transduction of CD34+ HSCs with alpharetroviral vectors.

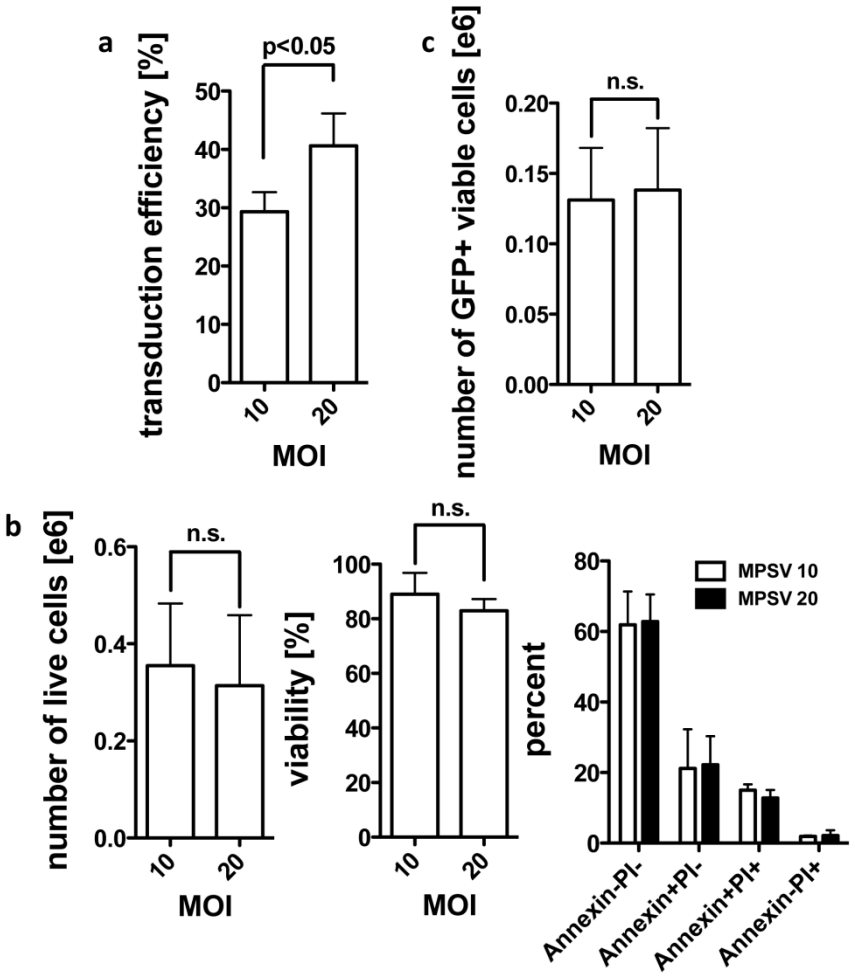

Figure 8: Increased MOI augments transduction efficiency but does not alter viability of CD34+ cells. (a) CD34+ cells were transduced with a multiplicity of infection (MOI) of 10 or 20. Six days after transduction, transduction efficiency was measured by GFP expression in flowcytometric studies (n=4). (b, c) Viability was assessed by Trypan blue and Annexin V/propidium iodide (PI) staining (n=4).

Retronectin, a recombinant fibronectin fragment, that we used to facilitate gene transduction into CD34+ cells, can be used in two different ways. Firstly, in the supernatant infection method (SNT) cells and retroviral supernatant are mixed, loaded on a Retronectin-coated plate and spun. Secondly, in the Retronectin-bound virus method (RBV) the virus is bound to a Retronectin-coated well by centrifugation, then the viral supernatant is removed and cells are added. With the RBV method, consistently higher transduction efficiencies of CD34+ cells were achieved (**Figure 9**).

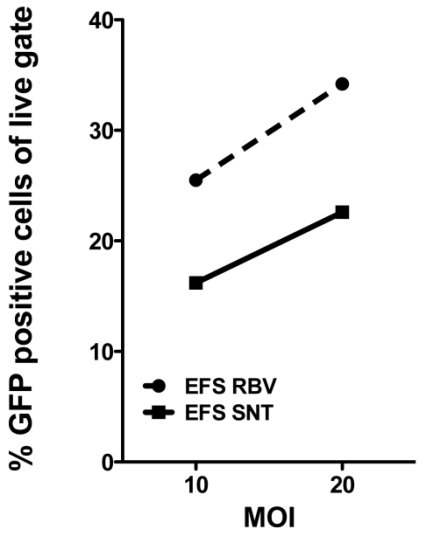

Figure 9: The Retronectin-bound virus method (RBV) yields higher transduction efficiencies than the supernatant infection method (SNT). CD34+ cells were transduced with EFS.EGFP alpharetroviral particles at a multiplicity of infection (MOI) of 10 or 20. Either cells and supernatant were mixed before spinning (SNT), or retroviral particles bound to the plate, supernatant removed and then cells added (RBV).

Effect of transduction procedure and transgene positivity of human UCB-derived CD34+ cells on proliferation and phenotypic differentiation pattern *in vitro*

Since frozen UCB CD34+ cells could be stored and used on demand, we compared transduction efficiency and proliferation rate of freshly isolated versus thawed CD34+ cells. Using alpharetroviral vectors, CD34+ cells were transduced and thereafter transferred onto OP9-DL1 feeder cells to induce preT development (**Figure 10**).

Figure 11a illustrates that transduction efficiency of CD34+ cells, transduced with either RD114/TR pseudotyped MPSV- or EFS-driven vectors, was comparable for both, fresh and thawed CD34+ cells. In the EFS group, transduction efficiency for fresh CD34+ cells was 9.2±2.27% (n=3) and 9.91±1.75% (n=3) for frozen CD34+ cells. For MPSV vector transduced CD34+ cells, transduction efficiency was 27±8.23% (n=3) for fresh and 30.6±5.37% (n=3) for frozen cells. Moreover, curves showing proliferation kinetics of fresh and thawed CD34+ cells were nearly superimposable (**Figure 11b**).

For *in vivo* applications, infusion of a high purity cell product is desired. Therefore, we sorted EGFP+ cells on day 12. As shown in **Figure 12a**, sorted cells expanded and remained 90% transgene positive for at least 24 days. By sorting we enriched for transduced cells, since their content in culture slightly reduced over time (17.3±6.7% on day 4 vs. 9.8±4.0 on day 40%, n=3) (**Figure 12b**).

Next, we assessed the effect of transduction on preT phenotype. It has been shown that a subset of *in vitro*-generated preTs co-expressing both, CD34 and CD7, has high engraftment potential after adoptive transfer (Awong *et al.*, 2013). As the percentage of CD34+ cells decreases with ongoing differentiation and the content of CD7+ is increasing, we asked whether transduction of CD34+ cells could possibly alter their differentiation pattern. Phenotypic comparison of GFP- and GFP+ populations showed comparable differentiation phenotypes on day 8, 16 and 24. Notably, the amount of *in vivo* engrafting CD34+ CD7+ preTs was comparable on day 8,

16 and 24 between GFP- and GFP+ cells (**Figure 13a**). The peak was seen on day 16 with 20.6±2.71% in the GFP- (n=3) and 27.03±4.14% in the GFP+ group (n=3) (**Figure 13**).

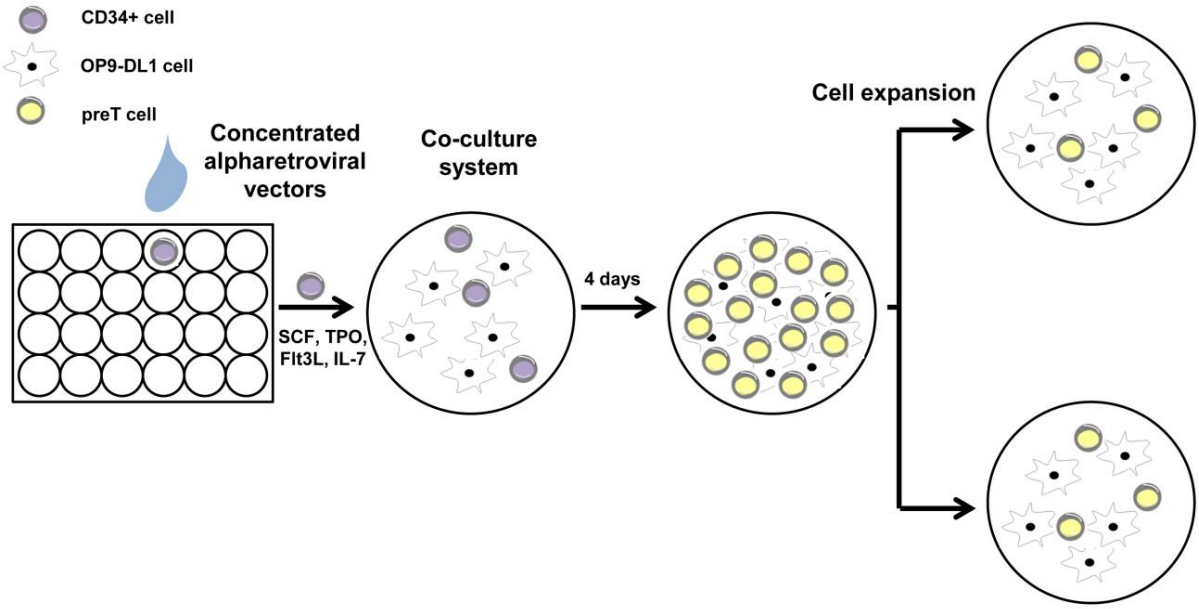

Figure 10: Co-culture of CD34+ cells with OP9-DL1 cells enables preT generation *in vitro*. CD34+ cells were transduced with alpharetroviral vectors and subsequently cultured on OP9-DL1 stromal cells with addition of SCF, TPO (both 20 ng/mL), Flt3L and IL-7 (both 10 ng/mL). Every four days, preTs were harvested and plated on new OP9-DL1 monolayers.

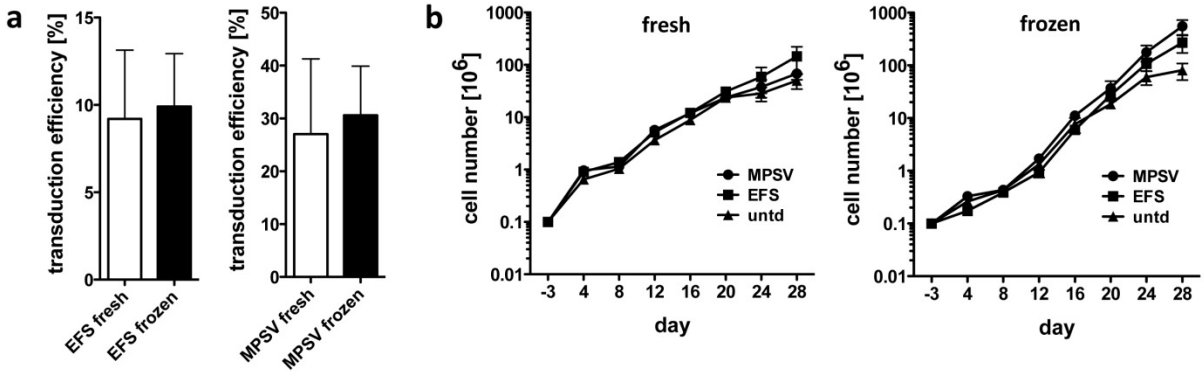

Figure 11: Transduction efficiency and expansion of fresh and frozen CD34+ cells are comparable. (a) Fresh or thawed UCB CD34+ cells were transduced with alpharetroviral vectors containing an EGFP reporter gene using an MOI of 10. Transduction efficiency was determined by flow cytometry after six days (n=3). (b) Transduced or control untransduced (untd) preTs were co-cultured with OP9-DL1 cells for 28 days. The proliferation rate of the cells was assessed every four days using Trypan blue for determination of viability (n=3).

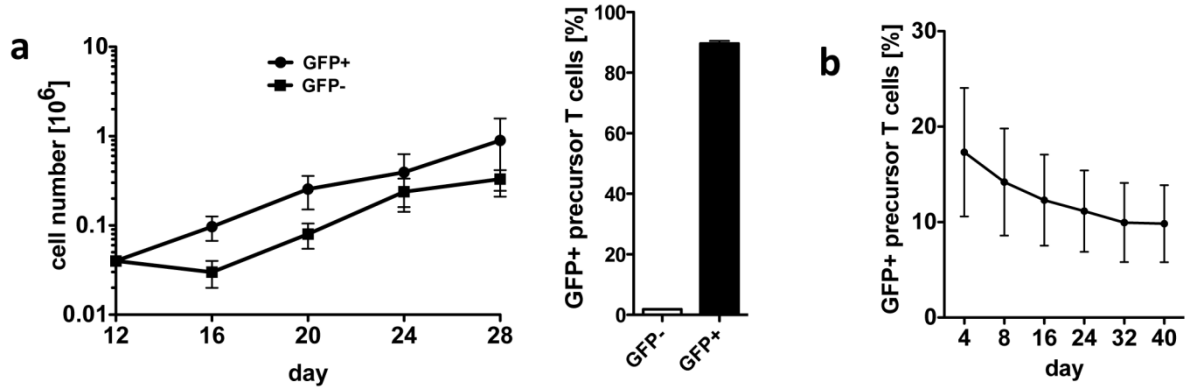

Figure 12: Sorting preTs enhances content of GFP+ cells for 24 days. (a) Transduced preTs were sorted for EGFP expression (GFP+) on day 8 and consecutively continued to be cultured on OP9-DL1 cells under comparable conditions for up to 28 days. EGFP expression was determined on day 32 (24 days after sorting) (n=3). (b) EGFP transgene expression of transduced but non-sorted preTs was determined over a period of 40 days (n=3).

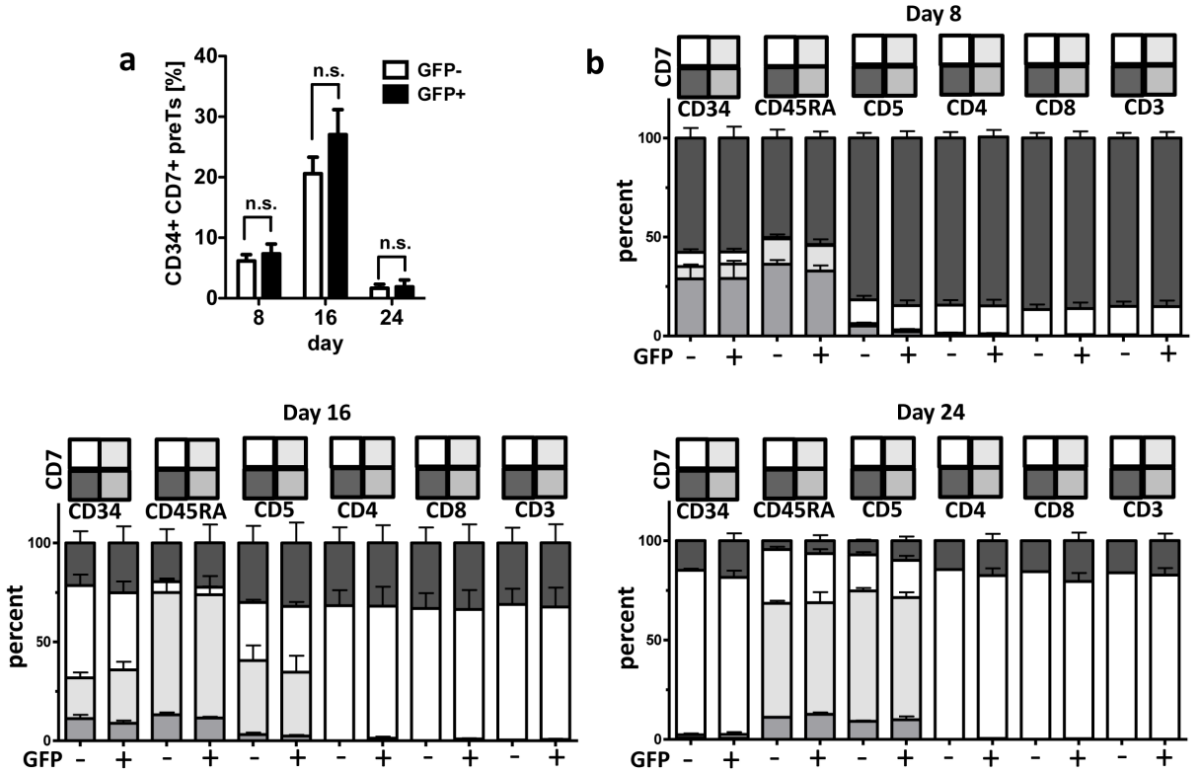

Figure 13: Transduction procedure and transgene positivity of human UCB-derived CD34+ cells do not impact the differentiation pattern towards preTs *in vitro.* (a) The percentage of non-transduced and transduced CD34+CD7+ preT cells was determined by flow cytometry on day 8, 16 and 24. (b) The phenotype of GFP- and GFP+ preTs was determined on day 8, 16 and 24. Gray-colored bars depict subpopulations of preTs.

Effect of transgene expression strength on efficacy of iCasp9-induced apoptosis

Adoptive cell transfer is associated with several safety concerns, for example on-target or off-target toxicities. To provide an effective safety switch for adoptive transfer of preT cells, we used the iCasp9 suicide gene technology. The iCasp9 cassette, which is comprised of a modified human caspase 9 gene fused to a human FK506-binding protein was additionally linked to a truncated human CD19 reporter gene via a 2A sequence and cloned into

alpharetroviral vectors under the control of EFS or MPSV promoters. To assess the functionality of the constructs, Jurkat cells were transduced and enriched for CD19 transgene positive cells by cell sorting. The dimerizing agent B/B Homodimerizer was added at various concentrations to induce apoptosis and the percentage of surviving transgene positive cells (CD19+ Annexin V-) was assessed by flow cytometry 72 hours later. Whereas the majority (90%) survived up to 500 nM of the dimerizing agent after transduction with the EFS-driven vector (viable cells between 69.77±1.79% and 92.4±0.70%, n=3), less than 10% survived even at the lowest dimerizer concentration of 0.05 nM (8.58±0.69%, n=3) after transduction with the MPSV-containing vector (**Figure 14**). These data emphasize the physiological relevance of promoter selection for sufficient expression of the iCasp9 suicide gene.

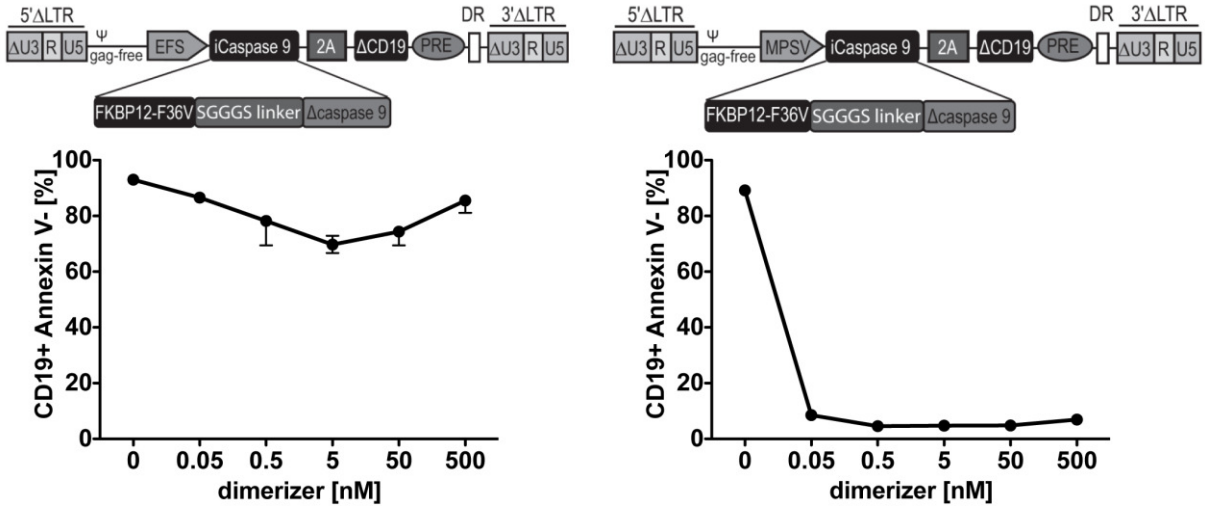

Figure 14: Transgene expression strength mediated by the MPSV promoter determines efficacy of iCasp9-induced apoptosis. iCasp9 containing the human FK506-binding protein (FKBP12) with an F36V mutation and a ΔCD19 reporter gene was cloned into an alpharetroviral backbone under the EFS or MPSV promoter. Human Jurkat cells were transduced with fresh retrovirus and expanded *in vitro*. Transgene positive cells were sorted based on ΔCD19 expression and cultured with increasing concentrations of a dimerizing agent. Three days later, cell viability was assessed based on Annexin V staining. The percentage of transgene positive cells that did not undergo apoptosis upon dimerizer administration (CD19+ Annexin V-) was used for comparison (n=3). Indicated are unique 5 (U5), repeat (R), and self-inactivating unique 3 (ΔU3) regions, long terminal repeat (LTR), packaging signal (ψ), woodchuck posttranscriptional regulatory element (PRE) and direct repeat element (DR).

A third generation CAR against CD123 as gene of interest in the alpharetroviral vector system for transduction of CD34+ cells

Due to the observed importance of promoter choice, we used the MPSV promoter for subsequent proof of principle experiments with alpharetroviral vectors. As potentially relevant gene of interest (GOI), we designed a third generation CAR. It consisted of a CD123-specific scFV being fused to CD28 and 4-1BB costimulatory molecules and a CD3ζ signaling domain. EGFP was used as a reporter gene (**Figure 15a**). We used this construct to transduce UCB CD34+ cells and GFP expression could be readily detected. Subsequently, after OP9-DL1 co-culture, we stained preTs for CAR expression and found that about 40% of GFP+ cells can be stained for the CD123 CAR (**Figure 15b**).

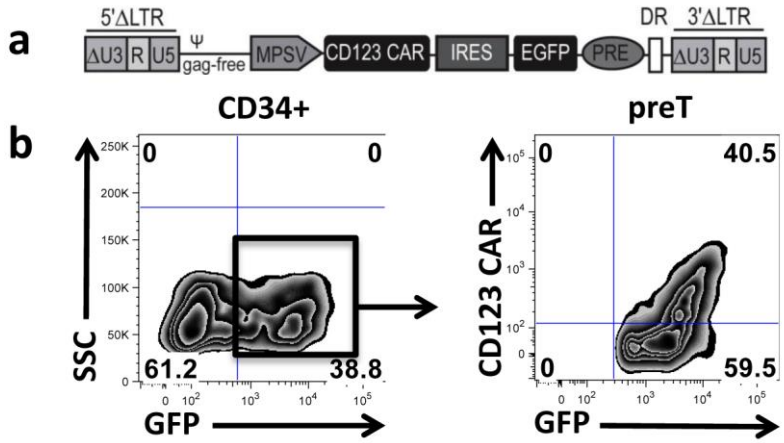

Figure 15: The CD123 CAR can be stained on preTs. (a) The CD123 CAR gene together with an EGFP reporter was cloned in an MPSV alpharetroviral vector. Indicated are unique 5 (U5), repeat (R), and self-inactivating unique 3 (ΔU3) regions, long terminal repeat (LTR), packaging signal (ψ), internal ribosomal entry site (IRES), woodchuck posttranscriptional regulatory element (PRE) and direct repeat element (DR). (b) Frozen CD34+ cells were transduced with RD114/TR-pseudotyped retroviral particles of this construct and subsequently preTs stained for CAR expression by flow cytometry (n=3).

Because CD123 is expressed by multipotent progenitor cells, we investigated whether CD123 CAR expression on UCB CD34+ cells and derived preTs would impact the expression of CD123, viability, and transgene expression of transduced preTs.

CD123 expression during co-culture was significantly reduced in the CAR.GFP-transduced group. This was mainly observed between day 4 and 8 of OP9-DL1 co-culture indicating some extent of fratricide (**Figure 16a**). Of note, CD123 expression was more prominently decreased in CAR.GFP+ compared to CAR.GFP- preTs (**Figure 16b**). There was no augmented Annexin V binding of CD123+ compared to CD123- preTs in the CAR.GFP-transduced group after day 8, making fratricide by CD123 CAR-expressing preTs unlikely from day 8 onwards (**Figure 16c**).

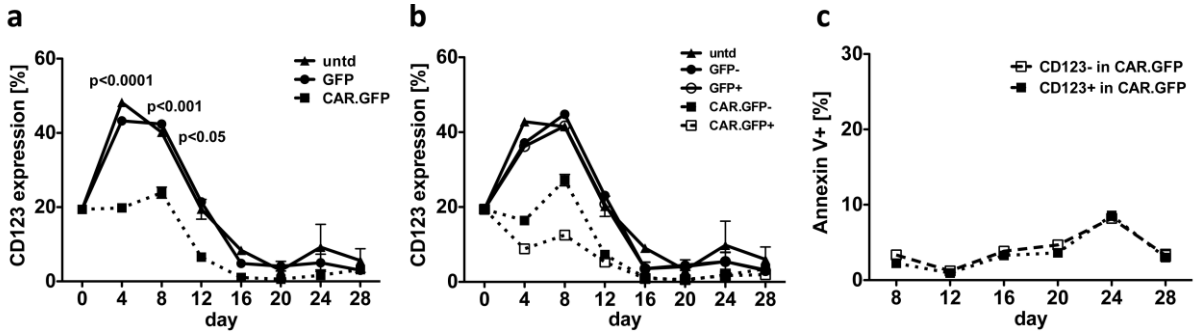

Figure 16: CD123 expression is reduced within CAR.GFP-transduced preTs. (a) Frozen UCB CD34+ cells were transduced with either the CD123 CAR.GFP or the GFP only construct. Untransduced (untd) cells were used as control. PreTs were assessed by flow cytometry for CD123 and Annexin V at indicated time points (n=3). CAR.GFP populations are depicted by dotted lines.

In line with this finding, the slow decrease of GFP transgene expression during time in the CAR.GFP-group showed similar kinetics when compared to GFP-only transduced preTs, leading to comparable cell yields at the end of the co-culture (**Figure 17a**). CD123 CAR expression of GFP+ transduced preTs also decreased from about 38% on day 4 to 28% on day 16. Most probably, this is due to a proliferative disadvantage of the respective cell

population after introduction of a relatively large gene cassette and not due to fratricide as demonstrated above (**Figure 17b**).

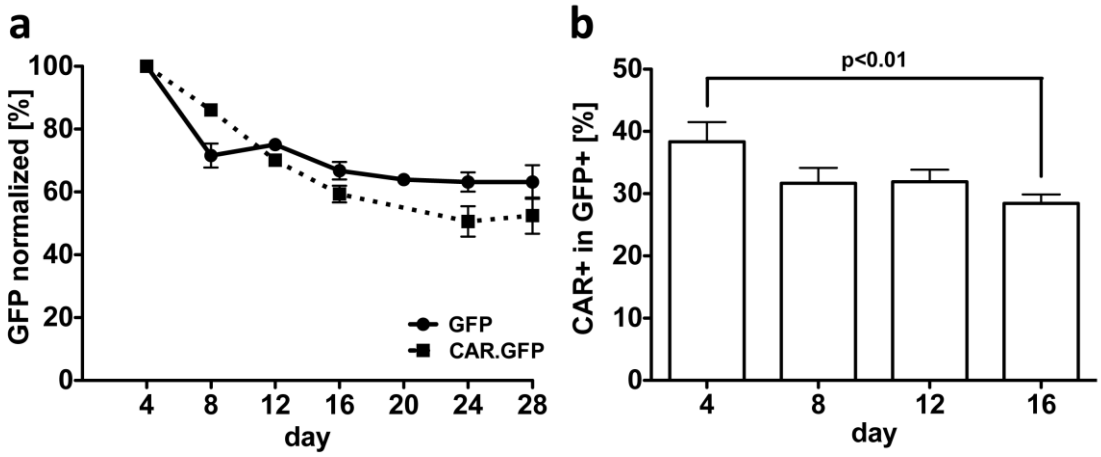

Figure 17: Transgene expression decrease is similar for CAR.GFP- and control GFP only-transduced preTs. Thawed UCB CD34+ cells were transduced with either the CD123 CAR.GFP or the GFP only construct (n=3). Expression of GFP and CD123 CAR was assessed during co-culture of OP9-DL1 cells and preTs. CAR.GFP groups are shown as dotted lines.

Functionality of the CD123 CAR and iCasp9 in human PBMCs

To evaluate the function of this new CD123 CAR, we transduced human PBMCs with an MPSV promoter-driven SIN alpharetroviral vector, which our proof of principle GOI was cloned into (**Figure 18a**). To generate target cells, 293T cells were transduced with retroviral vectors using the human CD123 sequence which had been linked to tdTomato via an IRES cassette. Two sorting rounds ensured over 98% CD123 expression. Non-transduced 293T cells were used as controls. Functionality was assessed by a fluorescence-based *in vitro* cytotoxicity assay and ELISA. CD123 CAR T cells strongly produced IFNγ upon stimulation on CD123+ target cells. Antigen negative target cells were spared demonstrating specific recognition of CD123. Untransduced PBMCs mediated a weak background response only, being comparable for both, antigen negative and antigen positive target cells (**Figure 18c**). More importantly, the CAR-transduced PBMCs effectively lysed target cells expressing the CD123 antigen, as evident by a nearly complete loss of the tdTomato signal (**Figure 18b**).

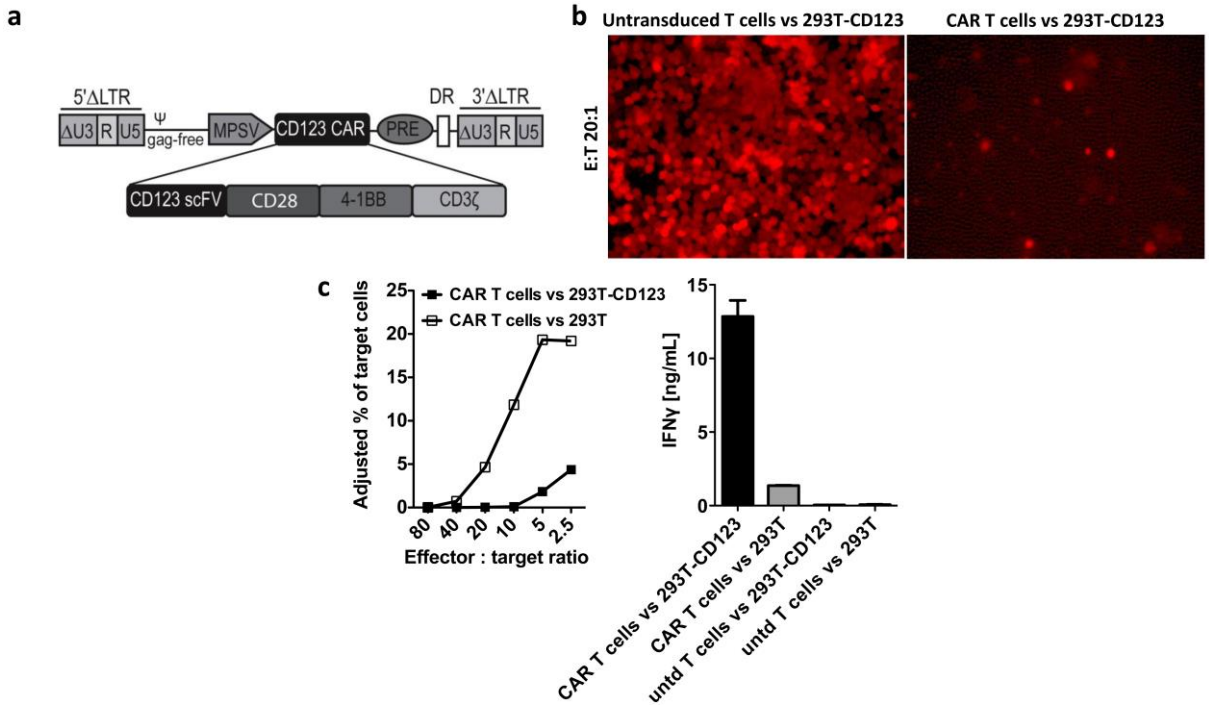

Figure 18: Mature human T cells expressing a third generation CAR against CD123 are functional *in vitro.* (a) Schematic figure of the MPSV.CD123 CAR construct. SIN alpharetroviral vectors encoding for a third generation CAR against the human surface molecule CD123 were generated. Indicated are unique 5 (U5), repeat (R), and self-inactivating unique 3 (ΔU3) regions, long terminal repeat (LTR), packaging signal (ψ), single chain variable fragment (scFV), woodchuck posttranscriptional regulatory element (PRE) and direct repeat element (DR). (b) Human PBMCs were activated with anti-CD3 and anti-CD28 antibodies for two days, transduced twice on consecutive days and expanded for four more days before being used in a cytotoxicity assay. TdTomato.CD123-transduced 293T cells were used as target cells. Decreasing numbers of untransduced (untd) or CAR-transduced PBMCs were cultured with target cells in triplicates for two days. TdTomato expression was used as a marker for surviving target cells in immunofluorescence microscopy studies. (c) Cytotoxicity of PBMCs was also determined by flow cytometry. The cell suspension containing effector and target cells was stained for CD3. The percentage of $CD3^{neg}$ cells (surviving target cells) was used as indicator for cytotoxicity (left panel). A mathematical correction was applied to eliminate the effect of increasing PBMC numbers on the percentage of surviving target cells in the cell mixture (adjusted % of target cells). T cells ($2x10^5$) and target cells ($2x10^4$) were incubated (effector:target ratio of 10:1) in 96-well plates. After 24 hours, supernatant was harvested and used in duplicates for an IFN-γ ELISA (right panel) (n=2). Untransduced (untd) T cells were used as control.

After having evaluated iCasp9 and the CD123 CAR in different vector cassettes, we cloned a clinically more relevant construct combining both GOIs under MPSV promoter control and used it to transduce PBMCs. After transduction, the CD123 CAR was specifically stained using a CD123 peptide in flow cytometric studies. In order to evaluate the functionality of this combined vector, apoptosis was induced using the dimerizer. No effect of the dimerizer on control CAR.GFP-transduced PBMCs was observed, showing the selectivity of dimerizer-induced apoptosis. Similar to iCasp9 only-transduced Jurkat cells demonstrated above, a dimerizer concentration of 0.05 nM was able to induce maximal apoptosis in CAR.iCasp9-transduced PBMCs (**Figure 19**). Apoptosis could also be induced in preTs transduced with this construct (**Figure 20**).

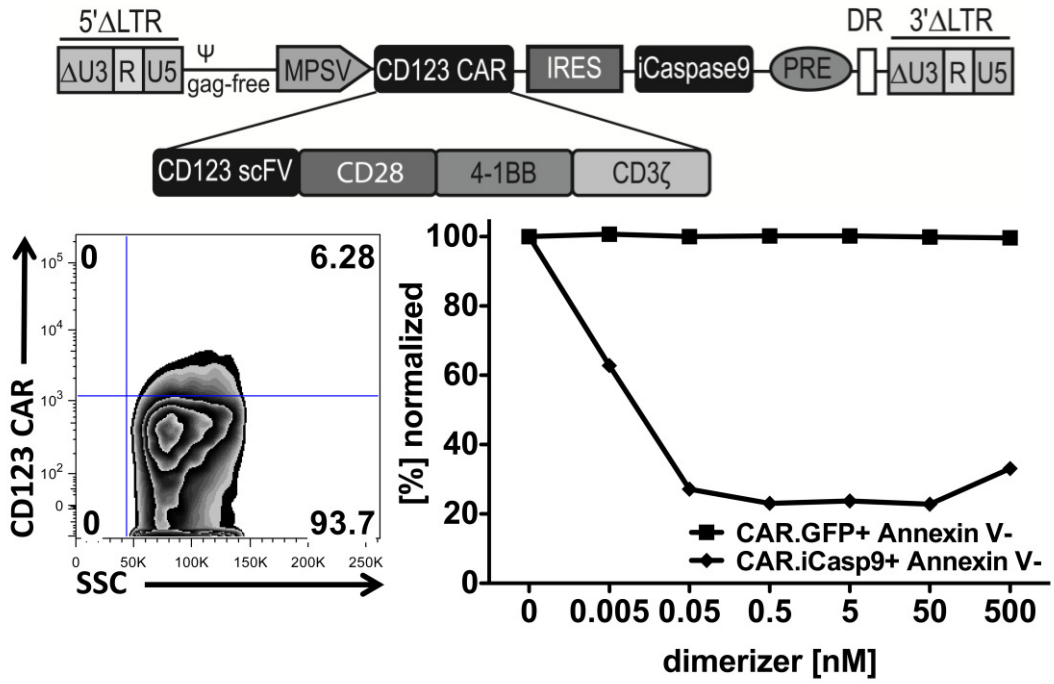

Figure 19: PBMCs transduced with a combined construct encoding CD123 CAR and iCasp9 can be stained for CAR expression and undergo dimerizer-induced apoptosis. Schematic figure of the MPSV.CD123 CAR.iCasp9 vector. Indicated are unique 5 (U5), repeat (R), and self-inactivating unique 3 (ΔU3) regions, long terminal repeat (LTR), packaging signal (ψ), single chain variable fragment (scFV), internal ribosomal entry site (IRES), woodchuck posttranscriptional regulatory element (PRE) and direct repeat element (DR). This bicistronic construct was used for transduction of human PBMCs. Transgene positive cells were stained for CD123 CAR by specific cell binding of a His Tag-labeled CD123 peptide. Transduced PBMCs were incubated with the dimerizer for 48 hours (n=3). CAR.GFP-transduced PBMCs were used as control group (n=3). Apoptosis was quantified by staining viable transgene positive (either CAR+ or GFP+) Annexin V- PBMCs.

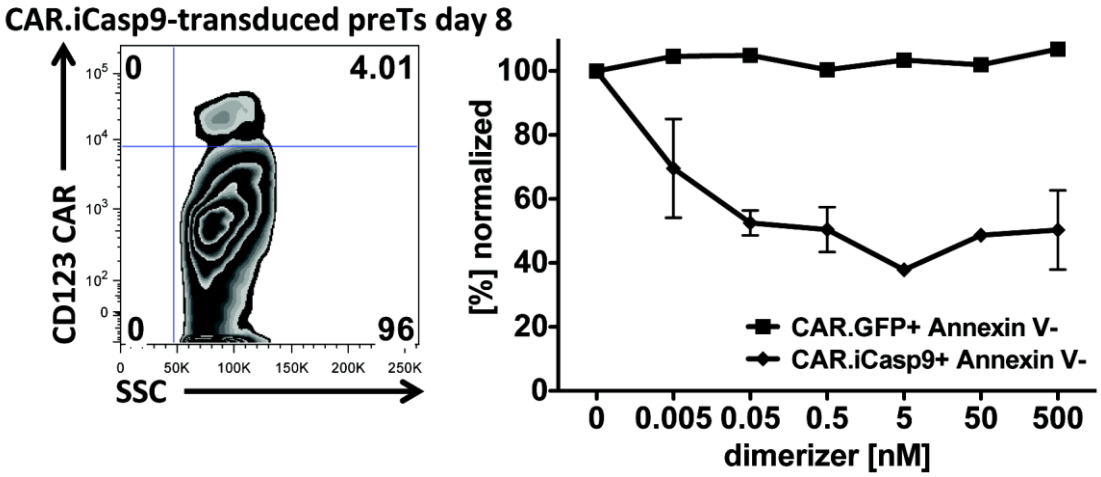

Figure 20: The CD123 CAR.iCasp9 construct is functional in preTs. UCB-derived CD34+ cells were transduced with the bicistronic alpharetroviral CD123 CAR.iCasp9 construct and consecutively differentiated into precursor T cells. By day 8 a distinct population of CAR+ preTs can be identified (left panel). As compared to CD123 CAR.GFP-transduced controls, apoptosis can be readily induced by addition of the dimerizer (right panel).

In vivo engraftment of CD123 CAR-expressing preTs

The phenotype of preTs was shown to determine thymic engraftment, which is necessary for subsequent release of mature T cells. Therefore, we investigated the influence of the CAR construct on *in vitro* phenotypic maturation of CD34+ cells by comparing an MOI of 10 to an MOI of 100. As control group, untransduced cells within the same culture population were used. Co-expression of CD34, CD7 and CD5 has been shown to facilitate thymic repopulation. When using the CD123 CAR encoding vector for transduction, CD34 co-expression on day 11 was significantly higher than on untransduced preTs. This indicates a differentiation delay caused by transduction. Comparing the lower and higher MOI, no difference was observed (**Figure 21a**).

PreTs were harvested on day 11 of co-culture, sorted by FACS for GFP expression and injected intrahepatically into irradiated (1 Gy) four day old NOD.cg-Prkdc^{scid}IL2rg$^{tm/Wjl}$/Sz (NSG) mice. Six weeks later, mice were sacrificed and thymi harvested. Extracted cells were then analyzed by flow cytometry for transgene GFP+ CD3 expressing thymocytes derived from human cells. Control mice, only receiving CB-derived HSCs, did not display thymic repopulation, whereas GFP+ CD3+ thymocytes were detected in GFP only- and CD123 CAR.GFP-transduced preT groups (**Figure 21b**). This underlines the engraftment capacity of preTs engineered with alpharetroviral vectors and encourages the further use of alpharetroviral vector platforms for adoptive T cell transfer studies in preclinical models of leukemia.

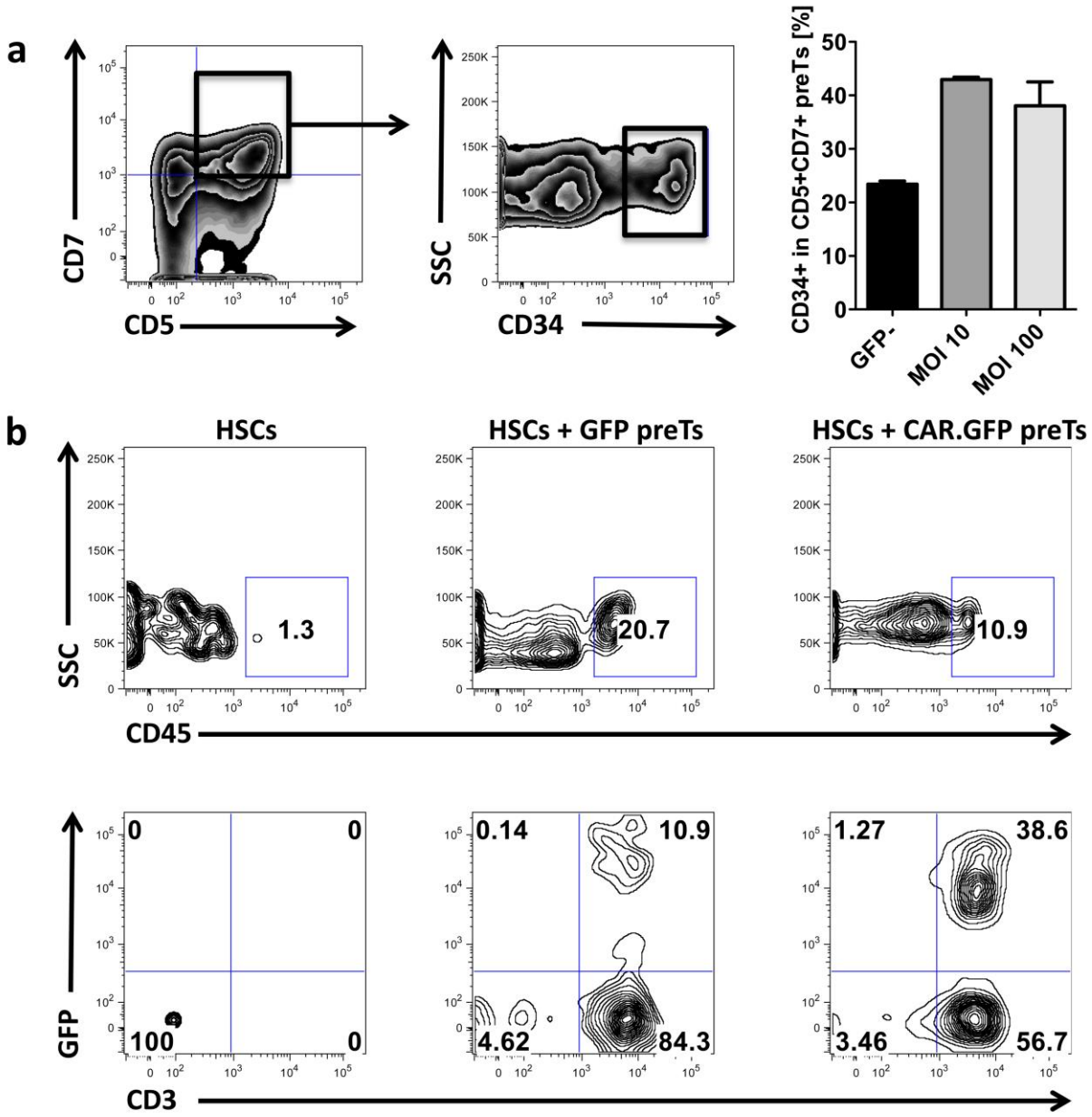

Figure 21: CAR-expressing preTs demonstrate thymic engraftment in NSG mice. (a) UCB-derived CD34+ cells were transduced with the CD123 CAR-containing alpharetroviral construct either using an MOI of 10 or 100. After transduction, cells were brought into the OP9-DLI co-culture system and phenotypically analyzed on day 11. Within the GFP+ population (transduced cells) CD5+CD7+ were gated on and assessed for CD34 co-expression. The GFP- fraction (non-transduced) served as control. CD34 co-expression was lower on non-transduced as compared to transduced CD5+CD7+ cells ($p<0.05$). No significant difference was found between MOI 10 and 100. Two independent experiments were performed. (b) $2x10^5$ GFP+ preTs (either transduced with GFP only or CAR.GFP) in combination with $0.2x10^5$ UCB-derived $CD34^+$ HSCs were intrahepatically injected into four day old irradiated NSG mice. Control mice were injected with HSCs only. Mice were boosted with the IL-7/M25 mixture every five days. Six weeks after injection, thymi were harvested and single cell suspensions were generated for flow cytometric analysis. The upper panel displays the percentage of cells derived from human origin as determined by CD45 expression in the different groups. After gating on this cell population, GFP and CD3 expression was determined (lower panel).

V Publication

Generation of genetically engineered precursor T cells from human umbilical cord blood using an optimized alpharetroviral vector platform

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Juwita Hübner contributed to experimental design, acquired and analyzed the data for Figs. 1-9, 10-13, 15-17 and 19-21, designed the figures and participated in writing the manuscript. Shahabuddin S. Hoseini participated in data acquisition and data analyses for Figs. 14 and 18 and in writing the manuscript. Julia D. Suerth helped acquiring and analyzing data for Fig. 7. Marcel Maluski and Jessica Herbst acquired and analyzed the data of Fig. 21, Qinggong Yuan and Michael Ott assisted in acquiring this data. Holger Maul, Arnab Ghosh, Britta Eiz-Vesper, Michael Heuser and Axel Schambach critically evaluated the data. In addition, Shahabuddin S. Hoseini, Julia D. Suerth and Dirk Hoffmann designed and cloned vector constructs used in this publication. Martin G. Sauer contributed to experimental design, evaluation of data, and writing of the manuscript.

original article

Generation of Genetically Engineered Precursor T-Cells From Human Umbilical Cord Blood Using an Optimized Alpharetroviral Vector Platform

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Retroviral engineering of hematopoietic stem cell-derived precursor T-cells (preTs) opens the possibility of targeted T-cell transfer across human leukocyte antigen (HLA)-barriers. Alpharetroviral vectors exhibit a more neutral integration pattern thereby reducing the risk of insertional mutagenesis. Cord blood-derived CD34+ cells were transduced and differentiated into preTs *in vitro*. Two promoters, elongation-factor-1-short-form, and a myeloproliferative sarcoma virus variant in combination with two commonly used envelopes were comparatively assessed choosing enhanced green fluorescent protein or a third-generation chimeric antigen receptor (CAR) against CD123 as gene of interest. Furthermore, the inducible suicide gene iCaspase 9 has been validated. Combining the sarcoma virus-derived promoter with a modified feline endogenous retrovirus envelope glycoprotein yielded in superior transgene expression and transduction rates. Fresh and previously frozen CD34+ cells showed similar transduction and expansion rates. Transgene-positive cells did neither show proliferative impairment nor alteration in their lymphoid differentiation profile. The sarcoma virus-derived promoter only could express sufficient levels of iCaspase 9 to mediate dimerizer-induced apoptosis. Finally, the CD123 CAR was efficiently expressed in CD34+ cells and proved to be functional when expressed on differentiated T-cells. Therefore, the transduction of CD34+ cells with alpharetroviral vectors represents a feasible and potentially safer approach for stem cell-based immunotherapies for cancer.

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INTRODUCTION

Cord blood (CB) is now a widely used source of hematopoietic stem cells (HSCs) for allogeneic hematopoietic stem cell transplantation (allo-HSCT).[1–3] The nature of its source excludes the possibility of returning to the donor to retransplant HSCs or T-cells to treat engraftment failure or relapse of malignancy. Thus, alternative T-cell sources to enhance immune reconstitution are needed. Over the last decade, coculture systems based on Notch ligand expressing bone marrow stroma cell lines have been developed allowing the differentiation and generation of T-cell-committed precursors (preTs).[4,5] These systems can be controlled to limit the differentiation of the precursors to early thymic preTs which upon cotransplantation into allogeneic recipients undergo further development *in vivo* including T-cell receptor (TCR) rearrangement, TCRβ-selection and both, positive and negative selection of developing thymocytes.[6,7] As a result, cotransplantation of preTs allows T-cell reconstitution of an immunosuppressed host across major histocompatibility complex (MHC)-barriers without the risk for graft-versus-host-disease while maintaining predominantly host-derived antigen presenting cell chimerism.[5]

The antitumor effects of preTs can be enhanced through genetic engineering with either chimeric antigen receptors (CARs) or TCRs against tumor-associated antigens.[8,9] However, whereas genetic engineering of mature T-cells using gammaretroviral vectors has remained demonstrably safe without serious adverse effects due to insertional mutagenesis, this remains a major safety concern when engineering HSCs and incompletely differentiated T-cells.[10,11] In contrast to gammaretroviral vectors, alpharetroviral vectors have a neutral integration pattern and can be readily designed to lack strong splice signals that might interfere with cellular mRNA processing.[12,13]

Here, we used an alpharetroviral vector system to genetically engineer human CB-derived CD34+ HSCs that were subsequently differentiated *in vitro* into preTs. We comparatively assessed the myeloproliferative sarcoma virus (MPSV) and the short form of the constitutively acting elongation factor 1α (EFS) promoter system in combination with either the vesicular stomatitis virus glycoprotein (VSVG) or a modified feline endogenous retrovirus glycoprotein

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(RD114/TR) envelope. We show that superior transduction and expression rates of the gene of interest (GOI) are physiologically highly relevant, especially if inducible caspase 9 (iCasp9) is used as a suicide gene. We observed that transducing CB-derived CD34+ cells with the alpharetroviral construct carrying a third-generation CAR against CD123 does slightly delay the *in vitro* differentiation process of preTs when using the OP9-DL1 coculturing system. The transduction efficiency and expansion patterns of preTs from fresh or previously frozen CB were comparable. We further demonstrate for the first time that T-cells expressing a CAR against CD123, that had been cloned into an alpharetroviral backbone, are functional and effective against CD123-expressing target cells. Altogether, we present a novel alpharetroviral system for potential clinical use when CB-derived CD34+ cells for the generation of preTs and T-cells are to be genetically engineered.

RESULTS

Human CB-derived CD34+ cells are differentiated into preTs *in vitro*

For generation of human preTs, CB was used as a source of CD34+ HSCs. CB samples were obtained from consenting mothers and CD34+ cells were isolated via two rounds of magnetic-activated cell sorting. The CD34+ purity was consistently higher (93 ± 3%) when two purification steps were performed (**Figure 1a**). Freshly isolated or thawed CD34+ cells were cocultured with the Notch ligand-expressing OP9-DL1 stromal cells having previously been shown to support *in vitro* generation of human preTs.[14] The kinetics of preT growth, which were comparable for both, fresh and frozen CD34+ cells, revealed slower cell proliferation up to day 12, and a more rapid cell growth until day 28 which was followed by a plateau phase. We observed an expansion rate of up to 750-fold until day 28 (**Figure 1b**).

To assess *in vitro* development, preTs were phenotyped by flow cytometry. As shown in **Figure 1c**, the expression of CD34 decreased over time and disappeared by day 24. The T-cell development markers CD45RA, CD7, and CD5 increased during the culture period. A small population of cells only started to express CD3 by day 40. We did not observe the appearance of CD4- and CD8-positive cells over the whole culturing course. Of note, CD34+ CD7+ progenitor T-cells that represent the thymus-engrafting population were most prominent on day 8 and had disappeared by day 24.[7]

Alpharetroviral vectors containing an MPSV promoter and an RD114/TR envelope deliver enhanced gene transfer into CB-derived CD34+ HSCs

To decrease the risk of insertional mutagenesis after transduction of CD34+ cells, we used self-inactivating (SIN) alpharetroviral vectors.[12,13] To improve gene transfer into CD34+ cells, we compared alpharetroviral vectors containing two different promoters, either using the EFS or the MPSV promoter. Respective retroviral particles were pseudotyped with either the VSVG or RD114/TR envelope. Enhanced green fluorescent protein (EGFP) was used as a reporter gene (**Figure 2a**). Using similar viral multiplicity of infection (MOI), transduction efficiency of CD34+ cells with RD114/TR- versus VSVG-pseudotyped vectors was up to twofold higher (percentage of EGFP+ cells: 55.8 ± 2.3 versus 31.1 ± 1.3 for the MPSV vectors and 37 ± 0.3 versus 16.9 ± 0.2 for the EFS vectors, respectively) (**Figure 2b,c**). As compared to EFS, MPSV-driven vectors resulted in up to fourfold increased transgene expression (EGFP mean fluorescence intensity: 45,484 ± 1,428 versus 13,720 ± 345 for the RD114/TR-pseudotyped and 55,035 ± 943 versus 13,274 ± 431 for the VSVG-pseudotyped vectors, respectively) (**Figure 2d**).

As the number of viral vectors integrating into the genome can increase the risk of insertional mutagenesis, we assessed the impact of vector MOI on transduction efficiency and mean vector copy number (VCN). As shown in **Figure 2e**, increasing MOIs do enhance transduction efficiency of transduced human CD34+ cells. This increase is more prominent at lower MOIs and reaches a plateau level at MOIs of more than 100. There was a linear correlation between transduction efficiencies of less than 25% and VCN.[15] For higher transduction efficiencies, this correlation becomes exponential. Collectively, these data suggest that at lower MOIs, an increase in transduction efficiency is associated with a proportional enhancement of VCN. Nevertheless, whereas higher MOIs are associated with modest increase in transduction efficiencies only, they result in a steep increase in VCN.

Transduction procedure and transgene positivity of human CB-derived CD34+ cells do neither impact the proliferation nor the differentiation pattern toward preTs *in vitro* when using EGFP as an insert only

Since frozen CB CD34+ cells could be stored and used on demand, we compared transduction efficiency and proliferation rate of freshly isolated versus thawed CD34+ cells. As GOI, a cassette containing EGFP only was used. **Figure 3a** illustrates that transduction efficiency of CD34+ cells, transduced with either RD114/TR pseudotyped MPSV- or EFS-driven vectors, was comparable for both, fresh and thawed CD34+ cells. Curves showing proliferation kinetics of fresh and thawed CD34+ cells were nearly superimposable (**Figure 3b**).

We sorted EGFP+ cells on day 12. As shown in **Figure 3c**, sorted cells expanded and remained 90% transgene positive for at least 24 days. By sorting, we enriched for transduced cells, since their content in culture slightly reduced over time (**Figure 3d**).

It has been shown that a subset of *in vitro*-generated preTs coexpressing both, CD34 and CD7, has high engraftment potential after adoptive transfer.[7] As the percentage of CD34+ cells decreases with ongoing differentiation and the content of CD7+ is increasing, we asked whether transduction of CD34+ cells could possibly alter their differentiation pattern. In contrast to the construct additionally carrying a CAR against CD123 (see **Figure 6**), phenotype comparison of EGFP+ and EGFP− populations showed comparable differentiation phenotypes on day 8, 16 (**Figure 3e,f**), and 24 (data not shown) if MOIs of 10 were used.

Transgene expression strength mediated by the MPSV promoter determines efficacy of iCasp9-induced apoptosis and allows efficient CD123 CAR expression in preTs

To provide an effective safety switch for adoptive transfer of preTs, we used the iCasp9 suicide gene technology. The iCasp9 cassette, which is comprised of a modified human caspase 9 gene fused to

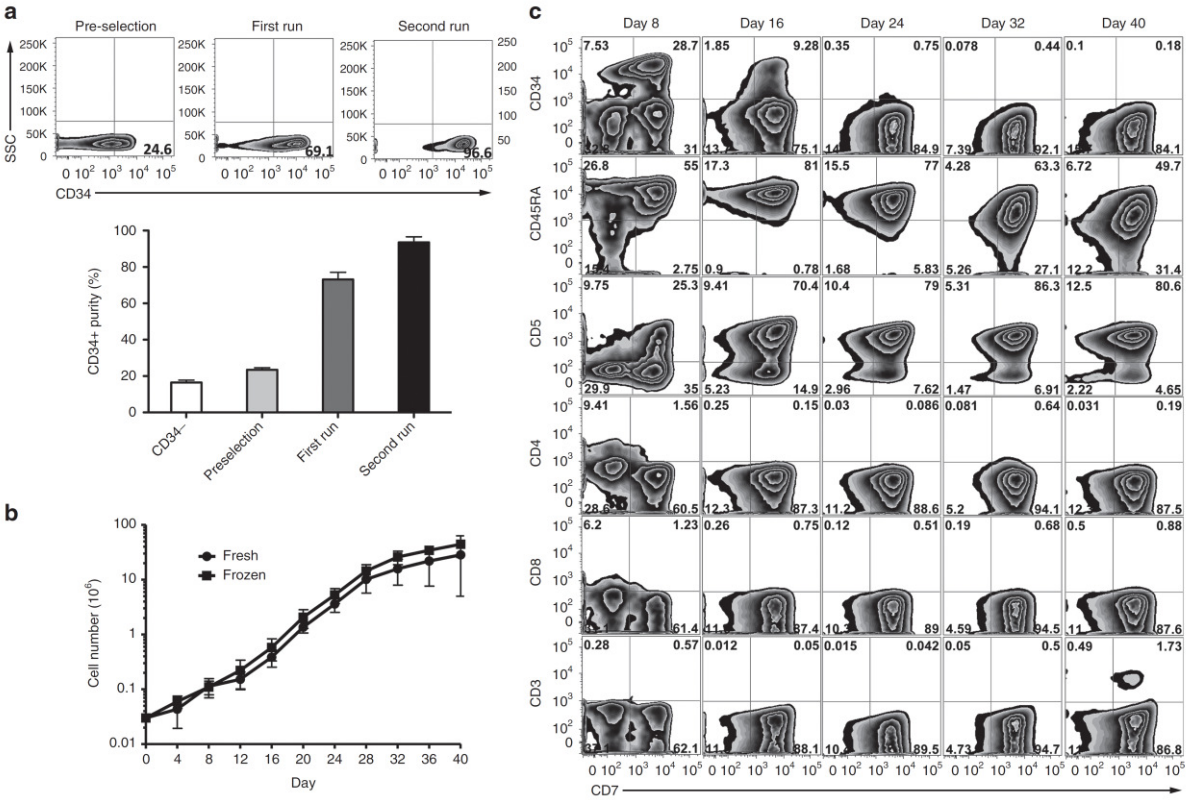

Figure 1 Human cord blood (CB)-derived CD34+ cells are differentiated into precursor T cells (preTs) *in vitro*. (**a**) Human CB samples were obtained from consenting mothers and CD34+ cells consecutively isolated via positive selection by magnetic-activated cell sorting (MACS). Purity was determined by flow cytometry after one or two rounds of MACS ($n = 2$). (**b**) Fresh or thawed CD34+ cells were cocultured on OP9-DL1 stromal cells in a cytokine cocktail for preT differentiation. The proliferation rate of the cells was assessed every 4 days ($n = 3$). (**c**) The lymphoid phenotype of the cells was determined by flow cytometry every 8 days for a period of 40 days. Results of a representative experiment are shown.

a human FK506-binding protein was additionally linked to a truncated human CD19 reporter gene via a 2A sequence and cloned into alpharetroviral vectors under the control of the EFS or MPSV promoter (**Figure 4a**). To assess the functionality of the constructs, Jurkat cells were transduced and enriched for CD19 transgene-positive cells by cell sorting. The dimerizing agent AP20187 was added at various concentrations to induce apoptosis and the percentage of surviving transgene positive cells (CD19+ Annexin V-) was assessed by flow cytometry 72 hours later. Whereas the majority (90%) survived up to 500 nmol/l of the dimerizing agent after transduction with the EFS-driven vector, less than 10% survived at dimerizer concentrations of 0.05 nmol/l after transduction with the MPSV-containing vector (**Figure 4a**). These data emphasize the physiological relevance of promoter selection for sufficient expression of the iCasp9 suicide gene.

Due to this observation, we decided to use the MPSV promoter for subsequent transduction experiments. We designed a third-generation CAR as a potentially relevant GOI. It consisted of a CD123-specific single chain variable fragment (scFV) being fused to CD28 and 4-1BB costimulatory molecules and a CD3ζ signaling domain as well as an EGFP reporter gene for first functionality assays. Umbilical cord blood (UCB) CD34+ cells were transduced with this construct and EGFP expression readily detected. Upon subsequent coculture with OP9-DL1 cells, we stained for CAR expression of preTs and observed that around 40% of EGFP+ cells can be stained for the CD123 CAR (**Figure 4b**).

Owing to the fact that CD123 is expressed on multipotent progenitor cells, we investigated whether forced CD123 CAR expression on CD34+ cells and preTs would impact CD123 expression, viability, and transgene expression of transduced preTs. In the CAR.GFP-transduced group, CD123 expression during OP9-DL1 coculture was significantly reduced. This effect was predominantly seen between day 4 and 8 of differentiation culture suggesting some degree of fratricide (**Figure 4c**). Of note, CD123 expression in CAR.GFP+ preTs was more markedly decreased than in CAR.GFP- preTs (**Supplementary Figure S1**). No increased Annexin V binding of CD123+ as compared to CD123- preTs in the CAR.GFP-transduced preT population could be demonstrated after day 8, which makes fratricide by CD123 CAR-expressing preTs unlikely from day 8 onwards (**Figure 4c**). In line with this finding, the slow decline of transgene expression over time in the CAR.GFP-transduced group did not differ in its kinetics from the GFP only-transduced group allowing for comparable cell yields at the end. CAR expression of EGFP+ preTs declined as well from around 38% on day 4 to 28% on

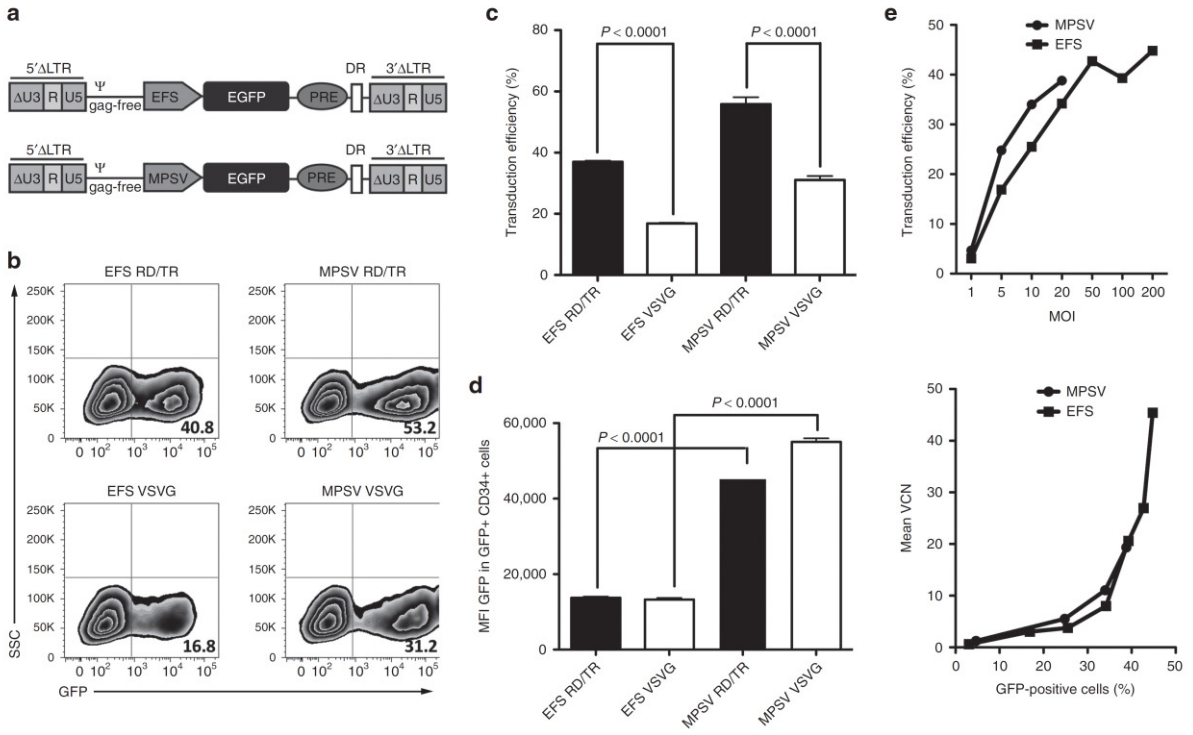

Figure 2 Alpharetroviral vectors containing a myeloproliferative sarcoma virus (MPSV) promoter and the modified feline endogenous retrovirus envelope glycoprotein RD114/TR deliver enhanced gene transfer into cord blood (CB)-derived CD34+ hematopoietic stem cells. (**a**) Self-inactivating (SIN) alpharetroviral vectors containing either an MPSV or an elongation-factor-1-short-form (EFS) promoter combined with an enhanced green fluorescent protein (EGFP) reporter gene were generated. Indicated are unique 5 (U5), repeat (R), and SIN unique 3 (ΔU3) regions, long terminal repeat (LTR), packaging signal (ψ), woodchuck posttranscriptional regulatory element (PRE) and direct repeat element (DR). (**b**) MPSV- and EFS-driven alpharetroviral vectors were pseudotyped with either vesicular stomatitis virus glycoprotein (VSVG) or RD114/TR and used to transduce CD34+ cells using an equal multiplicity of infection (MOI) of 100. (**c**,**d**) Transduction efficiency and mean fluorescence intensity (MFI) of EGFP were determined 6 days after transduction. Representative results of two independent experiments are shown ($n = 3$). (**e**) CB CD34+ cells were transduced with an increasing MOI using alpharetroviral MPSV- or EFS-driven vectors with the EGFP gene. After 6 days, transduction efficiency was determined by flow cytometry. Mean vector copy number (VCN) was assessed using quantitative real-time PCR for detection of PRE within the vector.

day 16. This is most likely caused by a proliferation disadvantage of the respective cell population after insertion of a relatively large gene cassette and not as a result of fratricide as shown above (Figure 4c).

Mature human T-cells expressing a third-generation CAR against CD123, or CD123 CAR and iCasp9 in combination are functional *in vitro*

For proof of principle experiments using a potentially relevant GOI, we evaluated the function of the new CD123 CAR and transduced human peripheral blood mononuclear cells (PBMCs) with the MPSV promoter-driven SIN alpharetroviral vector, into which our proof of principle GOI was cloned. Functionality was assessed by enzyme-linked immunosorbent assay and a fluorescence-based *in vitro* cytotoxicity assay. For targets, 293T cells were transduced with retroviral vectors using the human CD123 sequence which had been linked to tdTomato via an internal ribosomal entry site (IRES) cassette. Two sorting rounds ensured over 98% CD123 expression. Nontransduced 293T cells were used as controls. CD123 CAR T-cells strongly produced IFNγ upon stimulation on CD123+ target cells (Figure 5c). More importantly, these cells effectively lysed target cells expressing the CD123 antigen, as evident by a nearly complete loss of the tdTomato signal (Figure 5b). Antigen-negative target cells were spared demonstrating specific recognition of CD123 (Figure 5c). Nontransduced PBMCs mediated a weak background response only, being comparable for both, antigen-negative and antigen-positive target cells (Figure 5c).

After evaluating iCasp9 and the CD123 CAR in separate vector constructs, we designed a clinically more relevant combined gene cassette encoding both transgenes under the control of the MPSV promoter and used it for transduction of PBMCs. After transduction, the CD123 CAR could be specifically stained by flow cytometry using a CD123 peptide. To assess functionality of this combined construct, apoptosis was induced with the dimerizer. We did not observe any effects of the dimerizer on CAR.GFP-transduced PBMCs that were used as controls demonstrating the selectivity of dimerizer-mediated induction of apoptosis. A concentration as low as 0.05 nmol/l was sufficient to induce the maximal apoptosis rates in CAR.iCasp9-transduced PBMCs (Figure 5d). These functionality data encourage the further use of alpharetroviral vector platforms for adoptive T-cell transfer studies in preclinical models of leukemia.

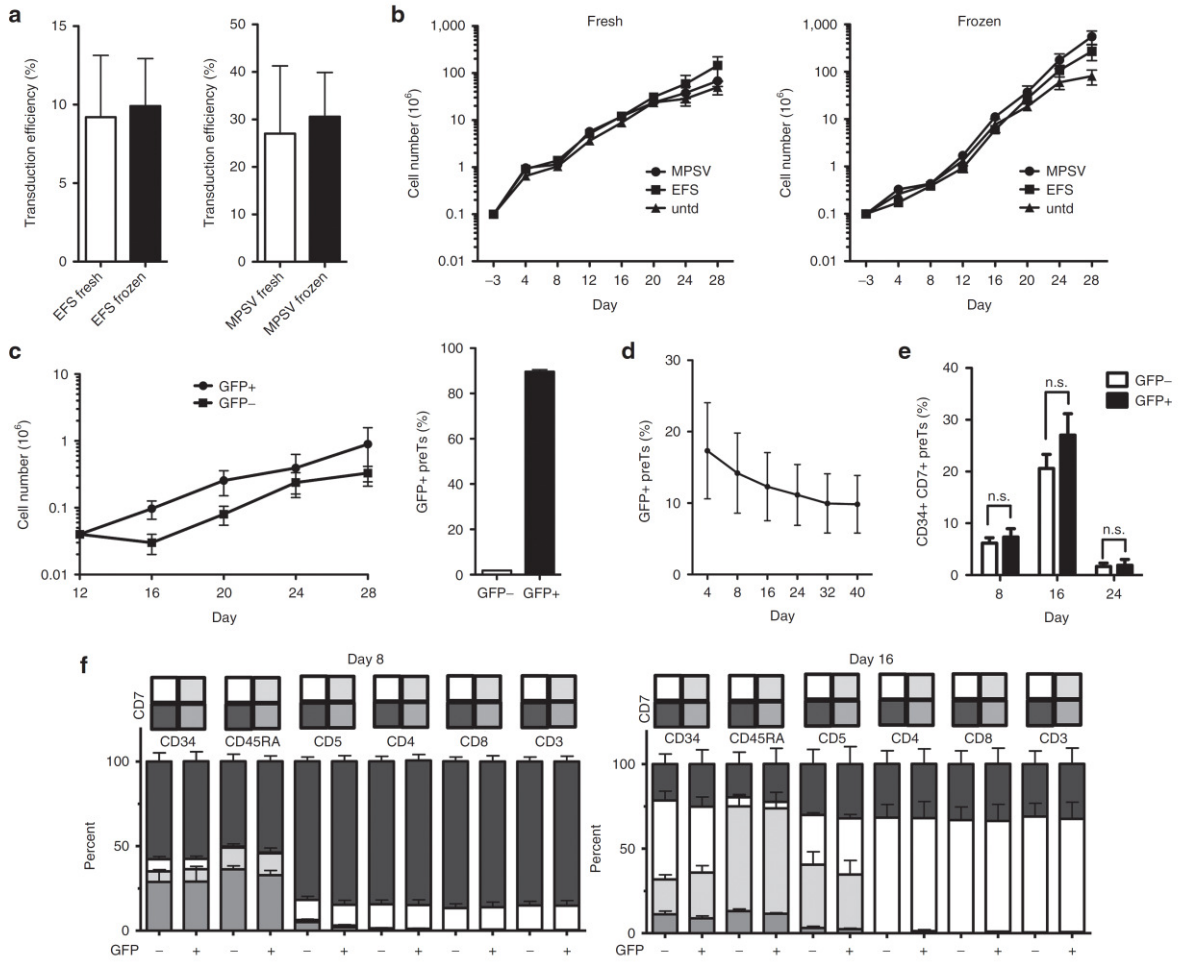

Figure 3 Transduction procedure and transgene positivity of human cord blood (CB)-derived CD34+ cells do neither impact the proliferation nor the differentiation pattern towards precursor T cells (preTs) *in vitro* when using enhanced green fluorescent protein (EGFP) as an insert only. (**a**) Fresh or thawed CB CD34+ cells were transduced with alpharetroviral vectors containing an EGFP gene serving as both, reporter and gene of interest. A multiplicity of infection of 10 was used. Transduction efficiency was determined by flow cytometry after 6 days (n = 3). Indicated are myeloproliferative sarcoma virus (MPSV) and elongation-factor-1-short-form (EFS) promoter. (**b**) Transduced or control untransduced (untd) preTs were cocultured with OP9-DL1 cells for 28 days. The proliferation rate of the cells was assessed every 4 days using trypanblue for determination of viability (n = 3). (**c**) Transduced preTs were sorted for EGFP expression (GFP+) on day 8 and consecutively continued to be cultured on OP9-DL1 cells under comparable conditions for up to 28 days. EGFP expression was determined on day 32 (24 days after sorting) (n = 3). (**d**) EGFP transgene expression of transduced but nonsorted preTs was determined over a period of 40 days (n = 3). (**e**) The percentage of nontransduced and transduced CD34+ CD7+ preTs was determined by flow cytometry on day 8, 16, and 24. (**f**) The phenotype of GFP- and GFP+ preTs was determined on day 8 and 16. Gray-colored bars depict subpopulations of preTs.

Equipping the alpharetroviral construct with the sequence of a third-generation CD123 CAR delays preT differentiation *in vitro* and allows for thymic engraftment and further differentiation *in vivo*

Since the phenotype of the preT-product has been shown to profoundly impact its thymic repopulating capacities (a prerequisite for further release of matured T-cell progeny into the periphery), we assessed the potential influence of the complete CAR-containing construct on *in vitro* differentiation of CD34+ cells in the OP9-DL1 coculture system by comparing our standard MOI of 10 to an MOI of 100. Nontransduced cells within the same culture were used as controls. CD34 on the early developing CD7+ CD5+ population has been demonstrated to be a sensitive differentiation marker and its coexpression facilitated robust thymic engraftment.[7] Using the CD123 CAR containing construct, coexpression of CD34 by day 11 was significantly higher than on the nontransduced control population. This suggests a differentiation delay induced by transduction. No differences were seen between the lower and the higher MOI (**Figure 6a**). PreTs harvested by day 11 of culture were sorted by fluorescence-activated cell sorting and consecutively injected intrahepatically into irradiated (1 Gy) 4 day old baby NSG (NOD.cg-Prkdc^{scid}IL2rg$^{tm/Wjl}$/Sz) mice.

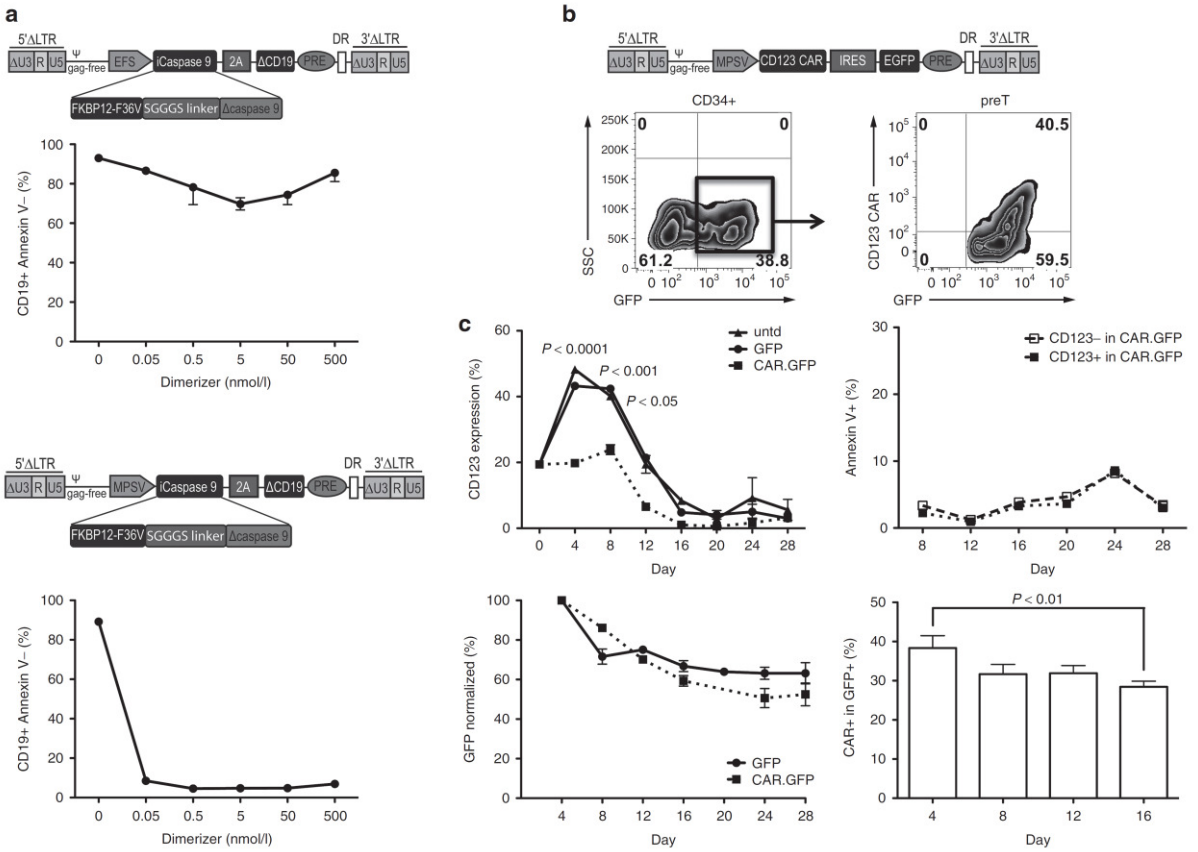

Figure 4 Transgene expression strength mediated by the myeloproliferative sarcoma virus (MPSV) promoter determines efficacy of inducible caspase 9 (iCasp9)-induced apoptosis and allows efficient CD123 chimeric antigen receptor (CAR) expression in precursor T cells (preTs). (**a**) iCasp9 containing the human FK506-binding protein (FKBP12) with an F36V mutation and a truncated ΔCD19 reporter gene was cloned into an alpharetroviral backbone under the elongation-factor-1-short-form (EFS) or MPSV promoter. Human Jurkat cells were transduced with fresh retrovirus and expanded *in vitro*. Transgene positive cells were sorted based on ΔCD19 expression and cultured with increasing concentrations of a dimerizing agent. Three days later, cell viability was assessed based on Annexin V staining. The percentage of transgene positive cells that did not undergo apoptosis upon dimerizer administration (CD19+ Annexin V-) was used for comparison ($n = 3$). (**b,c**) The CD123 CAR gene using an enhanced green fluorescent protein (EGFP) marker was cloned into an MPSV alpharetroviral backbone. Thawed CD34+ cells were transduced with modified feline endogenous retrovirus envelope glycoprotein (RD114/TR)-pseudotyped retroviral particles from the CAR.GFP, the GFP only construct or left untransduced (untd). At indicated time points during the OP9-DL1 coculture, preTs were assessed by flow cytometry for CD123, Annexin V, EGFP, and the CAR ($n = 3$). CAR. GFP groups are shown as dotted lines. Indicated are unique 5 (U5), repeat (R), and self-inactivating unique 3 (ΔU3) regions, long terminal repeat (LTR), packaging signal (ψ), internal ribosomal entry site (IRES), woodchuck posttranscriptional regulatory element (PRE) and direct repeat element (DR).

Six weeks after injection, thymi were harvested and analyzed for transgene-positive CD3 expressing thymocytes of human origin (**Figure 6b**). Whereas control mice, that had received CB-derived HSCs only, did not show any evidence of thymic engraftment at this time point, transgene positive CD3+ thymocytes could be readily detected in both treatment groups (CD123 CAR.GFP preTs or GFP only preTs) (**Figure 6b**). This demonstrates the engraftment capacity of alpharetrovirally-engineered preTs.

DISCUSSION

Redirecting the specificity of T-cells against tumor-associated antigens by genetically enforced expression of TCRs or CARs has recently boosted the field of adoptive T-cell transfer.[16–18] The use of second- and third-generation CARs has helped to resolve the long standing problem of insufficient *in vivo* T-cell persistence after transfer that was severely hampering its efficacy.[19,20] Nevertheless, important obstacles for a wider application are still remaining such as the necessity to produce a T-cell product on an individualized basis making this promising treatment approach hardly economically feasible. Although recent studies have successfully reported the use of autologous T-cells, it can be difficult to obtain suitable numbers of autologous cells in heavily pretreated patients.[21,22]

Human preTs might serve as the basis of a prefabricated cellular product that can be given even in a third party transplant setting across complete human leukocyte antigen (HLA) barriers without evoking graft-versus-host-disease. Their need to undergo thymic selection processes after cotransfer represents a potent filter preventing the release of graft-versus-host-disease-inducing alloreactive mature T-cells.[9] Alternative developments might even allow the use of post-thymic mature T-cells for multiple recipients. Elegant

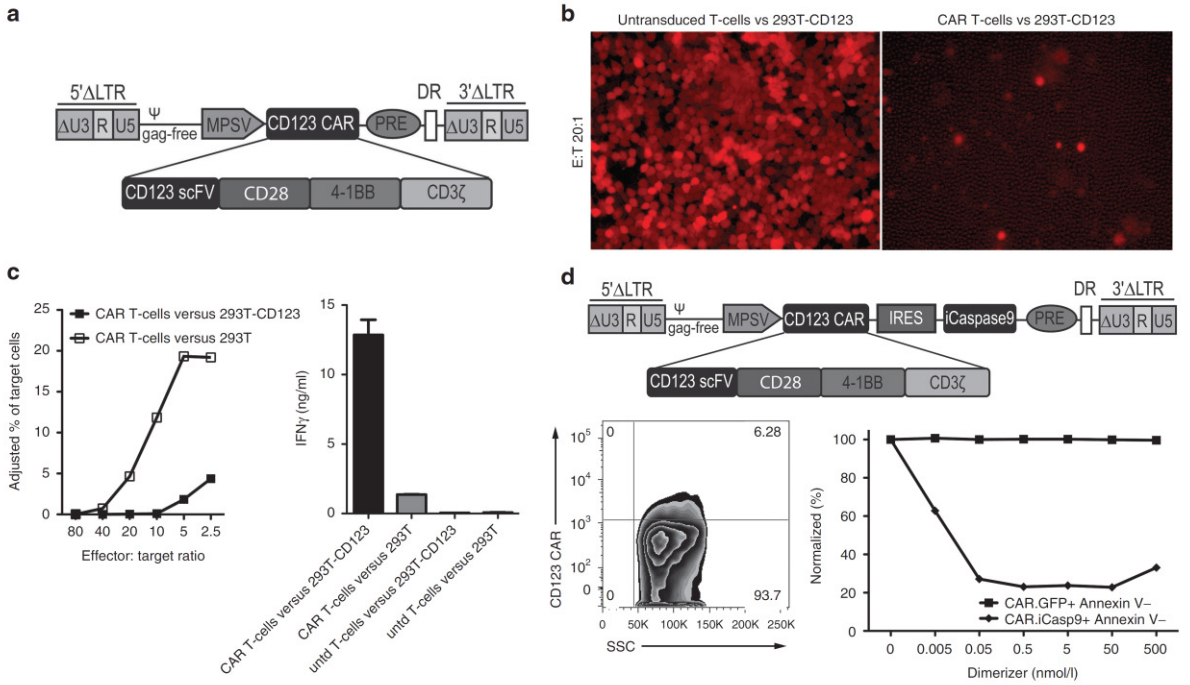

Figure 5 Mature human T-cells expressing a third-generation chimeric antigen receptor (CAR) against CD123, or CD123 CAR and inducible caspase 9 (iCasp9) in combination are functional *in vitro*. (**a**) Schematic figure of the MPSV.CD123 CAR construct. Self-inactivating (SIN) alpharetroviral vectors encoding for a third-generation CAR against the human surface molecule CD123 were generated. (**b**) Human peripheral blood mononuclear cells (PBMCs) were activated with anti-CD3 and anti-CD28 antibodies for 2 days, transduced on 2 consecutive days, and expanded for 4 more days before being used in a cytotoxicity assay. TdTomato.CD123-transduced 293T cells were used as target cells. Decreasing numbers of untransduced or CAR-transduced PBMCs were cultured with target cells in triplicates for 2 days. TdTomato expression was used as a marker for surviving target cells in immunofluorescence microscopy studies. (**c**) Cytotoxicity of the transduced effectors was determined by means of flow cytometry. Cell suspensions containing CAR-transduced effector cells and targets (either 293T cells only or 293T cells expressing CD123) at indicated ratios were stained for CD3. The percentage of viable $CD3^{neg}$ cells (surviving target cells) after incubation was used as an indirect parameter for cytotoxicity (left panel). Using an IFNγ release assay, T-cells (2×10^5) and target cells (2×10^4) were incubated at fixed effector:target ratios of 10:1 in 96-well plates. After 24 hours, supernatant was harvested and used in duplicates for an IFNγ enzyme-linked immunosorbent assay (right panel) ($n = 2$). Untransduced (untd) T-cells were used as control. (**d**) Schematic figure of the MPSV.CD123 CAR.iCasp9 vector. Human PBMCs were transduced with this bicistronic construct. Transgene positive cells with a functional CAR were identified by specific cell binding of a His Tag-labeled CD123 peptide. Transduced cells were incubated with the dimerizer for 48 hours. CAR.GFP-transduced PBMCs were used as control ($n = 3$). Apoptosis was quantified by staining viable transgene positive (CAR+ or GFP+) Annexin V-PBMCs. Indicated are unique 5 (U5), repeat (R), and SIN unique 3 (ΔU3) regions, long terminal repeat (LTR), packaging signal (ψ), myeloproliferative sarcoma virus promoter (MPSV), single chain variable fragment (scFV), internal ribosomal entry site (IRES), woodchuck posttranscriptional regulatory element (PRE) and direct repeat element (DR).

concepts are pursued wherein CAR-modified T-cells are subject to genetic editing and by doing so eliminating the expression of the endogenous αβ TCR.[23] Whereas we and others have shown that genetically engineered preTs can give rise to a long persisting tumor-specific T-cell population *in vivo*—a prerequisite for efficient antitumor efficacy[9]—this is, to our knowledge, still to be demonstrated for TCR-edited T-cells.

In vitro generation of human preTs from UCB has proven to compare favorably to their generation from bone marrow and peripheral blood stem cells.[4,14,24] T-cell potential of hematopoietic progenitors is known to decline with age as demonstrated in experiments conducted in fetal thymic organ cultures.[25] Since the demonstration of thymus-independent T-cell development *in vitro* using delta-like-1 by TM Schmitt and JC Zúñiga-Pflücker different human stem cell sources have been used on this system. Apart from studies with human UCB cells,[7,14] *in vitro* T-cell differentiation has been performed using embryonic stem cells,[26] adult bone marrow-derived hematopoietic progenitors,[24] and G-CSF-mobilized peripheral blood stem cells.[27] Work done by Patel *et al.*[28] comparing T-cell differentiation from various sources of CD34+ cells showed important differences in the kinetics and extent of proliferation prior to β-selection with a ten fold greater expansion of CB cells as compared to bone marrow cells. Therefore, different sources of CD34+ cells are not expected to yield a product of numerical comparability. Given the availability of CB, its biological proximity to fetal tissue, and the low degree of ethical concern as compared to other fetal stem cell sources, we decided to focus our studies on CB. In a next step, we were interested whether the use of frozen CB-derived preTs would result in comparable expansion and differentiation in an OP9-DL1 coculturing system. The fact that we could demonstrate superimposable expansion curves, both of fresh and thawed CB-derived CD34+ cells, supports the concept that frozen CB cells can serve for this technology.

Decreasing thymic function in elderly bone marrow transplant (BMT) recipients has raised concerns whether the cotransfer

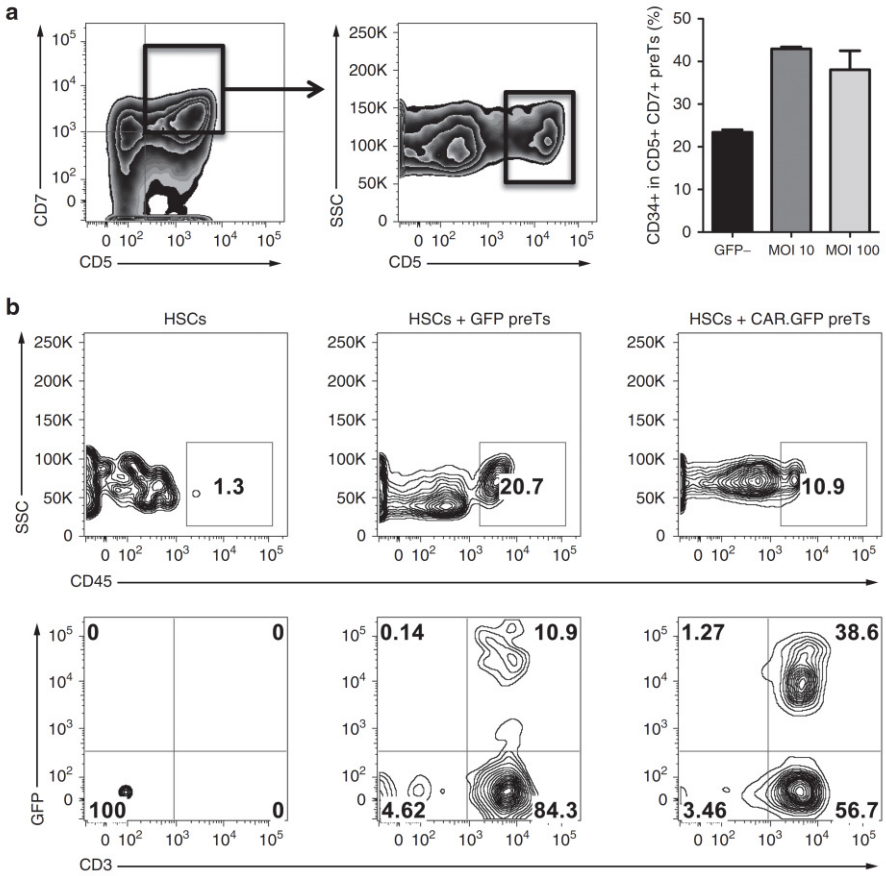

Figure 6 Equipping the alpharetroviral construct with the sequence of a third-generation CD123 chimeric antigen receptor (CAR) delays precursor T cell (preT) differentiation *in vitro* and allows for thymic engraftment and further differentiation *in vivo*. (**a**) Cord blood (CB)-derived CD34+ cells were transduced with the CD123 CAR-containing alpharetroviral construct either using a multiplicity of infection (MOI) of 10 or 100. Enhanced green fluorescent protein (GFP) was used as reporter gene. After transduction, cells were brought into the OP9-DL1 coculture system and phenotypically analyzed on day 11. Within the GFP+ population (transduced cells) CD5+ CD7+ were gated on and assessed for CD34 coexpression. The GFP- fraction (nontransduced) served as control. CD34 coexpression was lower on nontransduced as compared to transduced CD5+ CD7+ cells ($P < 0.05$). No significant difference was found between MOI 10 and 100. Two independent experiments were performed. (**b**) 2×10^5 transgene positive preTs (either transduced with GFP only or CAR.GFP) together with 0.2×10^5 CB-derived $CD34^+$ hematopoietic stem cells (HSCs) were intrahepatically injected into 4 day old irradiated NSG mice. Controls were injected with HSCs only. Mice were boosted with the IL-7/M25 mixture every 5 days. Thymi were harvested 6 weeks after injection, single cell suspensions were generated and analyzed by flow cytometry. The upper row shows the fraction of human-derived cells, which were obtained from the different treatment groups. After gating on this human-derived cell population, transgene-positive cells expressing CD3 can be found in the thymi of treated mice (lower row).

of preTs would sufficiently give rise to a respective post-thymic mature T-cell progeny. An increasing amount of evidence suggests that the potential for extrathymic T-cell development is strongly enhanced after BMT. It has been nicely shown that the extrathymic microenvironment, importantly the mesenterial lymphatic lymph node system of the intestine, can provide the required cues to harbor and further differentiate preTs into a functional extrathymically-derived T-cell pool. These data have underlined the potential of extrathymic T-cell development for T-cell reconstitution in patients with limited thymic function.[29]

Especially for lentiviral constructs, although being very efficient for transducing end-differentiated T-cells, the application of stable packaging cells for economical large scale vector production has proven to be extraordinarily difficult.[30,31] Therefore, for the production of genetically engineered preTs, gammaretroviral systems remain attractive since stable packaging cell lines can deliver consistency required for market authorization.[32] Furthermore, transducing a relatively small number of CD34+ cells for later differentiation into much larger numbers of preTs minimizes the amount of viral supernatant needed for transduction.

For the transduction of CB-derived hematopoietic precursors, we use a new generation of alpharetroviral SIN vectors since genotoxicity will be a major concern if genetically engineered preTs will be considered for clinical use. As comparatively shown by us in a serial murine transplantation model, the use of alpharetroviral vectors can result in sustained multilineage transgene expression being comparable to lentiviral and gammaretroviral SIN vectors.[12] In contrast to the latter two, alpharetroviral integration showed to

be less frequently occurring close to transcription start sites, CpG islands, and potential oncogenes. Of note, the propensity of the "extragenetic" genomic integration pattern of alpharetroviruses still suggested long-term transgene expression as demonstrated in serial transplant experiments. This extremely elegant comparative analysis of Suerth and co-workers was performed using the SFFV internal promoter for all three tested vector systems thereby modeling a "worst case scenario" for both, insertional gene activation and epigenetic silencing.[12,33–35] However, despite reported interlineage variability a trend versus lowest transgene expression rates was seen when using alpharetroviral vectors. Therefore, for the generation of genetically engineered preTs, we targeted previously enriched CB-derived CD34+ cells and comparatively assessed the use of two promoters (EFS versus MPSV), combining each with improved envelope glycoproteins, either VSVG or RD114/TR. The comparative study by Suerth and co-workers observed a reduced incidence of immortalization and a tendency for decreased fitness in immortalized cells when using alpharetroviral vectors with the GOI put under the SFFV promoter. Exchanging this internal "high risk" promoter with the EFS promoter abolished any evidence of genotoxic potential within the detection limits of their *in vitro* immortalization assay.

Engineering CB-derived CD34+ cells, we observe a significantly enhanced gene expression strength and transduction efficiency when using the MPSV promoter in combination with the RD114/TR envelope glycoprotein for pseudotyping the particles. Importantly, these differences were physiologically relevant when iCasp9 as a suicide gene was put under the respective promoter control. Adding the clinically applicable apoptosis-inducing dimerizer AP20187 *in vitro*, target cells containing the GOI under the control of the MPSV promoter only underwent rapid apoptosis at concentrations of the dimerizer being relevant for clinical studies.[36] Therefore, for further experiments (**Figure 4b**,**5**,**6**) alpharetroviral constructs containing the MPSV promoter were used only. In these studies, we could demonstrate that transduction had no impact on preT expansion rates regardless of whether CD34+ cells were used either freshly isolated or after storage in liquid nitrogen. This has important implications when evaluating a cell product with a potential for "off the shelf" use. Last, transduction of CB-derived CD34+ cells did not seem to influence the differentiation pattern along the different stages of preT development *in vitro*. However, this was the case only when EGFP alone was used as GOI. Adding a third-generation CD123 CAR to the construct caused maturation delay *in vitro* as demonstrated by a longer persistence of CD34 expression on CD5+ CD7+ early preTs. This is relevant since the human CD34+ CD7+ cellular equivalent to murine DN2 cells has the highest potential of thymic seeding after cotransfer and will consecutively give rise to a fully functional host MHC-restricted T-cell population.[5,14,37]

With CD123 being expressed on hematopoietic precursor cells, one might hypothesize that forced CD123 CAR expression on CD34+ cells and their further differentiated progeny might cause fratricide among CD123-expressing cells during *in vitro* culture. Indeed, this seems to be the case early in the differentiation process. However, this fratricide showed to be incomplete and decreases over time, most likely caused by decreasing levels of CD123 expression upon differentiation and an incomplete killing machinery of preTs. In this context, one needs to state, that some functions of an introduced construct can be functionally assessed only after preTs have undergone final maturation in the recipient. This holds specifically true for genes interfering with T-cell effector mechanism such as TCRs and CARs. It was therefore important to demonstrate in a humanized mouse model that the engineered preTs had indeed thymus engrafting potential with consecutive further T-cell developmental capacities *in vivo*.

Altogether, we apply a novel and improved alpharetroviral vector system for the generation of genetically engineered preTs. As proof of principle, we cloned three different genes of interest, either EGFP, the suicide gene iCasp9 or a CAR against CD123 into this construct. If once considered for clinical use, this system might have the potential to make engineering of preTs safer and economically feasible.

MATERIALS AND METHODS

Primary samples and cell lines. Human UCB samples (approximately 50 ml/sample), not eligible for banking, were obtained after written, informed consent by the child's mother. Procedures for the use of UCB for this study were reviewed and approved by the medical ethics committee of Hannover Medical School. CB mononuclear cells were isolated using Ficoll density centrifugation and CD34 selection was performed using a CD34 microbead kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purity of CD34+ cells was higher than 95% as determined by postenrichment flow cytometric analysis. CD34+ cells were cryopreserved in 50% fetal calf serum, 40% αMinimum Essential Medium, and 10% dimethylsulfoxide.

OP9-DL1 cells were cultured in αMinimum Essential Medium containing 20% heat-inactivated fetal calf serum. UCB CD34+ cells were stimulated for 36 hours in X-VIVO 10 medium containing the following human cytokines: stem cell factor (SCF, 100 ng/ml), thrombopoietin (TPO, 100 ng/ml), and FMS-like tyrosine kinase 3 ligand (Flt3L, 100 ng/ml) (all PeproTech, Rocky Hill, NJ). Stimulated CD34+ cells were transduced on Retronectin-coated 24-well plates. Cells were then transferred on 90% confluent OP9-DL1 cell monolayers containing 20% fetal calf serum, SCF (20 ng/ml), TPO (20 ng/ml; until day 24), Flt3L (10 ng/ml), and interleukin 7 (10 ng/ml). Every 4 days, preTs were harvested, passed through a 70 µm filter, and transferred to new OP9-DL1 cell monolayers.

Human embryonic kidney 293T cells and the fibrosarcoma cell line HT1080 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. TdTomato.CD123-expressing 293T cells were generated by transduction with a gammaretroviral vector encoding tdTomato and CD123 linked by an IRES sequence.

Mice. Animals in the experiments were used under protocols approved by the State Government of Lower Saxony, Germany. NOD.cg-Prkdc^{scid} IL2rg$^{tm/Wjl}$/Sz (NSG) mice were purchased from Charles River, housed, and bred in a pathogen free facility.

Hematopoietic cell transplantation. PreTs in combination with UCB-derived CD34$^+$ HSCs were intrahepatically injected in 4 day old irradiated NSG mice as previously described.[7] In brief, transgene-positive preTs were sorted on day 11 of OP9-DL1 coculture. 2×10^5 preTs together with 0.2×10^5 HSCs were resuspended in 30 µl PBS containing recombinant human interleukin 7 (rhIL-7) (2.5 µg) and the IL-7 antibody M25 (0.5 µg) and consecutively injected intrahepatically into irradiated (1 Gy) newborn mice. Controls were injected with HSCs alone. Mice were boosted with the IL-7/M25 mixture every 5 days. Thymi were harvested 6 weeks after injection for flow cytometric analysis.

Vector construction and cloning. We utilized SIN alpharetroviral vectors containing an MPSV or EFS promoter and a woodchuck post-transcriptional regulatory element (PRE).[38] EGFP was cloned into the

constructs and expressed via an IRES sequence. Cloning details are available on request.

Inducible caspase 9 was kindly provided by Prof. Malcolm K. Brenner, Baylor College of Medicine, Houston, TX, USA. It is comprised of a mutated human FK506-binding protein fused via an SGGGS linker to human caspase 9, and was linked by a 2A sequence to truncated human CD19 (ΔCD19). The dimerizer agent (Clontech, Palo Alto, CA) is a synthetic nontoxic FK506 analog that upon administration leads to aggregation and activation of inducible caspase 9 monomers and eventually induction of apoptosis.

A third-generation CD123-specific CAR containing the codon-optimized sequences for a CD123-specific scFV, the transmembrane region of the human CD28 molecule, the costimulatory signaling endodomains of CD28 and 4-1BB, and the CD3ζ signaling domain, was cloned into a SIN alpharetroviral backbone. EGFP or iCasp9 linked by an IRES sequence was cloned downstream of the CAR cassette.

Cell transduction. For transient viral vector production, 293T cells were transfected using a calcium phosphate transfection kit (Sigma Aldrich, Steinheim, Germany) with MPSV or EFS constructs. They were combined with plasmids encoding gag/pol and either the RD114/TR (described previously, provided by Prof. Els Verhoeyen, Lyon, France) or the VSVG envelope.[39] Retroviral supernatant was either freshly used or concentrated by ultra-centrifugation, immediately frozen in dry ice, and stored at −80 °C for further usage. The fibrosarcoma cell line HT1080 was used for determining the viral titer.

Before transduction, CD34+ cells were prestimulated for 36 hours in X-VIVO 10 serum free medium (Lonza, Basel, Switzerland) at a maximal density of 0.6×10^6/ml in the presence of SCF, TPO, and Flt3L (all 100 ng/ml; PeproTech).

For the transduction of Jurkat cells, PBMCs and CB-derived CD34+ cells, 24-well plates were coated with Retronectin (Takara, Otsu, Japan) overnight at 4 °C. Retroviral supernatant was added and plates were spin-oculated (490×g, 1 hour, 4 °C) to facilitate retrovirus binding to Retronectin. Subsequently, retroviral supernatant was removed and up to 15×10^4 cells were added per well.

Flow cytometry. The following fluorochrome-conjugated antibodies were purchased from BioLegend (San Diego, CA): CD3 (PerCPCy5.5), CD4 (Brilliant Violet 570), CD5 (Brilliant Violet 421), CD8 (PE), CD34 (PECy7), CD45RA (APCCy7), CD123 (APC), Annexin V (PE) or BD Biosciences (San Jose, CA): CD7 (APC), CD19 (PE), and Annexin V (APC). For CD123 CAR staining, human IL3 receptor α/CD123 protein with a His Tag (Sino Biological, Beijing, China) and an anti-His Tag antibody (APC) (R&D Systems, Minneapolis, MN) were used. Data were acquired using a FACSCanto or LSRII (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR). Untransduced cells were used as control.

q-RT-PCR for determination of vector copy numbers. Genomic DNA was isolated from transduced CD34+ cells and vector copy numbers determined by using the TaqMan system (Qiagen, Hilden, Germany). Quantitative PCR was performed on an Applied Biosystems (Darmstadt, Germany) Step One Plus real-time PCR.[13] The primers are specific for the vector PRE and the PTBP2 intron.[40] Vector copy numbers of CD34+ cell samples were analyzed as previously described.[41]

Induction of apoptosis. Cells transduced with vectors containing the iCasp9 and the ΔCD19 gene were sorted for CD19 using a FACSAria cell sorter (BD Biosciences). AP20187 (Clontech) was added at increasing concentrations. After 48–72 hours, cells were stained with Annexin V (APC or PE) and analyzed within 1 hour by flow cytometry for apoptotic cells. Alternatively, a construct encoding CD123 CAR and iCasp9 was used.

Generation of CAR T-cells and cytotoxicity assay. For the assessment of the CAR functionality *in vitro*, human PBMCs were isolated from blood samples of healthy volunteers using Ficoll-Paque PLUS reagent (GE Healthcare, Uppsala, Sweden) and were activated for 2 days with anti-CD3 antibody (50 ng/ml), anti-CD28 antibody (500 ng/ml) and IL-2 (25 U/ml). Cells were transduced on 2 consecutive days with alpharetroviral supernatant containing the CD123 CAR vector. After further expansion for 4 days, effector and target cells (293T cells expressing CD123 and tdTomato linked via an IRES sequence) were cocultured at indicated ratios for 2 days. Cytotoxicity was assessed by fluorescence microscopy or flow cytometry.

Enzyme-linked immunosorbent assay (ELISA). T-cells (2×10^5) and target cells (2×10^4) were incubated (effector:target ratio of 10:1) in V-bottom 96-well plates in the presence of IL-2 (25 U/ml) and IL-7 (5 ng/ml). After 24 hours, the culture supernatant was harvested and used in duplicates for an IFNγ ELISA (BioLegend).

Statistical analysis. Unless specified in the text, data were presented as mean ± standard error of the mean. The Student's *t*-test was used to determine the statistical significance of differences between samples. *P* values <0.05 were considered to be statistically significant.

SUPPLEMENTARY MATERIAL

Figure S1. CD123 expression is most prominently reduced in CAR. GFP+ preTs.

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VI Discussion

VI.1 *In vitro* generation of human preTs for adoptive transfer

ACT for cancerous malignancies has mainly exploited mature T cells. Even though the use of mature autologous T cells proved successful, the reduced number of functional T cells from intensively pretreated tumor patients impedes clinical application (Brentjens *et al.*, 2013). Alternatively, an allogeneic donor can be matched, but then MHC compatibility is required. Therefore, ACT is highly individualized which represents an obstacle to fit it into current practices of cancer treatment. In addition, it is labor-intensive and requires expertise in laboratory procedures. Using preTs might allow their adoptive transfer as an "off-the-shelf" product over MHC barriers because they still have to be selected in the thymus of the recipient as shown in a murine transplantation model where maturation of alloreactive T cells was successfully prevented (Hoseini *et al.*, 2015).

We have shown that human preTs can be efficiently generated from highly purified UCB CD34+ cells. In contrast to other groups, we did not observe the emergence of mature T cells expressing CD4 and/or CD8 (van Lent *et al.*, 2007). This might be due to different culture conditions such as relatively high concentrations of SCF and IL-7. For murine cells, it was observed that this prevents development from the double negative to the double positive stage (Wang *et al.*, 2006). In another study, after five weeks of co-culture in freshly reconstituted lyophilized medium 22% of the cells were CD4/CD8 double positive compared to only 0.5% in the ready-to-use medium group (Six *et al.*, 2011). Even though mature T cells may emerge during OP9-DL1 co-culture, adoptive transfer of these cells bears the risk of impaired negative selection of self-reactive T cells, because OP9-DL1 cells presumably have a limited capability to present self-antigens (van den Brink *et al.*, 2004). This also favors use of *in vitro* generated preTs instead of mature T cells.

We used UCB as a source for preT generation, but *in vitro* differentiation was also shown from other origins such as postnatal thymocytes, bone marrow and peripheral blood (Schmitt *et al.*, 2002; De Smedt *et al.*, 2004; Awong *et al.*, 2009; Van Coppernolle *et al.*, 2009). However, only UCB is a widely accessible source of HSCs without invasive procedures on donors, which encourages its use. In addition, we have demonstrated comparable transduction efficiency, proliferation and phenotype kinetics in fresh and frozen CD34+ cells transduced with an EGFP construct. This further strengthens the potential as a readily available cell product. Recently, a pyrimidoindole derivate, UM171, was shown to be efficient for expansion of UCB CD34+ cells *ex vivo* (Fares *et al.*, 2014). We were interested in investigating how this expansion would affect the proliferation and phenotype during OP9-DL1 co-culture and found similar kinetics compared to untreated CD34+ cells (**Figure 22**). Therefore, this procedure could further increase availability of UCB CD34+ cells and large-scale generation of human preTs.

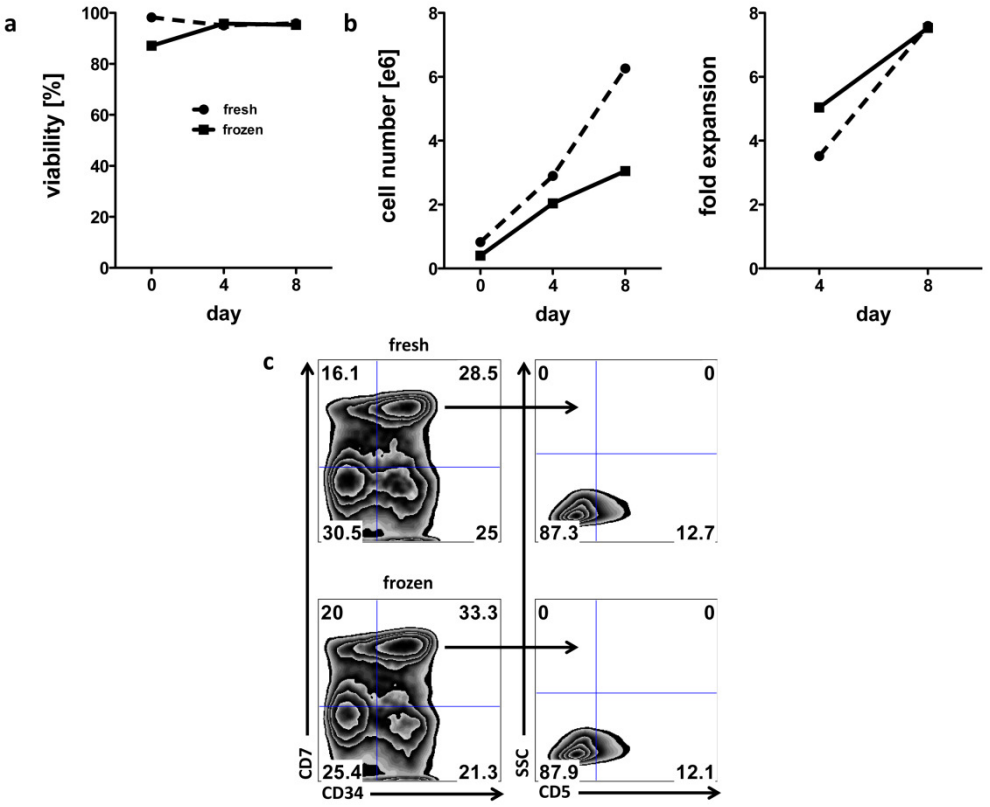

Figure 22: CD34+ cells expanded in UM171 expand in subsequent OP9-DL1 culture and develop a preT phenotype. (a, b) CD34+ cells were expanded for eleven days with UM171 and either directly used for OP9-DL1 co-culture or previously frozen away. Viability and cell number were assessed by Trypan blue. (c) Phenotype of preTs was determined by flow cytometry with CD34, CD7, and CD5 staining.

Another potentially unlimited resource is programming somatic cells into induced pluripotent stem cells (Takahashi *et al.*, 2006), but it has been shown that factors for reprogramming, like c-myc, induce genetic instability and mutations and therefore this might present a safety risk (Louis *et al.*, 2005).

There are not only various HSC sources, but also several possibilities to differentiate these *in vitro*. In our experiments, preT differentiation was driven by genetically modified murine OP9-DL1 feeder cells. This impedes transfer to human application as it is a xenogenic system and recipients might mount an immune reaction. It might be preventable by using autologous primary fibroblasts that could generate self-tolerant and self-MHC restricted T cells after transfer. However, primary human fibroblasts made to express DL-4, another Notch ligand, showed very limited support of human T cell differentiation, which could not be further enhanced when myelopoiesis was blocked (Mohtashami *et al.*, 2013). Another attempt is to generate feeder cell free culture systems. UCB CD34+ cells were cultured on immobilized DL-1 (Ohishi *et al.*, 2002) or DL-4 (Reimann *et al.*, 2012) and generated preTs transferred to immunodeficient mice where they gave rise to mature T cells.

VI.2 Genetically engineering cord blood CD34+ cells with alpharetroviral vectors

Next, we used alpharetroviral vectors to transduce UCB CD34+ cells and showed that the MPSV promoter and RD114/TR envelope ensure improved gene transfer.

Gammaretroviral vectors are still most widely used of all retroviral vector systems. This is partly due to the fact that generation of stable packaging cell lines to upscale vector particle production is difficult for lentiviral vectors (Ikeda *et al.*, 2003; Throm *et al.*, 2009). However, gamma- and lentiviral vectors still show an unfavorable integration pattern with gammaretroviral ones integrating close to transcription start sites and proto-oncogenes and lentiviral ones next to actively transcribed regions (Mooslehner *et al.*, 1990; Scherdin *et al.*, 1990; Schroder *et al.*, 2002; Wu *et al.*, 2003; Hematti *et al.*, 2004; Wagner *et al.*, 2005; Beard *et al.*, 2007; Cattoglio *et al.*, 2007; Montini *et al.*, 2009). Recently, the alpharetroviral vector integration pattern was observed to be relatively neutral with respect to the mentioned genomic structures (Narezkina *et al.*, 2004; Hu *et al.*, 2008). In addition, it was shown in a mice transplantation model that transgene expression is long-term and comparable to the aforementioned established viral vector systems (Suerth *et al.*, 2012). However, the alpharetroviral vectors were prone to lead to the least transgene expression and we therefore decided to compare two different promoters regarding their transgene expression strength. We used the constitutive cellular EFS promoter, because it is already being integrated into clinical grade vectors and is less likely to activate nearby genes. This was shown in gammaretroviral and alpharetroviral SIN vectors, where the genotoxicity was reduced below the detection level of an *in vitro* immortalization assay when compared to the retroviral promoter SFFV (Zychlinski *et al.*, 2008; Suerth *et al.*, 2012). Even though the retroviral MPSV promoter exhibits higher enhancer activity in a plasmid-based enhancer assay, which is a potential drawback (Zychlinski *et al.*, 2008), we could show that transgene expression strength driven by this promoter was significantly higher and important when used with clinically relevant genes and therefore nonetheless favors its use.

We not only compared two promoters, but also two envelopes. VSVG is a widely used envelope known for stability, broad tropism, and high titers (Burns *et al.*, 1993). However, human beings may evolve immune reactions against VSVG-pseudotyped cells. Therefore, other envelopes have been developed. In 2002, RD114/TR was created and lentiviral vectors pseudotyped with this envelope showed increased transduction in human peripheral blood lymphocytes and CD34+ cells compared to VSVG (Sandrin *et al.*, 2002). We also confirmed this in CD34+ cells with alpharetroviral vectors. Comparable to lentiviral vectors, RD114/TR transduced CD34+ cells at lower MOI than VSVG and with comparable VCN (Di Nunzio *et al.*, 2007). We were further able to improve transduction efficiency by applying the RBV infection method, thereby possibly removing inhibitory molecules that decrease the efficiency of retroviral transduction. We also compared viability of transduced CD34+ cells and did not observe a difference between the two promoters and two envelopes six days after transduction. However, we did not continue the culture beyond that time point and might have missed the toxic effects of VSVG as observed in lentiviral vectors (Di Nunzio *et al.*, 2007).

Then, we investigated the impact of transduction in fresh versus frozen cells on transduction efficiency, proliferation, and phenotype and found comparable results for MPSV- and EFS-driven GFP only vectors. We used a small MOI of 10, assuming that each insertion has the same likelihood of causing a pro-leukemogenic event and that the amount of retroviral insertions is of decisive importance (Fehse *et al.*, 2004; Modlich *et al.*, 2005). Particularly, the number of CD34+CD7+ preTs was similar, which is important because this population

represents the cell product engrafting and inducing thymic reconstitution in an immunodeficient mouse model (Awong *et al.*, 2009; Awong *et al.*, 2013) and might therefore be equivalent to murine DN2 cells that undergo selection processes in the recipient thymus (Zakrzewski *et al.*, 2006).

VI.3 iCasp9 and CD123 chimeric antigen receptor for alpharetroviral gene transfer

For proof of principle experiments we used EGFP as a reporter gene. Then, we chose a more clinically relevant gene: iCasp9. Much progress has been made in designing safer gene transfer systems, however the risk of side effects remains and using suicide genes may improve their safety profile. iCasp9 has been widely used in clinical trials (Di Stasi *et al.*, 2011) and might also be beneficial for purposes other than abrogating adverse events. For example, inducing apoptosis while T cells are in secondary lymphoid organs would present a second role as cancer vaccine since this might increase the amount of antigens for DCs to present and thereby enhance immunogenicity. Originally, iCasp9 was developed in the laboratory of Malcolm Brenner in 2005 and they showed that retrovirally transduced and murine stem cell virus promoter-driven GFP^{high}-selected T cells were more sensitive to the dimerizer (Straathof *et al.*, 2005). In the alpharetroviral preT setting, we were also able to demonstrate the relevance of transgene expression strength. Only the MPSV promoter mediated iCasp9-induced apoptosis, even at low and clinically relevant dimerizer concentrations (Di Stasi *et al.*, 2011). Nonetheless, about 10% of transduced Jurkat cells survived the dimerizer. In an autologous primate transplantation model, remaining cells were shown to upregulate Bcl-2, an anti-apoptotic protein (Barese *et al.*, 2015). However, iCasp9 responsiveness could be restored upon expansion and reactivation of T cells in vitro (Tey *et al.*, 2007; Di Stasi *et al.*, 2011). As an alternative, a dual suicide gene approach or using a CD19-drug conjugate to eliminate residual transduced cells after survival of the dimerizer might improve the safety strategy. Nevertheless, eliminating all transduced cells also abrogates anti-tumor effects. In order to reduce toxicity and maintain long-term anti-tumor function, one possible strategy, however only valid for solid tumors, consists of only expressing the CAR at the tumor site, for instance by making use of tumor-related metabolic conditions such as hypoxia (Allen *et al.*, 2011). Other attempts to enhance safety do not focus on suicide genes, but rather the CARs themselves. Firstly, dual targeting by introducing two different CARs that provide complementary killing and co-stimulation signals and that only kill if both CARs specifically bind their respective antigen has shown promising results (Kloss *et al.*, 2013). A limitation to this approach is that antigen expression on human cancer cells is usually heterogenous. Secondly, inclusion of a second "inhibitory" CAR (iCAR) could avoid on-target off-tumor toxicity. To achieve this, an activatory CAR binds the tumor antigen, whereas an iCAR recognizes a second antigen being expressed on normal tissue. In the iCAR, CD3ζ is replaced by a CTLA-4 or PD-1 moiety which is stronger than the activatory CD3ζ signal and therefore leads to target antigen sparing, as shown in preclinical studies (Fedorov *et al.*, 2013).

As a second clinically relevant gene, we generated a third generation anti-CD123 CAR as potential AML target and showed its functionality in immunofluorescence, cytotoxicity, and IFN-γ studies. Introducing a TCR or CAR

into T cells broadens the spectrum of tumor antigens to be targeted. Genetic engineering of human HSCs and subsequent differentiation on OP9-DL1 cells was first performed with TCRs against p53 (Zhao *et al.*, 2007), and melanoma, CMV and minor histocompatibility antigens (van Lent *et al.*, 2007). Based on our knowledge, we demonstrate for the first time alpharetroviral transduction of CD34+ HSCs with a CAR, in our case against AML. Two main targets have been investigated for CAR development against AML, primarily CD33. The CD33 CAR turned out to convey potent killing but physiologic hematopoietic reconstitution was hampered because of CD33 expression on these cells (Marin *et al.*, 2010). Next, focus was put on CD123, the IL-3 receptor α chain, which, like CD33, is overexpressed on AML, but is less present on hematopoietic stem and progenitor cells (Jin *et al.*, 2009). Another advantage of targeting CD123 is the possibility to impact leukemia stem cells, which are mainly liable for chemoresistance and relapse (Snauwaert *et al.*, 2013). *In vitro*, a second generation CD123 CAR, based on a lentiviral vector platform, demonstrated functional activity and little *in vitro* toxicity against common myeloid progenitors from UCB (Mardiros *et al.*, 2013) and a first generation one showed improved sparing of normal hematopoietic cells and reduced killing of CD123+ monocytes and endothelial cells (Tettamanti *et al.*, 2013). *In vivo*, third generation CD123 CAR T cells were studied in NOD/SCID/IL-2r-γnull mice engrafted with primary human AML cells. Robust anti-cancer activity was observed, even after secondary transplantation. Furthermore, normal hematopoietic stem and progenitor cells were mostly spared as seen by engraftment of CB CD34+ cells (Pizzitola *et al.*, 2014). However, with another, second generation, CD123 CAR normal human myelopoiesis in immunodeficient mice got eliminated (Gill *et al.*; 2014). As an alternative, other AML antigens investigated for CAR development include cell surface associated Mucin 1 (Wilkie *et al.*, 2008) and folate receptor β (Lynn *et al.*, 2015).

Specific targeting and activation of CARs is of outmost importance. With our CD123 CAR we observed low antigen-unspecific IFN-γ production *in vitro*, however *in vivo* behavior remains to be assessed. High antigen-unspecific IFN-γ production is unfavorable because it leads to earlier T cell apoptosis (Muhlbauer *et al.*, 2006). One possibility to reduce this is inactivation of phosphorylated tyrosines in immunoreceptor tyrosine-based activation motifs (ITAMs), which can be found three times in CD3ζ. It was reported that changing tyrosine to phenylalanine in the first and third ITAM decreases unspecific IFN-γ production (Kochenderfer *et al.*, 2010).

Collectively, we have shown that alpharetroviral vectors represent a potentially effective therapeutic tool for genetic modification of human HSCs, their differentiation into preTs and engraftment in NSG mice. Further research should assess the functionality of these preTs after ACT in humanized mice models (Traggiai *et al.*, 2004) in order to show validity of iCasp9 and CAR transfer into human HSCs for cell-based immunotherapy.

Juwita Hübner

Generation of genetically engineered precursor T cells from human umbilical cord blood using an optimized alpharetroviral vector platform

Summary

Retroviral engineering of hematopoietic stem cell-derived precursor T cells (preTs) opens the possibility of targeted T cell transfer across MHC barriers. Using alpharetroviral vectors that exhibit a more neutral integration pattern can thereby reduce the risk of insertional mutagenesis. We transduced cord blood (CB)-derived CD34+ cells and differentiated them into preTs *in vitro.* Two promoters, elongation factor 1 short-form (EFS) and a myeloproliferative sarcoma virus variant (MPSV), in combination with two commonly used envelopes, vesicular stomatitis virus glycoprotein (VSVG) and a modified cat retrovirus glycoprotein (RD114/TR), were comparatively assessed for transduction. Green fluorescent protein or a third generation chimeric antigen receptor (CAR) against CD123 was used as gene of interest. Furthermore, the inducible suicide gene Caspase 9 (iCasp9) has been validated. Combining the sarcoma virus-derived promoter with a modified feline endogenous retrovirus envelope glycoprotein yielded in superior transgene expression and transduction rates. Fresh and previously frozen CD34+ cells showed similar transduction and expansion rates. Transgene positive cells did not show proliferative impairment. Only the sarcoma virus-derived promoter could express sufficient levels of iCasp9 to mediate dimerizer-induced apoptosis. Finally, the CD123 CAR was efficiently expressed in CD34+ cells and preTs and proved to be functional when expressed on differentiated T cells. Furthermore, CAR-expressing preTs showed *in vivo* thymic engraftment in mice. Therefore, the transduction of CD34+ cells with alpharetroviral vectors represents a feasible and potentially safer approach for stem cell-based immunotherapies for cancer.

Juwita Hübner

Generation of genetically engineered precursor T cells from human umbilical cord blood using an optimized alpharetroviral vector platform

Zusammenfassung

Retrovirale Vektoren können zur genetischen Modifikation von hämatopoietischen Stammzellen verwendet werden. Daraus generierte Vorläufer-T-Zellen erlauben potenziell MHC-unabhängigen Transfer von Antigen-spezifischen Zellen. Die Verwendung von alpharetroviralen Vektoren ermöglicht hierbei eine Verringerung des Risikos für insertionale Mutagenese, da diese ein neutraleres Integrationsspektrum zeigen. Wir nutzten alpharetrovirale Vektoren zur Transduktion von CD34+ Zellen aus Nabelschnurblut und differenzierten diese anschließend *in vitro* zu Vorläufer-T-Zellen. Für die Transduktion wurden zwei Promotoren, die kurze Form des Elongationsfaktor 1 Promotors (engl. elongation factor 1 short form, EFS) und eine Variante des myeloproliferativen Sarkom-Virus Promotors (engl. myeloproliferative sarcoma virus, MPSV) verwendet. Als Hüllproteine wurden das Glykoprotein des Vesikulärstomatitis-Virus (engl. vesicular stomatitis virus glycoprotein, VSVG) und ein Glykoprotein eines modifizierten endogenen Katzenretrovirus (RD114/TR) verglichen. Getestete Transgene waren grün fluoreszierendes Protein oder ein chimärer Antigenrezeptor (engl. chimeric antigen receptor, CAR) der dritten Generation spezifisch für CD123. Außerdem zeigten wir die Funktionalität der induzierbaren Caspase 9 (iCasp9) als Sicherheitsmechanismus. Die Kombination des Sarkom-Virus abgeleiteten Promotors mit dem modifizierten Katzenretrovirus-Hüllprotein führte zu höherer Transgenexpression und Transduktionseffizienz. Dabei zeigten frische und aufgetaute CD34+ Zellen vergleichbare Transduktions- und Proliferationsraten. Zellen, die positiv für das Transgen waren, zeigten keine Einschränkung in der Expansion im Vergleich zu untransduzierten Zellen. Des Weiteren war nur der Sarkom-Virus abgeleitete Promotor in der Lage ausreichende Mengen von iCasp9 zu exprimieren, um nach Zugabe des Dimerisierungsreagenz in den Zellen Apoptose zu induzieren. Das dritte getestete Transgen, der CD123 CAR, konnte effizient in CD34+ Zellen und Vorläufer-T-Zellen exprimiert werden. Seine Funktionalität wurde in ausdifferenzierten T Zellen gezeigt. Zudem besiedelten CAR-exprimierende Vorläufer-T-Zellen den murinen Thymus *in vivo*. Zusammenfassend konnten wir zeigen, dass die Transduktion von CD34+ Zellen mit alpharetroviralen Vektoren eine realisierbare und potenziell sicherere Methode für stammzellbasierte Immunotherapien bei Krebs sein kann.

VII Appendix

VII.1 List of Abbreviations

ACT	adoptive cell immunotherapy
AML	acute myeloid leukemia
Ann V	Annexin V
CAR	chimeric antigen receptor
CB	cord blood
CML	chronic myeloid leukemia
CRISPR	clustered regularly interspaced palindromic repeat
CTLA-4	cytotoxic T lymphocyte antigen 4
DC	dendritic cell
DLI	donor lymphocyte infusion
DR(E)	direct repeat element
EFS promoter	intron-less short form of the human elongation factor-1α promoter
EGFRt	truncated human EGF receptor
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FDA	United States Food and Drug Administration
Gag	retroviral structural proteins
GOI	gene of interest
GVHD	graft versus host disease
GVL	graft versus leukemia effect
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
iCAR	inhibitory chimeric antigen receptor
iCasp9	inducible caspase 9
IFN-γ	interferon-γ
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITR	inverted terminal repeat
IR	internal repeat
IRES	internal ribosomal entry site
LAG-3	lymphocyte activation gene-3
LTR	long terminal repeat
mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MAGE-A3	melanoma-associated antigen-A3
MART-1	melanoma antigen recognized by T cells 1
MFI	mean fluorescence intensity
MOI	multiplicity of infection
MPSV	myeloproliferative sarcoma-virus variant
n.s.	not significant
PBMCs	peripheral blood mononuclear cells
PBS	primer binding site
PD-1	programmed death-1
PI	propidium iodide
Pol	retroviral enzymatic proteins
PolyA	Poly(A) tail
PPT	polypurine tract

preT	precursor T cell
qRT PCR	quantitative reverse transcription polymerase chain reaction
R	repeat region
RBV	Retronectin-bound virus method
RD114/TR	modified envelope glycoprotein derived from endogenous cat retrovirus
RPMI	Roswell Park Memorial Institute
scFv	single chain variable fragment
SCID	severe combined immunodeficiency
SIN	self-inactivating
SNT	supernatant infection method
SPR	short palindromic repeats
SSC	sideward scatter
TCR	T cell receptor
T_{CM}	central memory T cell
T_{EFF}	effector T cell
T_{EM}	effector memory T cell
T_{N}	naïve T cell
T_{SCM}	stem cell memory T cell
TIL	tumor-infiltrating lymphocyte
Tim-3	T cell immunoglobulin and mucin domain-containing protein 3
T7	T7 RNA polymerase
TCR	T cell receptor
UCB	umbilical cord blood
UTR	untranslated region
U5	unique 5 region
VCN	vector copy number
VEGF	vascular endothelial growth factor
VSVG	envelope glycoprotein derived from vesicular stomatitis virus
PRE	woodchuck posttranscriptional regulatory element
ΔU3	self-inactivating 3'U3 region
Ψ	packaging signal

VII.2 References

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VII.4 Curriculum Vitae

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VII.5 Erklärung zur selbstständigen Verfassung der Dissertation

Ich erkläre, dass ich die der Medizinischen Hochschule Hannover zur Promotion eingereichte Dissertation mit dem Titel „Generation of genetically engineered precursor T cells from human umbilical cord blood using an optimized alpharetroviral vector platform" in der

Klinik für Pädiatrische Hämatologie und Onkologie
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unter Betreuung von:

Wissenschaftliche Betreuung der Dissertation:	Prof. Dr. Martin Sauer
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mit der Unterstützung durch: Dr. Dr. Shahabuddin S. Hoseini, Dr. Dr. Julia D. Suerth, Dr. Dirk Hoffmann, Marcel Maluski, Jessica Herbst, Dr. Holger Maul, Dr. Dr. Arnab Ghosh, Dr. Qinggong Yuan, Prof. Dr. Michael Ott, Prof. Dr. Michael Heuser

ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Die Gelegenheit zum vorliegenden Promotionsverfahren ist mir nicht kommerziell vermittelt worden. Insbesondere habe ich keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.
Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Weiterhin versichere ich, dass ich den beantragten Titel bisher noch nicht erworben habe.
Ergebnisse der Dissertation wurden in folgendem Publikationsorgan veröffentlicht: Molecular Therapy.

Hannover, den _____

_____
(Unterschrift)