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Optimization of suicide gene therapy in stem cell transplantation with special reference to the selection marker OuaSelect

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Till mamma och pappa för mina rötter och mina vingar

ABSTRACT

For many patients suffering from leukemia and other hematological malignancies, allogeneic hematopoietic stem cell transplantation (HSCT) represents the only curative approach. However, an optimal HSCT procedure is yet to be developed. Transplant-related morbidity and mortality are severe complications arising from this treatment modality. Donor lymphocytes have been recognized to contribute to tumor cell eradication, a graft-versus-leukemia (GvL) effect. However, they can also cause graft-versus-host-disease (GvHD), a major contributor to transplant-related morbidity and mortality. A challenge of allogeneic HSCT is to prevent GvHD without loosing the GvL effect. Suicide gene modification of the donor lymphocytes has been proposed as a means of exploiting their beneficial effects, and allowing their safe elimination in the event of causing significant GvHD. In the ex vivo preparation of cells for suicide gene therapy, a selection step to purify genetically modified cells is necessary. The selection marker used in early gene therapy trials was the neomycin resistance gene (NeoR). Our first study aimed at marking the stem cell graft in autologous HSCT for multiple myeloma (paper I). The retroviral vector used encoded NeoR. The extension of ex vivo cell processing to encompass the seven days required for NeoR selection is not applicable for stem cell gene transfer protocols. Hence, selection was not performed. The results of this study showed that gene marking of stem cells was safe and marked cells were detected until end of study, 5 years after transplantation. In addition, the data suggested that it is the inability to achieve complete eradication of residual tumor cells after chemotherapy, rather than residual tumor cells contaminating the graft that constitute the source of malignant cells causing relapse after autologous HSCT. Selection of gene marked cells prior to transplantation could have strengthened the results, since it would have greatly increased the ratio of marked cells transplanted.

In paper II the development of a new selection marker OuaSelect is described. OuaSelect is the human Na+, K+ ATPase alpha 1 subunit with two amino acid substitutions - Q118R and N129D. It confers two orders of magnitude increased resistance to the cardiac glycoside ouabain. In paper II and III we demonstrate that >98% pure populations of genetically modified cells can be obtained within only 48 hours of selection. OuaSelect uniquely combines affordable and highly efficient selection with high yield and short selection time. Moreover, the high similarity to the native human protein minimizes the risks for immunogenicity.

Herpes simplex thymidine kinase (HSVtk)/ganciclovir (GCV) is the most frequently used suicide gene system. HSVtk expression of donor lymphocytes and GCV administration can abrogate GvHD in the setting of allogeneic HSCT. In paper III we show that protein fusion allows for highly correlated co-expression and function of OuaSelect and HSVtk in retroviral vector transduced lymphocytes.

Limitations to the HSVtk/GCV system include toxicity at administered doses of GCV and a rather slow response rate to prodrug administration. In paper IV, we could demonstrate improvements of the HSVtk/GCV system with a codon optimized A168H HSVtk mutant (TK.007). We observed a higher rate of GCV induced cell death for TK.007 in hematopoietic cells. In addition, a significantly higher GCV sensitivity and bystander effect was observed in cancer cell lines, allowing equal efficiency at lower GCV doses. In summary, the TK.007 suicide gene potentially represents a valuable improvement to the clinical use of HSVtk.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

I. Evren Alici, Bo Björkstrand, **Alexandra Treschow,** Alar Aints, C I Edvard Smith, Gösta Gahrton, M Sirac Dilber. Long-term follow-up of gene-marked CD34+ cells after autologous stem cell transplantation for multiple myeloma. *Cancer Gene Therapy 2007 (14) 227-232*

II. **Alexandra Treschow**, Christian Unger, Alar Aints, Ulrika Felldin, Johan Aschan, M Sirac Dilber. OuaSelect, a novel ouabain-resistant human marker gene that allows efficient cell selection within 48 h.
Gene Therapy. 2007 (14) 1564-1572

III. **Alexandra Treschow**, Ulrika Felldin, Axel Schambach, Evren Alici, Boris Fehse, Gösta Gahrton, Christopher Baum, M Sirac Dilber. Optimal expression and function of OuaSelect and HSVtk by fusion transgene cassette design. Manuscript

IV. Ellen Preuss, **Alexandra Treschow**, Jurgen Otte, Ulrika Felldin, Daniela Brucher, Evren Alici, Gösta Gahrton, Boris Fehse, M Sirac Dilber. A novel codon-optimized HSVtk(A168H) mutant [TK.007] for suicide gene therapy Submitted to *Human Gene Therapy*

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1 LIST OF ABBREVIATIONS

ADA	adenosine deaminase
AICD	activation induced cell death
APC	antigen presenting cells
AZT	azido-3′-deoxythymidine
CD	cytosine deaminase
CIDs	chemical inducer of dimerization
CMV	cytomegalovirus
co	codon-optimised
CTLA-4	cytotoxic T-lymphocyte antigen 4
DLI	donor lymphocyte infusion
dLNGFR	truncated low affinity nerve growth factor receptor
EBNA-1	Epstein-Barr virus nuclear antigen-1
EBV	Epstein-Barr virus
EGFP	enhanced green fluoresecent protein
FADD	Fas-associated death domain protein
FC	fluorocytosine
GAr	glycine alanine repeat
GCV	ganciclovir
GITR	glucocorticoid-induced tumor necrosis factor receptor
GMP	good manufacturing protocol
GPI	glycosylphosphatidylinositol
GvHD	graft-versus-host disease
GvL	graft-versus-leukemia
HLA	human leukocyte antigen
HPT	hygromycin phosphotransferase
HSCT	hematopoietic stem cell transplantation
HSVtk	Herpes simplex virus-1 thymidine kinase
IFN-α	interferon-alpha
IRES	internal ribosome entry site
KIR	killer cell immunoglobuline-like receptor
LAK	lymphokine activated lymphocyte
LPD	lymphoproliferative disease
mAb	monoclonal antibody
MACS	magnetic activated cell sorting
MLR	mixed lymphocyte reaction
MM	multiple myeloma
m.o.i.	multiplicity of infection
MoMuLV	Moloney murine leukemia virus
MSC	mesenchymal stem cell
NeoR	neomycin phosphotransferase II
NK	natural killer
OuaSelect	human Na+, K+ ATPase alpha 1 Q118R/N129D
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PHA	phytohaemagglutinin

RCR	replication competent retrovirus
RIC	reduced-intensity conditioning
RT	reverse transcriptase
SCID	severe combined immuno-deficiency
TBI	total body irradiation
TCR	T cell receptor
TNF	tumor necrosis factor
TP	triphosphate
Treg	regulatory T cell

2 GENERAL INTRODUCTION

2.1 ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION AND DONOR LYMPHOCYTES

2.1.1 The development of hematopoietic stem cell transplantation

As an introduction, this section will recapitulate the experimental background, early clinical successes and problems encountered in the development of HSCT as a treatment modality. Several comprehensive reviews, among them the Nobel lecture by E.D. Thomas in 1990 describe the process and major milestones of HSCT development.[1-3]

The use of HSCT in medicine arrived in the wake of the first atomic bomb explosion and the efforts put forward for protection of atomic warfare. In 1949, mice could be protected from lethal photon beam irradiation by shielding their spleens with led.[4] In 1951, Lorentz and colleagues demonstrated that intravenous infusion of marrow cells into mice and guinea pigs also protected against lethal irradiation.[5] The first proofs that the protection came from the transplanted cells and not from a substance produced by the cells beneficial for the recovery of the transplanted animals came in 1955.[6] Main and colleagues showed in mice that recipients of allogeneic bone marrow later accepted skin grafts from the donor strain. It meant that donor immune cells had been established in the recipient, and were to account for tolerance of the transplanted skin. Rapidly, several investigators followed with reports documenting the recovery after irradiation being attributed to donor-derived cells.[7-9]

These findings had an enormous importance, not only for the biological implications: the fact that all hematopoietic tissues could be regenerated from transplanted allogeneic stem cells after lethal irradiation, but also because they raised the possibility to offer allogeneic HSCT as a treatment to patients with various severe hematological diseases. HSCT could potentially be used for treatment of patients with acquired bone marrow failure from irradiation, inherited genetic disorders (sickle cell disease and immunodeficiencies), acquired marrow failure (severe aplastic anemia) and hematological malignancies (leukemia).[2] In the case of leukemia, the efficacy of drug treatment improved, since the dose of cytotoxic drugs used for chemotherapy could be increased to levels beyond sublethal doses due to bone marrow toxicity.

The first attempt in patients with terminal cancers showed that engraftment could occur – hematopoietic mature cells from the graft appeared in peripheral blood.[10] However, all patients died from their diseases. Thus, this and other attempts of translating the discovery into clinical practice failed to prove persistent engraftment or cure of disease and complication were severe, as reviewed in [11]. The patients that did engraft developed fatal “wasting syndrome” (at that time also referred to as “secondary disease”) with diarrhea, weight loss and skin changes[12], now known as Graft-versus-Host Disease (GvHD)[13]. As a result, there was a general distrust in the possibility to use allogeneic HSCT in the clinic.

The major breakthroughs came with subsequent work on dogs. In dog, the importance of in vitro histocompatibility typing for transplantation outcome was recognized.[14] In 1954, Miescher had recognized that antibodies induced by transfusions or pregnancy reacted with antigens on white blood cells.[15] Later,

Dausset and van Rood and colleagues used such antibodies to describe human leukocyte antigen (HLA) groups.[16, 17] This discovery led to the development of techniques for defining tissue antigens that was critical to the development of HSCT. Conditioning regimens providing sufficient host immunosuppression for graft acceptance in addition to tumor cell killing, as well as effective drug regimens to control GvHD were also developed in dog.[18, 19]

The first matched transplants, translating the discoveries from dog, were made in the late 1960's. There was a combination of factors that together lead to the success in the treatment of leukemia with allogeneic bone marrow transplantation.[20-22] One factor was the use of HLA matched sibling donors. The others were the combination of total body irradiation (TBI) and cyclophosphamide for conditioning treatment to facilitate engraftment, in addition to post-transplant immunosuppressive treatment with the anti-metabolite methotrexate to suppress GvHD development. From that time, the aim was to apply maximally tolerated doses of chemo- or radiotherapy conditioning to patients in order to eliminate the leukemia cells. The rationale for this approach was confirmed by the fact that an increased dose of the conditioning regimen reduced the probability of relapse after transplantation.[23-25] The allogeneic bone marrow graft was considered mainly as a non-tumor contaminated source of hematopoietic stem cells that restored hematopoiesis.

2.1.2 Graft-versus-Host Disease

Despite HLA matching and the use of post-transplant methotrexate treatment, approximately half of the patients developed GvHD [11] consistent with observations in studies on dogs.[19] A major improvement in patient survival and GvHD prevention came with the use of methotrexate combined with the T cell activation inhibitor cyclosporine A [26]. These drugs have remained the most widely used method for GvHD prevention. However, GvHD is still a major cause of transplant-related morbidity and mortality. The standard grading system includes alterations in the skin, gut and liver. Mild GvHD (Grade I) is associated with little morbidity, while the median incidence of moderate to severe acute GvHD (grade II to IV) is about 40%, ranging according to risk factors from 10 to 80%.[27] Once it occurs its difficult to treat. Steroids are generally the first-line treatment of established GvHD, but the response to treatment is on average 30-50%.[28, 29] Patients not responding to steroid treatment have a poor prognosis and GvHD grade IV has a mortality rate greater than 90%. Clearly, new and effective regimens to contain GvHD are greatly needed. With such methods in hand, even HLA-haploidentical grafts[30] could be applicable and allow allogeneic HSCT to evolve into a widely accessible and effective treatment modality for hematological malignancies. In addition, it could be considered more feasible also for patients with non-malignant hematopoietic diseases.

A novel approach to treat severe acute GvHD was recently developed by Le Blanc, Ringdén and coworkers at the Karolinska University Hospital Huddinge. Allogeneic mesenchymal stem cells (MSC) were infused into patients with severe steroid-refractory GvHD.[31] The MSCs were obtained from adult bone marrow of HLA-identical siblings, haploidentical family donors, or mismatched unrelated donors and cultured for up to four passages ex vivo prior to infusion. The doses of infused cells were 0.6 – 9 x 106/kg, and no side effects or ectopic tissue formation were observed. Clinical GvHD disappeared completely in six out of eight patients. In a subsequent

multicenter phase II study of 55 patients, 30 patients (55%) showed complete responses and no signs of toxicity with the administration of up to five doses of 0.4 – 9 x 106/kg MCSs was reported.[32] Moreover, the response rate was not related to donor HLA match. Thus, infusion of ex vivo cultured allogeneic MSCs could be an effective approach to treat severe GvHD.

In the post-transplant period, patients are profoundly immuno-supressed as a result of the conditioning chemotherapy, GvHD and its treatment. Thus, they are at great risk of opportunistic infections. Cytomegalovirus (CMV) disease is difficult to cure and constitute a major cause of transplant-related mortality.[33] Bacterial and fungal infections can be controlled with antibiotics.

2.1.3 A paradigm shift in allogeneic hematopoietic stem cell transplantation

Tumor eradication in patients transplanted with allogeneic hematopoietic stem cells can only partly be achieved with high-dose conditioning regimens. Already in 1960, demonstrated in a mouse model of leukemia[34], it was evident that extremely large doses of TBI are needed to kill every tumor cell in vivo. These doses far exceeded what is applicable for human patients. Also in the 1960's, it was shown that leukemic mice receiving allogeneic grafts in comparison to syngeneic grafts had better survival and greater tumor reduction rates.[35]

Mathé and coworkers, pioneers in the field, recognized the importance of immunologic anti-tumor effects exerted by donor cells. They hypothesized that residual leukemic cells after conditioning could be totally eradicated by this immune reaction and that a permanent control of the disease could be achieved.[36] Reports followed, that human recipients of allogeneic HSCT who developed GvHD were less likely to suffer from leukemic relapse,[37, 38] confirming the hypothesis and earlier mouse studies.

The importance of donor T cells in the GvL reactions was shown in patients receiving T cell depleted allogeneic bone marrow cells. They had significantly higher rates of relapse than patients receiving grafts including T cells.[39] Moreover, the probability of relapse was associated with the degree of HLA disparity between donor and recipient, and with the number of T cells in the graft transfused. [39] In the 1990's, the power of the GvL effect was directly demonstrated with infusions of donor lymphocytes in patients with leukemia relapse after allogeneic HSCT. A new remission could be achieved without additional chemotherapy, radiotherapy or HSCT.[40-43] As a consequence, this resulted in a broader use of donor lymphocyte infusions (DLI) as a treatment option for leukemia, lymphoma and myeloma. Moreover, recognizing the importance of the GvL effect in allogeneic HSCT led to the development of reduced-intensity conditioning regimens (RIC).[44-46]

The recognition of the GvL effect has shifted the aim of HSCT towards an immunotherapeutic approach. But without a reduction of the tumor burden achieved by the chemotherapy prior to the transplantation, it would be like trying to dry the ocean with a hair dryer, to use the words of George Klein, Professor Emeritus at Karolinska Institutet.

The non-myeloablative conditioning of RIC suppresses the recipient immunity sufficiently to allow donor hematopoietic stem and effector cells to engraft. The aim of

the subsequent allogeneic HSCT is to generate a platform of induced stable donor chimerism in the patient and exploit the GvL response. Observations of anti-tumor responses in patients after RIC HSCT have demonstrated donor lymphocyte induced GvL effects.[47-49] However, in RIC HSCT the tumor eradication relies almost entirely on the GvL effect and a failure to achieve complete remission is frequently observed.[50, 51] Still, the reduced transplant related mortality has made HSCT a treatment option for a broader patient group, including older patients and those with compromised organ function. Adoptive immunotherapy with donor lymphocytes can be used in cases of relapse or preemptively in patients with high relapse risk.

2.1.4 Graft-versus-leukemia reactions

Although the term graft-versus-leukemia reaction implies a specific killing of leukemic cells exerted by donor cells, there is currently no conclusive evidence that GvL reactions are specific.[52] In contrast, the GvL effect is more likely part of the outcome of a generalized immune response against the host. For instance, donor T cells with specificity for minor histocompatibility antigens that are selectively expressed on hematopoietic cells including leukemic clones could generate an effective GvL response.

Donor T cells reacting against HLA-peptide complexes on recipient cells drive GvHD. GvHD can also occur from the recognition of minor histocompatibility antigens on matched recipient HLA molecules. It is also indirectly mediated by cytokines (in the early days it was attributed to a “cytokine storm”). It probably contributes to a pro-inflammatory microenvironment that decreases the threshold of T cell receptor (TCR) activation and promotes the expansion of self-antigen specific T cells.[52] Thus, GvHD is not required for a GvL reaction, but a strong GvL reaction can be induced and sustained with GvHD. The possibility to exploit the GvL effect for tumor eradication in the absence of GvHD remains a challenge in allogeneic HSCT optimization.

Distinct subsets of donor lymphocytes contribute to GvL effects, including T cells and natural killer (NK) cells. T cells mainly exert GvL effects in an antigen-specific, HLA- restricted way, while NK cells kill as a consequence of cell to cell contact and activation delivered by activation of killer cell immunoglobulin-like receptors (KIRs), C-type lectins, and natural cytotoxicity receptors. Currently, T cells are the cells of choice considered for allogeneic immunotherapy, but an appreciation for the relative contribution of NK cells to the GvL effects and potential implications thereof have begun to emerge[53, 54] Adoptive transfer of autologous lymphokine activated lymphocytes (LAK cells) to patients with advanced solid tumors were tested in the 1980’s, but the results were disappointing.[55, 56] New techniques have been developed for ex vivo expansion and activation of NK cells, and significant cytoxicity against autologous myeloma cells can be achieved.[57] Thus, NK based DLI represent new immunotherapeutic possibilities.

The generation of tumor specific T cells by genetic engineering is an interesting immunotherapeutic cancer therapy strategy.[58] However, it will not be discussed here.

2.2 T CELL SUICIDE GENE THERAPY

2.2.1 Rationale for "suicide gene" mediated control of GvHD

Limitations with standard DLIs have prompted researchers to develop methods by which the beneficial effect on immune reconstitution, engraftment, GvL and antiviral activity can be exploited while GvHD can be controlled. Gene therapy has opened up new possibilities of improving allogeneic HSCT. One approach is to incorporate a conditional suicide gene into the genome of donor lymphocytes prior to their administration to the patient. The suicide gene can be activated in the case of severe GvHD by administration of a prodrug. Prodrug conversion by the suicide gene product leads to the specific and selective elimination of alloreactive donor lymphocytes and abrogation of GvHD. Suicide gene modified T cells can be used as DLIs, infused into patients to treat residual disease, relapse, or to ward off opportunistic infections. They can also be administered as an add-back with T cell depleted HSCT.

2.2.2 T cell suicide gene therapy

Suicide gene therapy was one of the first T cell gene therapy applications explored. Clinical evaluations of the method started in the 1990's. Bonini and colleagues reported on the first pilot study including 8 patients in 1997.[59] Other early studies of suicide gene modified donor lymphocytes to treat relapse or Epstein-Barr virus (EBV) related lymphoproliferative disease (LPD) after allogeneic HSCT were soon following.[60, 61] Prophylactic DLI doses for acute leukemic relapse following reduced intensity conditioning ranges between 1 - 10 x 10^6 $CD3^+$ cells/kg.[62] The threshold for allogeneic donor lymphocyte causing GvHD is often above 10^6 cells/kg.[63] In the Bonini study, the cells were administered in escalating doses ranging from 1 x 10^5 to 4 x 10^7/kg. Two patients developed acute GvHD. GCV treatment resulted in the disappearance of gene modified lymphocytes and clinical symptoms of GvHD. One patient with chronic GvHD responded only with a reduction of gene-modified cells to prodrug administration, leading to residual GvHD.[59] In the Link study, higher doses were used (0.1 – 2.5 x 10^8/kg for HLA-identical sibling donor and 0.01 - 1 x 10^8 for recipients of grafts from unrelated donors). Moreover, the prodrug was only administered in the case of steroid-refractory GvHD. Out of 6 patients treated, one developed chronic GvHD that did not respond to conventional treatment, but resolved after 5 days of GCV.[64] In the Champlin study, 23 patients were receiving doses of 5 - 8.6 x 10^7/kg. Only one patient developed GvHD that resolved without treatment. Thus, no patients were administered the prodrug.[61] This poor outcome of the study led to the decision to stop it and put similar studies on hold in the US. Tiberghien and colleagues reported on the use of suicide gene-modified donor lymphocytes with T cell depleted HSCT to enhance engraftment and GvL at high risk for GvHD.[65] Out of 12 patients three developed acute GvHD ≥ grade II. Two patients responded to GCV alone whereas the third required additional steroids. One patient developed chronic GvHD and did not respond until after three weeks of prodrug administration. [66]

The clinical relevance of manipulated donor lymphocytes is dependent on the safety and functionality of the infused cells. These early clinical trials provided valuable information with respect to feasibility of the approach; no acute toxicity was observed after gene modified cell administration. Moreover, the cells were detectable

for months and were sensitive to prodrug administration. Suicide gene modification of donor T cells was also shown to be clinically relevant since a GvHD response was associated with prodrug administration. However, a worrisome observation was made that the incidence of GvHD was much lower than expected. Moreover, 3 out of 12 patients in the Tiberghien trial developed EBV LPD.[66] In a similar study with unmanipulated DLIs, the rate of EBV LPD was 9,5 %.[67] As a result, concerns were raised regarding the ability of the manipulated lymphocytes to maintain their immunological reactivity after *ex vivo* processing.

The concept of suicide gene therapy initially appeared to be a relatively simple application of retroviral gene transfer technology, since lymphocytes are readily accessible, well defined, and can be expanded *in vitro*. However, the results from the first clinical attempts revealed that a better understanding of lymphocyte prerequisites were necessary for clinical application of this strategy. The potential impact of the *ex vivo* processing and vector/transgene effects on T cells required to be addressed systematically.

The *ex vivo* cell processing consists of several procedures; T cell culture conditions, T cell activation, transduction, and selection. Equally important factors that come into play post-transfusion are safety, feasibility (suicide gene activation) and immunogenicity. Here follows a brief description how these processed and factors have been addressed.

2.2.3 Cell culture protocols and T cell activation

Ex vivo processing has been shown to play a pivotal role in disturbing T cell functionality. [68-71] Alterations that have been observed include loss in antiviral potential, [72] loss of the naïve T cell subset,[70, 73] and a skew in T cell receptor beta-chain repertoire, indicating preferential expansion of a limited number of T cell clones[74]. These effects resulted to a greater extent from the culture itself, rather than from transduction and selection. As a result, new cell culture and T cell activation protocols have been developed and optimized. Surrogate parameters to determine *in vitro* immune competence, such as the T cell receptor repertoire[74, 75] cytokine production, immunological phenotype[70] and frequency of antigen-specific T cells,[69] have been used in protocol development. The functional characteristics of T cells can also be tested *in vivo.* The cellular trafficking and homing pattern of cells can be monitored[76] as well as the capacity of the cells to induce GvHD on mouse models of allogeneic HSCT.[77, 78]

Retroviral vector transduction requires T cell activation since retroviruses only can access the chromosomal DNA for integration during mitosis when the nuclear membrane breaks down.[79] In the first clinical study, donor peripheral blood mononuclear cells (PBMCs) were activated with the mitogen phytohaemagglutinin (PHA).[59] Since PHA is not applicable in good manufacturing protocol (GMP) procedures of clinical products, it was replaced with the anti-CD3 monoclonal antibody (mAb) OKT-3 (Jansen-Cilag) and recombinant IL-2. This activation protocol has been used in several subsequent studies[60, 80, 81]

T cell CD3 stimulation in vitro can inhibit proliferation by activation induced cell death (AICD).[82] Low levels of CD3/TCR receptor engagement can activate memory T cells and result in a proliferative response but is insufficient for effective activation of naïve T cells.[83] In contrast, high levels of CD3/TCR receptor engagement provide a

robust activation of naïve T cells while causing activation AICD of memory cells.[84, 85] Thus, the level of signaling can lead to very different outcomes, depending on the maturation state of T cells. CD3 stimulation in combination with CD28 co-stimulation reduces AICD. Thus, to avoid massive AICD by activation of T cells for retroviral transduction, the protocols were extended to include an anti-CD28 mAb. The addition of an anti-CD28 mAb to the activation protocol has also been found to preserve the T cell receptor repertoire distribution.[74]

The presence of CMV- and EBV-specific T cells is of great importance to protect patients from post-transplant infectious complications. The assessment of anti-EBV responses became particularly relevant from the high incidence of EBV LPD reported in one of the first clinical trials.[86] The addition of anti-CD28 mAb to anti-CD3 mAb and IL-2 activation supports the transduction and survival of CMV- and EBV-specific T cell clones in the bulk culture.[87, 88]

In *in vivo* responses to viral challenges, the magnitude of CD8+ T cell response is usually stronger and more rapid than that of CD4+ cells.[89] Similarly, *in vitro* activation of T cells with anti-CD3 and IL-2 leads to preferential CD8+ T cell expansion.[90] The CD8+ fraction cycles more rapidly, thus is more susceptible to viral entry. As a result, CD8+ cells are more readily transduced and expanded than CD4+ T cells. This gives an outcome of a reversed or at least reduced CD4/CD8 ratio in the final cell population after transduction and selection. CD28 co-stimulation enhances CD4+ T cell proliferation[91] but activation with soluble CD3 and CD28 mAbs still does not preserve the roughly 60:40 CD4:CD8 ratio at culture initiation.[73] It is not known how the potency of DLI following HSCT could be affected by alterations in the relative numbers of CD4+ and CD8+ T cells, but *in vitro* alloproliferation of CD8+ T cells is dependent on the presence of CD4+ T cells,[92] and efforts have been made to maintain the ratio of CD4/CD8 in fresh PBMCs. Lately, coating cell sized beads with anti-CD28 and anti-CD3 mAbs have become the preferred choice with the argument that bead-mediated T cell activation could create a more physiological situation where the bead is mimicking an antigen presenting cell (APC). Reducing the dose of IL-2 limits the expansion advantage of CD8+ cells over CD4+ cells. In a recent study with novel beads coated with anti-CD3, anti-CD28 and anti-CD2 (co-stimulatory molecule) mAbs (Miltenyi) and low dose (20 U/ml) Il-2, a maintained CD4/CD8 ratio between transduced and non-transduced cells was achieved.[93]

Lentiviral suicide gene vectors have also been tested. IL-2, IL-15 or IL-7 cytokine stimulation allows lentiviral vector transduction and has been shown to preserve the T cell phenotype. In addition, cytokine stimulation protocols also preserve antiviral activity and alloreactivity better than strongly mitogenic anti-CD3, anti-CD28 + IL-2 activation protocols.[94, 95] It remains to be seen if these promising approaches better maintain the desired functional characteristics of *ex vivo*-manipulated cells.

A subpopulation of CD4+ T cells that express constant high levels of CD25, the high affinity IL-2 receptor alpha-chain, in addition to the forkhead/winged helix transcription factor Foxp3 are described as T regulatory cells (Treg)[96, 97] and play an instrumental role in the maintenance of immunological self-tolerance. They are essential for the active suppression of autoimmunity[97, 98] and are critical in transplantation tolerance.[99] Co-administration of donor CD4+CD25+ Tregs with marrow grafts can prevent GvHD in mice.[100, 101] In humans, Foxp3 mRNA expression negatively correlates with severity of GvHD following HSCT.[97] However, increased levels of both CD4+CD25+ and CD8+CD25+ donor cells after

HSCT has been shown to represent a significant risk factor for acute GvHD in humans. [102] Thus, CD25 and Foxp3 expression in the HSCT/DLI settings seems to be complex. In fact, phenotypic and functional assessment of human Tregs has been hampered by the difficulty to distinguish between CD25+ and/or Foxp3+ activated and regulatory T cells. *In vitro* activation of T cells induces the expression of CD25 on both CD4+ and CD8+ T cells and these cells expand strongly.[93, 103] In addition, activated T cells are able to temporarily up-regulate Foxp3, or express other Treg markers in addition to Foxp3 (cytotoxic T-lymphocyte antigen 4 (CTLA4) and glucocorticoid-induced tumor necrosis factor receptor (GITR)) without exhibiting any suppressive activity.[104-106] In addition, continuous *in vitro* activation of CD4+CD25- cells results in transient Foxp3 expression and functional suppression that is reverted again after 7 days.[107] Thus, it seems that a suppressive functionality is not an exclusive feature, but all activated T cells can attain a transient regulatory state [108] Clearly, the characterization of regulatory T cells, and the impact of *in vitro* activation on suppressive features, in addition to possible implementations of Tregs in the setting of HSCT warrant further studies.

It is highly desirable to maintain the highest possible alloreactivity of suicide gene-modified donor lymphocytes in order to provide a powerful GvL response. Albeit CD3/CD28 co-stimulation plus IL-2 improve EBV reactivity and T cell clonality, activated and expanded lymphocytes still have a decreased alloreactivity to fresh cells, assessed by *in vitro* mixed lymphocyte reaction (MLR).[109] The mechanism leading to impaired alloreactivity is unclear, but the duration of culturing and expansion are important factors. [110, 111] Recently it was shown that the impaired *in vitro* alloreactivity was not due to Treg suppressive activities or preferential expansion, but rather resulted from exhaustion by *ex vivo* expansion.[106, 112] Thus, it seems that limiting the expansion and *ex vivo* culture duration time would have to be considered in the design of cell processing protocols in the future.

2.2.4 Suicide genes

Suicide gene therapy is based on the conversion of a non-toxic prodrug into a toxic compound by a gene-encoded system. Ideally, the system should be rapid and effective even at low substrate doses, since biodistribution of the prodrug can vary. Moreover, the toxicity should be specific. Only the gene-encoded system should be capable of prodrug conversion. In 1986, Moolten and colleagues first showed the feasibility of this approach as an anti-cancer strategy transferring the Herpes simplex virus-1 thymidine kinase (HSVtk) gene to tumor cells and demonstrating selective chemo-sensitivity to ganciclovir (GCV).[113] In contrast to mammalian thymidine kinases, the HSVtk is capable of phosphorylating certain nucleoside analogs, such as GCV to nucleoside monophosphates. The nucleoside monophosphate is subsequently phosphorylated by cellular kinases to nucleoside triphosphate (GCV-TP) which is incorporated into the nascent DNA strand during DNA synthesis. These nucleotides give rise to base-pair mismatches, DNA fragmentation, sister chromatid exchange and lethal genome instability.[114]. In addition, GCV-TP inhibits the cellular DNA polymerases, thereby blocking further DNA synthesis.[115]

As a cancer gene therapy approach, the HSVtk/GCV system has been widely studied for a variety of tumor types, including glioma, neuroblastoma, and prostrates cancer.[116, 117] However, gene delivery and thereby clinical efficacy remains one of

the main challenges that need to be solved for this approach to translate it into routine clinical practice. Currently, a phase III clinical trial is ongoing, targeting operated glioma with replication deficient adenoviral vector delivered HSVtk[1]. Of note, it is the first clinical phase III trial in gene therapy to meet primary endpoints and French authorities have allowed the treatment to be prescribed to certain patients even though the approach has not yet been approved for general use.

The HSVtk/GCV system is most effective in situations of rapidly dividing cells, high expression levels of HSVtk, and relatively high concentrations of GCV. Therefore, T cell suicide gene therapy represents an attractive application for this system. T cells are readily manipulated *ex vivo*, and retrovirus vectors based on the long terminal repeat (LTR) of the strongly lymphotrophic Moloney murine leukemia virus (MoMuLV) permits high levels of transgene expression.[118] Only proliferating cells expressing HSVtk are sensitive to GCV- induced cell death, since DNA replication occurs during cell division. In cancer gene therapy, this can be a major obstacle, but an *in vivo* selectivity of GCV toxicity could be of considerable clinical relevance in T cell suicide gene therapy. Only (allo)-activated proliferating HSVtk expressing donor lymphocytes would be eliminated with GCV administration, while the non-affected non-dividing HSVtk expressing cells could potentially contribute to subsequent immune competence.

The first clinical trial of T cells employing a retroviral vector encoding a suicide gene (HSVtk) was an autologous adoptive immunotherapy approach, transducing HIV-gag specific CD8+ T cells *ex vivo* and re-infusing the cells back into HIV infected patients. There was no acute toxicity observed, but the cells were rapidly eliminated in 5 out of 6 patients by immune-mediated clearance of transduced cells.[119].

The transfer of suicide gene modified donor lymphocytes in the setting of allogeneic HSCT has been investigated in a number of clinical phase I/II studies.[60, 65, 80, 81, 120-122] In fact, all clinical trials of T cell suicide gene therapy up until now have been based on the HSVtk/GCV system.[123]

Despite a promising proof-of-principle, the system has several shortcomings that have become apparent with its clinical use. First, the prodrug ganciclovir (Cymevene) is an anti-viral agent that is widely used for Herpes virus infections. The use of GCV for treatment of CMV infections in patients included in a suicide gene therapy trial, resulted in the undesired elimination of gene modified donor lymphocytes.[59] Fortunately, there are now indications that other anti-viral drugs have comparable efficacy and safety features, and thus may substitute GCV for CMV treatment in this particular patient cohort.[124] Second, GCV is relatively toxic in administered doses (maximum 10mg/kg/day), particularly to bone marrow and myelopoiesis.[125, 126] Consequently, prolonged administration may lead to profound immuno-suppression. Significant improvements could arise from engineered HSVtk mutants, designed to increase specificity and activity towards the prodrug, [127-129]. Alternatively, major benefits could be achieved with the introduction of new nucleoside analogues with higher affinity for HSVtk and fewer side-effects than GCV.[129-132] Third, the rate of cell death is rather slow. Rates of GCV induced cell elimination to abrogate GvHD

[1]http://investors.arktherapeutics.com/servlet/HsPublic?context=ir.access&ir_option=RNS_NEWS&item=65925600515789&ir_client_id=4553&transform=newsitem_new; 11/03/2008

reported *in vivo* require on average 5-6 days, but ranges from one day to three weeks.[59, 86] A significant improvement in the rate of GCV induced cell death *in vitro*, in addition to improved expression levels have been observed with the A168H HSVtk mutant.[129](Preuss *et al.,* submitted) A more rapid rate of cell killing would be advantageous, in particular for the elimination of modified donor lymphocytes in order to abrogate severe acute GvHD causing high patient morbidity and mortality.

Cytosine deaminase (CD)/5-fluorocytosine (5-FC) is an alternative cell suicide system, that also produces a toxic nucleotide analogue. Cytosine deaminase derived from either yeast or bacteria can be used. The applicability of CD as a suicide gene was first shown by Mullen in 1992[133] and has been investigated by several research groups since. It has been studied for feasibility in cancer gene therapy in addition to T cell suicide gene therapy [117, 134]. Despite encouraging preclinical results, the treatment with CD/5-FC alone may at best lead to partial response and combination of several genes or treatment modalities could increase the efficiency of this gene therapy strategy.[117, 135]

Due to the limitation of immunogenicity, described in more detail below, more recent alternative combinations are exclusively based on human genes. Among them is the engineered thymidylate monophosphate kinase (tmpk) combined with the prodrug 3′-azido-3′-deoxythymidine (AZT), a drug widely used in HIV therapy[136, 137]. The rate limiting step in the conversion of AZT to its toxic triphosphate AZT-TP, is catalyzed by tmpk. The engineered tmpk allows the prodrug conversion to accelerate in transduced cells, resulting in effective specific cell killing.[137]

A system has been developed utilizing the concept of receptor dimerization by the small natural FK506 molecule. It is based on Fas or Fas signaling mediators that have been designed to respond to non-toxic lipid-permeable ligands termed chemical inducers of dimerization (CIDs). Target proteins are fused to one or more CID-binding domains so that they become non-covalently cross-linked after interaction with CIDs.[138] Thomis and colleagues designed a chimeric protein containing the membrane-anchored intracellular domain of Fas fused to two modified copies of the FK506-binding protein FKBP12.[139] Cross-linking of the Fas receptor through binding of AP1903, a bivalent synthetic FK506 molecule, triggers the apoptotic death signal. Effective killing of gene modified lymphocytes was also demonstrated by the same concept and dimerization of the Death Effector Domain of the Fas-associated death domain-containing protein (FADD).[140]. However, the activation of apoptosis by plasma membrane proximal inducers such ass Fas and FADD can be blocked by cellular inhibitors of apoptosis acting on downstream signaling molecules. Up-regulation of apoptosis inhibitors occur in the long-term maintenance of memory T cells, and these cells may therefore inadvertently be spared from suicide induction.[141] Importantly, it is also a frequent early event in malignant transformation. As a response, caspase 9, acting downstream of cellular inhibitors, has also been engineered for CID sensitivity.[142] The suicide gene, termed $iCasp9_M$ allows for highly effective killing (>99%) both *in vitro* and *in vivo*. Interestingly, complete elimination is achieved already three days after a single dose of the CID AP20187. A clinical protocol for haploidentical HSCT and transduced donor T cells, based on the $iCasp9_M$ suicide gene, is now being developed.[143]

Monoclonal antibodies now have applications in virtually all areas of biology and medicine. This technology has also been exploited in the development of suicide genes and selection markers in gene therapy. CD20 is a membrane protein, restricted to the B

cell lineage and expressed on B cells from pre-B cell to memory B cell stages. Due to its restricted expression, it has been used as a target for monoclonal antibody treatment of B-cell malignancies. Rituximab, a human/mouse chimeric monoclonal anti-CD20 antibody has been in use since the 1990´s for B-cell lymphomas. Introna and colleagues demonstrated the possible application of ectopic expression of CD20 from a retrovirus vector and use of Rituximab for *in vivo* elimination of transduced T cells in addition to purification by immuno-selection.[144] One obvious drawback of this system is that Rituximab administration also inadvertently depletes normal B expressing CD20.

2.2.5 Selection marker genes

A selection step enriching the gene modified population to high purity prior to infusion is needed, since infused donor T cells lacking an integrated suicide gene vector will escape suicide gene activation. A consequence of infusing non-transduced donor T cells would be the risk of provoking an uncontrollable GvHD. Therefore, current suicide gene vectors have been designed to constitutively co-express a selection marker.

Initially, the bacterial neomycin phosphotransferase II gene (NeoR) was used.[59, 60, 65] The NeoR requires at least 7 days of selection.[145] The need to culture cells for an extended period of time in selection medium imposes a major limitation to bacterial derived antibiotic resistance genes for clinical use. As discussed earlier, the extent of *ex vivo* culture duration negatively correlates with maintained functional characteristics of T cells. Moreover, G418 selection per se is toxic to primary cells.[69]

Another finding from the early clinical trials was that not all patients could fully benefit from the genetically modified T cell approaches due to specific immunity directed against the engineered cells.[119, 146] Further studies on this problem, has revealed that the induction of immune reactions against gene modified cells is one of the most important factor that influences the efficacy of gene therapy.

The immunogenicity concerns led to the replacement of NeoR with wild-type and engineered human surface marker proteins and antibody-based cell sorting techniques. The truncated low affinity nerve growth factor receptor (dLNGFR)[147] has been adopted by several researchers and has been used extensively in preclinical and clinical studies. It is included in the HSVtk suicide gene vector now under clinical phase III evaluation.[123] There are no adverse events reported from its clinical use, and it appears to be a safe candidate for clinical applications.[148] However, concerns have arisen from *in vitro* and *in vivo* studies that dLNGFR could promote transformation.[149, 150] Yet, its growth promoting role is limited by a dependence of the co-expression of a tyrosine receptor kinase gene and the presence of neurotrophins.[151]

The truncation of the cytoplasmic region renders the protein comparable to existing anti-apoptotic decoy receptors of the tumor necrosis factor (TNF) family, inhibiting pro-apoptotic intracellular pathways.[152] The restricted use of dLNGFR in cell types that lack co-operation signals should reduce its risk profile. For instance, the transformation mechanisms may not apply to T cells. Still, additional studies are required to address these points in depth. Interestingly, a proposed safety adjustment of the current protein is to replace the dLNGFR by an engineered dLNGFR that is made unable to bind its ligands, yet leaves the antibody epitope used in cell sorting procedure unaltered.[153]

Truncated CD34 (tCD34) is a surface marker that has been developed in order to take advantage of existing, commercially available and clinically approved immuno-selection materials and methods.[154] CD34 is physiologically expressed on hematopoietic stem and progenitor cells and systems to enrich for CD34+ cells *ex vivo* have been developed for the enrichment of stem and progenitor cells, e.g. for HSCT purposes. The protein signals through phosphorylation at an intracellular protein kinase C recognition site.[155] An alternative splice variant to the full-length protein exists naturally that lack the intracellular region including the recognition site for protein kinase C, and functional signaling.[156] A great advantage of this marker and the methods used, is that high purities of transduced cells (97-99%) can be reached.[157] However, a recent study demonstrated that ectopic expression of murine CD34 and tCD34 in mouse hematopoietic cells *in vivo* affects trafficking of cells.[76] These results give some apprehensions about using this marker in vectors targeting lymphocytes.

Thy-1 (CD90) is expressed in all thymocytes and T cells in mice, but in humans it is only expressed on a small fraction of fetal thymocytes, between 10-40% of CD34+ and <1% of CD3+CD4+ lymphocytes in peripheral circulation.[158] It has been proposed as a selection marker and has been introduced into T cells, dendritic cells and CD34+ cells without any apparent toxicity.[159, 160] However, both ligands and functions of Thy-1 in the human immune system remain unidentified, and further studies are needed to address potential phenotoxicity. Of note, Thy-1 is an extracellular protein anchored to the cell membrane by Glycosylphosphatidylinositol (GPI). GPI anchored proteins, such as Thy-1, can be shed from the membrane by phospholipase-C.[161] Thus, albeit probably applying only to a small fraction, both false positive and false negative selection is possible with the use of this marker. Therefore, Thy-1 does not appear to be the perfect choice for hematopoietic cells.

The CD20 suicide gene candidate described earlier, can also function as a selection marker.[144] Immuno-selection of CD20 transduced cells is rather inefficient. Yields of 50% and purities of 77-98% at most have been reported.[162] As Thy-1, CD20 is expressed on a subpopulation of human T cells.[163] Hence, there is a risk for false positive selection. Given that most cell surface molecule expressed on a cell participate in the functions of that cell, it is possible that CD20 plays a role in that specific subpopulation. Further investigations are needed to elucidate possible effects of ectopic CD20 expression in other T cells.

CD19 is a B cell surface molecule that assembles with the antigen receptor. It is critical for the regulation of B cell development, activation and differentiation.[164] A truncated CD19 (tCD19) has recently been developed as a selection marker, with potential tyrosine phosphorylation sites removed from the intracellular domain.[143] High purities (98%) were achieved but the average yield was merely 31%. Toxicity related to transgene expression that interfere with cellular decisions of proliferation and differentiation, could manifest with undesirable transformation promotion events. Even though T cells do not express the CD19 associated B cell receptor, special attention should be paid to the use of molecules such as CD19.[151] For instance, it has been shown CD19 improves EBV induced transformation of B cells.[165]

Bacteria derived selection markers such as NeoR and hygromycin phosphotransferase (HPT) inactivate antibiotics through phosphorylation and have been used extensively in molecular biology research. Chemical selection is highly efficient and cost-effective but selection is time consuming. Aints and colleagues made a major

breakthrough by the development of a chemical selection marker based on OuaR, the L799C mutated alpha 1 subunit of the rat Na^+, K^+ ATPase.[166, 167] The expression of OuaR from plasmids and retroviral vectors allowed for highly efficient (99%) and affordable selection with ouabain in only 24 hours of incubation.

The Na^+, K^+ ATPase is an oligomeric protein composed of alpha subunits, beta subunits and FXYD proteins. It is ubiquitously expressed and maintains the sodium and potassium gradients in the cell, thus controls basic cellular homeostasis. It has also tissue specific roles, such as excitability of neuronal and contractile tissues and sodium re-absorption in renal epithelia.[168] The catalytic alpha subunits hydrolyze ATP and transport the cations. Beta subunits act as chaperones for alpha subunit expression and function, in addition to having a regulatory role.[169] FXYD proteins have a regulatory role and are expressed in distinct tissues, e.g. brain and heart. Alpha subunits are represented by 4 isoforms 1-4.[170] All tissues express the major alpha 1 isoform, while the others are tissue specific. The alpha subunits possess a binding site for cardiac glycosides such as digoxin and ouabain. These drugs have for centuries been used for treatment of congestive heart failure. Binding of digoxin or ouabain in nanomolar ranges leads to increased contractive force of heart muscles. The effects observed from nanomolar range exposure result either directly from the partial inhibition of the pumping activity, or as a result of protein-protein interactions and intracellular signaling.[171, 172] In micromolar ranges, ouabain completely blocks the pumping activity in human cells. The blocking disrupts ion homeostasis and rapidly induces apoptosis via activation of caspase-3, cytochrome C release from mitochondria and the generation of reactive oxygen species.[173, 174]

The Na^+, K^+ ATPase/ouabain selection system was recently adapted to the human alpha 1 subunit, for possible implementation in clinical gene therapy.[175]

The engineered human ouabain resistant alpha 1 (OuaSelect) carry two amino acid substitutions, Q118R and N129D. An equally efficient selection to the rat gene has been demonstrated (99%), albeit at an extension to 48 hours selection. The longer selection time depends on a lower ouabain resistance level of OuaSelect compared to the rat OuaR. Further studies are needed to address its applicability in clinical gene therapy.

2.2.6 Immunogenicity

The induction of immune reactions against genetically modified cells is one of the most important factors that can influence the efficacy of gene therapy. An increasingly recognized problem in lymphocyte targeting gene therapy is immune reactions against vector encoded transgenes. Such reactions can result in rapid elimination of genetically engineered cells. Non-human proteins, such as HSVtk but also altered human proteins are potentially immunogenic. Repeated infusions of HSVtk transduced T cells to HIV infected patients lead to rapid clearance of genetically modified cells.[119]

Patients undergoing allogeneic HSCT have severely compromised immune systems. HSVtk positive lymphocytes delivered as post-transplant DLI can be detected for long-term and eliminated with GCV administration.[59] Yet, immune reactions against HSVtk have been observed.[146, 176] It is possible that immune reactions depend on the functional state of the immune system. It has been proposed that early post-transplant immune incompetence could be utilized for delivery of potentially immunogenic lymphocytes.[177] The profound immuno-supression associated with the

early post-transplant period would be sufficient to dampen immunity to transgenes expressed by infused lymphocytes. Later infusion when the immune system has recovered to some extent would provoke efficient immune responses against immunogenic transgenes. However, although persistence of transgene positive cells can be observed long-term after transplant, rapid elimination of HSVtk transduced lymphocytes has been observed even when administered at the time point of HSCT.[121] In addition, induction of an immune response against the enhanced green fluorescent protein (EGFP) transgene has also been observed as a consequence of HSCT of EGFP transduced CD34+ stem cells in primates.[178] Further studies are needed to identify efficient strategies to circumvent immune responses against transgenes in the setting of HSCT and T cell suicide gene therapy.

Switching transgene expression on and off is an attractive approach. The suicide gene expression would then only be turned on at the time point of desired suicide activation and the problem of transgene immunogenicity circumvented. Moreover, the inducible gene product could be toxic per se (e.g. diphtheria toxin) making prodrug administration redundant. Various inducible gene expression systems have been developed. Recently several mechanisms were combined into one system; cre-lox site-specific recombination, RNA interference and tetR and lacl Escherichia coli repressor systems. Firm control of gene expression was possible with this combined system.[179] Although this system is probably not directly applicable in clinical gene therapy, it can be used to study a number of clinically relevant processes. The modulation of translation termination with aminoglycoside antibiotics is clinically feasible. However, it is not likely to be stringent enough, resulting in leaky expression in the off state.[180] Efficient gene expression silencing is important, in particular for toxic genes. A single molecule of diphtheria toxin is sufficient to kill a cell.[181] Manipulation of mRNA splicing by antisense oligonucleotides is another approach that could potentially be used to control expression. This method was recently shown to locally restore dystrophin expression in patients with Duchenne's muscular dystrophy.[182]

Interestingly, constitutive HSVtk expression while avoiding an antigen directed immune response could potentially be achieved by fusing the glycine alanine repeat (GAr) of the Epstein-Barr virus nuclear antigen-1 (EBNA-1) to the HSVtk coding sequence.[181] The GAr reduces proteosomal degradation of the GAr-HSVtk protein and presentation of the protein on MHC molecules. However, it remains to be shown if this approach fully prevents immune responses against the HSVtk.

2.2.7 Insertional mutagenesis and retroviral vectors

Gene therapy is defined as the introduction of genes into a person's cells, undertaken to prevent or treat disease. Treating cells with naked DNA does not enable efficient and stable gene transfer, whereas viruses represent a naturally efficient delivery device of genes. As a result, many virus types have been exploited in the development of gene transfer technologies, among them retroviruses.[183] Retroviral based vector delivery is currently the method of choice to deliver sustained expression of introduced transgenes to hematopoietic cells. The introduced genes are stably integrated into the host cell genome, and could theoretically provide a life long effect. Beside promising results from the T cell suicide gene therapy approach,[123] clinical benefit from the introduction of genes by retroviral vectors have also been

demonstrated for patients with X-linked severe combined immune deficiency (SCID-X1) and adenosine deaminase (ADA) deficiency dependent SCID.[184, 185]

At the same time, evidence for a severe dose-limiting side effect of retroviral gene delivery has become apparent. Transformation promotion by untargeted integration of the vector near proto-oncogenes, resulting in their up-regulated expression, represents a major concern for stem cell targeting.[186, 187] So far, no indication of transformation of retroviral vector transduced lymphocytes has been reported. A recent *in vivo* study was designed to provoke insertional mutagenesis in mouse stem cells and lymphocytes by cooperation of introduced transgene with the insertion site.[188] The results showed transformation of stem cells, while no transformation events were observed in genetically modified lymphocytes. Thus, although insertional mutagenesis is a demonstrated severe side effect in retroviral vector targeting of stem cells, its relevance to effector cells such as lymphocytes remains to be an open question. Still, T cell retroviral vectors designed for clinical use should obviously be rigorously tested in a variety of assays before entering the clinic.

2.2.8 Retroviral vector design

Retroviruses are lipid-enveloped viruses, containing two copies of single stranded genomic RNA. Infection with a retrovirus starts with the recognition of a surface receptor allowing fusion of the virus with the cell membrane and receptor-mediated endocytosis. The viral RNA is reversely transcribed into linear double stranded DNA by the viral enzyme reverse transcriptase. The reverse transcriptase is packaged into the virus particle and released with the infection. Integration occurs during mitosis, when the nuclear membrane is disintegrated and the chromosomes accessible. The integrated provirus generates new RNA genomes that are packages into core particles budding off from the cell surface. Retroviral vectors are limited by the inability to transduce non-dividing cells. Lentiviruses, such as human immunodeficiency virus (HIV) belong to the retrovirus family and can infect both dividing and non-dividing cells. This feature makes lentiviral vectors attractive for gene delivery and lentiviral vector development is ongoing since the introduction more than a decade ago.[189]

Retroviral vectors and virus particle packaging systems have been investigated and developed for clinical use for about 20 years. General challenges for vector development is to minimize the risk of generating replication competent retroviruses (RCRs), to obtain adequate vector titer, and achieve long-term transgene expression at appropriate levels. Several components are needed for retroviral vector production.[79] One is packaging cells, providing the gag-pol and env in *trans*. Gene products from gag-pol include the structural proteins and replication enzymes of the virus. The env gene encodes for the envelope that gives target specificity of the virus. Another component is the vector backbone. Retrovirus vectors consist of replication defective retroviruses with critical *cis*-acting viral elements. These include a promoter and a polyadenylation signal, a viral packaging signal (Ψ), signal for reverse transcription, and short, inverted repeats for integration. The last component is the gene(s) of interest and strategies for gene expression. Transgene size is a limiting factor since the cloning capacity of retroviral vectors is limited to 8 kilobases. Single genes can be expressed from the 5'LTR promoter, replacing the retroviral coding sequences. Co-expression of multiple genes can be achieved by bi-directional or multiple promoters, alternative splicing, and by an internal ribosome entry site (IRES).[190, 191] In addition, co-

expression is also achievable by direct fusion of multiple genes. The gene products can remain ligated, generating chimeric proteins or can be cleaved by a connective 2A peptide sequence.[192]

Sustained long-lasting transgene expression is the most important feature of retroviral vector mediated gene transfer. Beside effects related to vector insertion and LTR driven expression, the transgenes themselves can influence expression stability. Gene expression is regulated on many different levels, and the coding sequence can contain elements influencing RNA stability and translation efficiency.[193, 194] Activities of cryptic splice sites within the HSVtk transgene have been found, generating deleted vectors and lost transgene activity.[195] Directed mutagenesis can be used to disrupt cryptic splice sites.[196] Alternatively, synthetic, software generated transgene sequences (e.g. GeneArt GeneOptimizer) can substitute native gene sequences to remove cryptic splice sites. Such codon optimized sequences can also enhance titers and expression levels of retroviral vector encoded transgenes.[197](Treschow et al., manuscript)

3 AIMS OF THIS THESIS

The main objective of this thesis was to optimize T cell suicide gene therapy vectors with the means of genetic engineering. The specific aims addressed in each study included are listed below.

I) To analyze the long term effect of retroviral gene transfer to hematopoietic stem cells and investigate the potential contribution of contaminating tumor cells in autologous stem cell grafts to MM relapse.

II) To develop a new selection marker gene that would be suitable for clinical gene therapy applications.

III) To optimize the design of a retroviral vector co-expressing the OuaSelect selection marker gene and the HSVtk suicide gene.

IV) To study the effects of codon-optimization and introduction of the A168H mutation on the HSVtk suicide gene.

4 RESULTS AND DISCUSSION

4.1 GENE MARKING IN AUTOLOGOUS HSCT IN MULTIPLE MYELOMA (PAPER I)

At the time the clinical trial described in this study was conducted, multiple myeloma (MM) was an incurable hematological malignancy. Still today, high dose chemotherapy followed by autologous hematopoietic stem cell transplantation improves the outcome compared to conventional chemotherapy, but the overall complete response rate is < 50% and median duration of remission after treatment is about 2-3 years[198].

Autologous stem cells are able to reconstitute hematopoiesis after myeloablative chemotherapy for the malignant disease. However, disease relapse occurs in about 80%[199], and it was unclear whether small numbers of viable clonogenic tumor cells, contaminating the stem cell graft, contribute significantly to relapse.

The treatment for multiple myeloma was clearly insufficient and improvements of the current treatment modality, as well as the development of new and better cures, were urgently needed. The development of gene marking procedures for hematopoietic stem cells was important for such strategies, in order to understand the biology of transplanted cells.

The primary aim of this study was to analyze the safety of gene marking of autologous stem cell for transplantation. The secondary aims were to assess the persistence of gene marked cells long term in the patients and to study the potential contribution of re-infused remaining tumor cells in the graft to subsequent relapse. Two earlier marking studies had been conducted in which bone marrow nucleated cells were transduced with retroviral vectors encoding the NeoR gene. In one, an AML study, four out of 12 patients relapsed and in two of them the marker gene was present in the tumor cells.[200] In the other study, conducted on neuroblastoma patients, five patients relapsed and marked tumor cells were observed in four of them[201]. One gene marking study had already been conducted on multiple myeloma[202], but the low transduction efficiency achieved warranted further studies.

Eight multiple myeloma patients intended to undergo high-dose chemotherapy and autologous hematopoietic stem cell transplantation were enrolled in the study. Six received grafts including gene-modified CD34+ cells. Two patients died before treatment.

Supernatant of the retroviral vector G1Na encoding the bacterial selection marker NeoR was used to transduce half of the autologous bone marrow harvest. The other half was left untreated and later infused together with gene marked cells.

CD34+ cells were sorted before two rounds of transduction at an m.o.i. of 5 in the presence of stem cell factor, IL-3 and IL-6 and basic fibroblast growth factor. Transduced cells were assessed by colony assay with or without G418 selection medium. The transduction efficiency was low (range 0,43-5,1%) and the number of transduced CD34+ cells transplanted was between 0,1-0,5 x 10^6 in a total infused graft of 1,95-5,66 x 10^6 cells/kg. The patients were monitored at intervals up to 5 years after transplant for (I) presence of marker gene by nested PCR, (II) expression of the transgene by reverse transcriptase PCR and (III) transgene activity by colony forming assays in the presence of G418.

Table 1. Separation, transduction and transplantation of CD34+ cells.

Patient #	1	2	3	4	5	6	7	8
CD34+ Seperation	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)
Total cell count before seperation $x10^6$	5500	42000	43500	33000	47100	37500	14200	21480
$CD34^+$ before seperation $x10^6$	7	NS	374	1683	636	994	328	752
$CD34^+$ after seperation $x10^6$	5	NS	262,9	304	180	213,2	49,1	187,6
Transduced cell count $x10^6$	10,8	104,2	85,6	308	52,1	97	73,2	126
Number of $CD34^+$ cells for transduction $x10^6$	1,9	NS	51,5	268	39,2	70,8	19,5	77,2
Living cell count post transduction $x10^6$	5,1	7,2	30,1	61,6	11,8	39	71,8	85,5
Number of colonies $/10^6$ cells	13600	8000	34600	10600	77600	30300	32300	183300
Number of transduced colonies $/10^6$ cells	300	NA	2000	NA	300	NA	666	1300
Transduction efficacy (% colonies)	2,37	NA	5,1	NA	0,43	NA	2,06	0,73
Transduced cells for transplantation	104280	NA	NA	NA	39775	NA	1370000	591300
Transduced cells $x10^4$ / kg body weight	0,16	NA	NA	NA	0,062	NA	1,91	0,75
Total number of cells transplanted $x10^6$	4792 (BM)	NA	NA	368	138	228	140,5	263
Total number of cells $x10^4$ / kg body weight	7487 (BM)	NA	NA	566	216	300	195	337

The low transduction efficiency achieved of CD34+ cells resulted in a low number of infused marked cells, but transgene positive cells were observed until the end of the study. The amount of marked cells in the bone marrow in three patients was determined by quantitative PCR to be less than 1 in 10^5 at the latest time point. Transgene activity of the NeoR gene product was observed up to 6 months post transplant. No expression was detected after six months, explained either because it was below the threshold for detection by RT-PCR (1 in 10^5), because the expression had been silenced by promoter methylations, or from the NeoR in cells where it was expressed having elicited an immune response in the recipient, resulting in the elimination of those cells, or combinations of the above events.

Table 4. Colony assays and NeoR PCR analysis in bone marrow cells following autologous bone marrow transplantation

Patient #	1	4	5	6	7	8
Time period	Positive colonies					
3 weeks	2%	1%	4%	1%	1%	1%
3 months	NA	1%	5%	1%	5%	1%
6 months	2%	2%	2%	0%	1%	0%
	PCR / Nested PCR (N)					
3 weeks	+	-/N+	NA	+	+	+
3 months	+	NS	-/N+	-/N+	+	+
6 months	+	-/N+	-/N+	-/N+	+	-/N-
	Nested PCR (N)					
1 year	+	+	+	D	+	-
2 years	+	D	+		+	D
3 years	+		+		+	
4 years	NA				+	
5 years	+		D		NA	
Biopsy	-	NA	-	NA	NA	-

Bone marrow cells were also harvested from the patients at the time point of relapse. Malignant cells were immuno-selected using the BB4 antibody before marker gene analysis. No marking was observed in bone marrow cell samples of patients in relapse. Noteworthy, the transgene was detected in the bone marrow of the same patients in the remission period that followed the relapse. Due to the low marking rate, the data are suggesting, rather than proving that it is the inability to achieve complete eradication of residual tumor cells after chemotherapy, rather than the remaining tumor cells in the re-infused graft that constitute the source of malignant cells causing the relapse.

Selection prior to transplantation could have been advantageous, since it would have greatly increased the ratio of marked cells transplanted. Moreover, co-infusion with non-transduced cells could have impaired the engraftment of transduced cells. A better engraftment could perhaps have been achieved with a later infusion of non-manipulated cells. It could have resulted in a more robust marking ratio of cells, thereby facilitating detection and monitoring of marked cells. In this study we used the prokaryotic selection marker NeoR since there were no other better selection markers available at this time. With the use of the NeoR selection marker, the antibiotic selection proceeds in a timescale of over a week. Since the duration of the *ex vivo* procedures had to be kept within a few days for the stem cells not to loose their pluripotency, selection was not possible in this study. Studies have shown that prokaryotic NeoR can cause immunogenicity *in vivo*[59, 146] Beside, toxicity in terms of alterations in cell function after the lengthy selection have also observed[69].

Therefore, new selection markers that would be applicable in these types of studies are desirable.

4.2 THE DEVELOPMENT OF A NOVEL SELECTION MARKER (PAPER II)

Based on the observations in Paper I, the aim for this study was to address the limits of current *ex vivo* cell selection systems and develop a new, improved selection marker and selection regimen that would be suitable for clinical applications.

The human Na^+, K^+ ATPase and its inhibitor drug ouabain were an attractive candidate system, since ouabain is a clinically used cardiac glycoside that, at toxic levels, rapidly induces cell death in exposed cells. The target for ouabain is the alpha 1 subunit of Na^+, K^+ ATPase, but the specific binding site and blocking mechanisms are not yet fully understood. The rat alpha 1 is three orders of magnitude more resistant than the human alpha 1, yet 98% similar on protein level. Therefore, we hypothesized that humanization (synthesizing using recombinant DNA technology to circumvent the clinical problem of immune response to foreign antigens) of the rat alpha 1 could generate a chimeric alpha 1 protein with as much human alpha 1 identity as possible, that would be adequately resistant to be used for positive cell selection with ouabain.

First, hybrid genes of the rat and human Na^+, K^+ ATPase subunits were constructed. Plasmids expressing the gene constructs as fusion genes with EGFP were introduced into cell lines by transient transfection followed by ouabain exposure. The first 130 amino acids were identified to be critical to conferring resistance to 10 M ouabain, which is two orders of magnitude increased resistance relative to human alpha1. All ouabain resistant chimeric proteins contained the rat N-terminus, while all construct with the human N-terminus were sensitive to 10 M ouabain.

Having identified a critical region in the alpha 1 for ouabain sensitivity, site-directed mutagenesis of the human gene was performed to achieve the same level of ouabain resistance with a minimal number of amino acid alterations. Again the constructs were analyzed by transient transfection and ouabain exposure of cell lines. Two amino acid substitutions, Q118R and A129D, were identified as sufficient for the increased ouabain resistance. We termed the $hNKAa1_{Q118R/A129D}$ gene OuaSelect.

Over-expression of the wild-type gene did not confer ouabain resistance. Transient co-transfection of an EGFP-OuaS plus an anti-EGFP shRNA expressing plasmid into U2OS cells lead to a loss of resistance, confirming that the mutant was conferring the increased ouabain resistance.

Retroviral vectors were constructed with the EGFP-OuaSelect fusion gene. Cell lines and primary human lymphocytes were transduced and analyzed for ouabain sensitivity. Transduction efficiencies of 23.3 – 34.2 % were achieved in lymphocytes of three healthy donors. Recovery rates of, on average, 82% of transduced cells were achieved with 48 hours of exposure to 1 M ouabain. Unmodified cells were eliminated to >99%. Thus, the OuaSelect gene confers resistance also to stably transduced primary human cells and an efficient purification of genetically modified cells is possible within 48 hours of selection.

Toxicity is an important aspect of transgenes and cell selection regimens. EGFP-OuaSelect transduced and selected Jurkat cells were cultured in parallel with normal Jurkat cells for three weeks and showed similar expansion rates. The turnover rate of non-transduced primary lymphocytes, compared to OuaSelect expressing cells +/-

ouabain exposure did not differ. The morphology of transduced cells or subcellular localization of OuaSelect was not perturbed by the introduction of the two amino acid substitutions. Thus, no apparent signs of toxicity were found.

In summary, this study describes the development of a novel human selection marker, the ouabain resistance gene (OuaSelect), which has the potential for rapid *in vitro* selection of target cells with high recovery. We showed that cells, including primary human T cells, transduced with a retrovirus vector encoding for the OuaSelect could be efficiently selected with ouabain in 24-48 hours and pure populations of selected cells could be obtained.

4.3 OPTIMAL VECTOR DESIGN FOR OUASELECT AND HSVTK CO-EXPRESSION (PAPER III)

A number of conditional suicide genes have been proposed, but the most extensively investigated gene for clinical suicide gene therapy is the Herpes simplex virus-1 thymidine kinase gene (HSVtk). Cells expressing HSVtk can be killed by ganciclovir (GCV), a drug used to treat herpes virus infections. (see general intro.)

The aim of this study was to optimize the design of a retroviral vector co-expressing the OuaSelect selection marker gene and the herpes simplex virus thymidine kinase (HSVtk) suicide gene. Several aspects, such as titer, functionality and concordance are important to consider when aiming at expressing two or more genes from a single vector, since low titer, imbalanced protein expression and decreased transgene functionality can result from suboptimal constructs. Therefore, a panel of vectors with co-expression variations that are relevant for clinical vector design, such as direct fusion, an internal ribosomal entry site (IRES) or viral derived 2A "self-cleaving" peptide linkers, were constructed and screened.

Control vectors in which the red fluorescent protein DsRed-Express replaced the HSVtk gene and in which EGFP was fused to OuaSelect were included in the panel to facilitate vector evaluation. Moreover, since no anti-HSVtk monoclonal antibody is available, the hemagglutinin epitope was tagged to the C-terminal of HSVtk to allow antibody mediated protein visualization (western blotting and confocal microscopy).

First, we analyzed to what level a synthetic, fully codon optimized OuaSelect (OuaSelect*) sequence could improve titer and expression level in target cells. We also compared the SF91 retroviral backbone used in paper II, with the RSF91 vector that has been shown to improve RNA expression in producer cells and titer levels. In the RSF91, we also replaced the pre element with the pre* element, a shorter version including a safety mutation. Codon optimization of the OuaSelect and the use of an improved vector backbone increased titer and gene expression.

HeLa, Jurkat and K562 cell lines were transduced with the co-expression vectors and analyzed for transduction efficiency and expression level by flow cytometry. OuaSelect expression level was slightly reduced with the addition of a second gene into the vector, regardless of mode of co-expression. DsRed expression was low and only a fraction of the cells were EGFP/DsRed double positive, even after selection.

OuaSelect functionality was assessed by survival of transduced cells in 1 M ouabain. Direct fusion with HSVtk at the first position and IRES mediated co-expression generated functional OuaSelect expression, while direct fusion in the reverse order and 2A peptides resulted in minimal OuaSelect functionality.

HSVtk functionality was assessed on transduced and selected Jurkat cells in a ganciclovir dose response assay. HSVtk activity was achieved with all vector variants. The results showed that the direct fusion construct with HSVtk on first position (HSVtk-OuaSelect) demonstrated highest ganciclovir sensitivity in combination with concordant ouabain resistance.

Finally, primary human lymphocytes were transduced with either the HSVtk-OuaSelect or the EGFP-OuaS.IRES.HSVtk containing vector. The cells were analyzed for transgene functionality by ouabain resistance and ganciclovir sensitivity (Figure 6B). The recovery rate after ouabain selection was better for the fusion construct. Again, the fusion construct showed better ganciclovir sensitivity than the IRES construct, confirming cell line results. In summary, we were able to construct a vector that offers a novel attractive selection marker/suicide gene combination for T cell suicide gene therapy.

The SF91 long terminal repeat has previously been shown to allow high expression in myeloid cells and lower levels in lymphoid cells. In this study, we observed a similar pattern with the RSF91 vector. Since long term functional expression is required for suicide gene therapy, a promoter supporting high expression levels in lymphoid cells should be advantageous in a vector aimed for clinical use.

4.4 SUPERIOR KILLING CHARACTERISTICS OF THE NOVEL HSVTK WITH THE A168H INTRODUCED MUTATION (PAPER IV)

The Herpes simplex virus thymidine kinase (HSVtk) gene together with its prodrug ganciclovir (GCV) is the currently most widely used tool for both adoptive immunotherapies with suicide gene-modified donor lymphocytes after allogeneic stem cell transplantation and cancer gene therapy. The system has also been among the most successful gene therapy strategies so far. However, there are several limitations of the current HSVtk/GCV system that have become apparent with its clinical use.

The aim of this study was to investigate the possibility that an engineered HSVtk gene ($HSVtk_{A168H}$) could further enhance its performance as a suicide gene/prodrug system. The shortcomings of the system that were targeted for improvements were I) GCV toxicity at administered doses, II) several days of GCV exposure required to reach maximum killing efficiency, III) *cis*-acting elements perturbing ganciclovir sensitivity, and IV) cytotoxic activity at high expression levels, probably due to HSVtk's affinity for endogenous thymidine.

The $HSVtk_{A168H}$ mutant was previously shown to have a strongly reduced affinity to endogenous thymidine compared to the wild type enzyme and was therefore an interesting candidate for reducing cytotoxicity of the gene in the suicide gene therapy setting. Indirect effects of a lower toxicity could be that higher expression levels in the cells would be tolerated and lead to higher sensitivity to GCV induced cell killing. A solution to the problem with *cis*-acting elements was the generation of a fully synthetic codon optimized coding sequence.

In this study, we developed a synthetic codon-optimised (co)HSVtk gene, introduced the amino acid substitution A168H and compared the two new variants with the viral wild-type (wt) HSVtk gene for (I) expression level, (II) functionality and (III) sustained functional expression long term. The genes were introduced into retroviral and lentiviral vectors also encoding reporter/selection marker genes. The HSVtk gene variants were fused to either the tCD34 or the OuaSelect selection marker in retroviral

MP71 vectors and to the EGFP/NeoR opt combined reporter/selection marker gene in lentiviral vectors.

Myeloid (K562) and lymphoid (PM1, Jurkat, CEM) cells were transduced with retroviral vectors followed by either magnetic sorting (MACS) or ouabain selection. The cells were subsequently assessed for HSVtk functionality by exposure to different concentrations of GCV and analyzed at intervals for app. 1 week. We found strongly improved killing kinetics for the coHSVtk(A168H) mutant as compared to both wild-type HSVtk and coHSVtk in both myeloid and lymphoid cells. The expression levels were also markedly higher for the HSVtk(A168H). Long-term studies (3 mo) also showed a better sustained functional expression for the A168H mutant.

Primary human T cells of seven donors were transduced with either tCD34 or OuaSelect vectors, selected and assessed for ganciclovir sensitivity. The results confirmed the observations obtained in cell lines. Two days exposure lead to significantly improved killing efficiency of the coHSVtk(A168H). Thus, the rate of cell killing of the system could be improved.

We also assessed the potential of the HSVtk variants in killing solid tumor cancer cells. Glioblastoma (U44 and G62), lung cancer (A549), and colon cancer (SW620) cell lines were transduced with lentiviral vectors, selected in G418 and analyzed for ganciclovir sensitivity. Again, the highest killing rates after GCV-treatment were observed for the co-HSVtk A168H gene. Moreover, significantly lower GCV doses were sufficient for effective GCV induced killing as compared to the wild type variants.

Since it is not feasible to achieve an introduction of the HSVtk gene into the whole cell population of a tumor *in vivo*, the successful eradication of tumors depends on the ”bystander effect” phenomenon, by which the HSVtk can affect also neighboring cells in which the gene is not present. From a therapeutic point of view, the bystander effect might have a significant value to cancer gene therapy. The bystander effect of the A168H mutant was assessed by mixed cultures of unmodified and HSVtk positive cells, and a one log increase in bystander GCV induced killing was achieved with the A168H mutant compared to wild type HSVtk.

In summary, we provide evidence that the novel codon-optimised HSVtk A168H gene shows improved efficacy as compared to wild type HSVtk and coHSVtk and we termed the gene TK.007. This gene represents a novel promising candidate for various types of suicide gene therapy.

4.5 GENERAL DISCUSSION

The ability to purify populations of cells genetically modified with retroviral and/or lentiviral vectors is important in the analysis of gene transfer effects. It is also often of uttermost significance for the safety and effectiveness of the gene therapy approach.

Low transduction rates obtained in early stem cell marking studies could result in a marking rate below detection limit with available monitoring techniques and an inability to monitor the transduced cells and their progeny. Moreover, in therapeutic gene therapy approaches, low transduction rates could be inconvenient, since competition from unmodified infused cells can dilute the therapeutic effect of the transduced cells. In either case, low transduction efficiency can be compensated with a cell selection procedure. Cell selection systems have a number of criteria to fulfill in order to be applicable for clinical use. A low mutagenic activity of the selection agent

in addition to low immunogenicity and low phenotoxicity of the marker gene are prerequisites for all applications. Other features required are more application specific.

We did not observe any marking of tumor cells in relapse when transducing autologous CD34 purged bone marrow or peripheral blood harvest from multiple myeloma patients and infusing the cells together with an untreated fraction of the grafts. These findings suggest that it is the inability to complete eradication of residual tumor cells after chemotherapy, rather than the remaining tumor cells in the re-infused graft that constitutes the source of malignant cells causing the relapse. We did observe gene marking in the bone marrow until five years post transplant, at the end of the follow-up study. Transgene activity could only be monitored up to six months post-transplant by real-time PCR. After six months, the marking rate fell below detection level of the PCR method. A higher transduction level could have resulted in a higher marking rate and make transgene expression monitoring long term possible. Moreover, the low transduction rate (range 0.43-5.1%) must be taken into consideration when acknowledging the absence of marked tumor cells at relapse.

In vitro cell selection by ouabain exposure is the most rapid chemical selection method available. It is possible that the 24-48 hours ouabain selection time could be implemented in transduction protocols for hematopoietic stem cells. Increased transduction rates in addition to selection of genetically modified stem cells would improve *in vivo* marking rates and thereby the outcome of gene marking as well as therapeutic gene transfer studies.

Today, the efficiency (ratio of successfully targeted cells in the population) of viral vector-mediated gene transfer to T cells *ex vivo* using high titer supernatants and optimized protocols could reach 90-95%. However, much lower levels are optimal due to the inherent risk of insertional mutagenesis with this treatment modality. Currently, the standard approach is to obtain transduction efficiencies that would result in not more than one integrated proviral copy per modified cell, achievable with <30% transduction rates. This approach necessitates a purification step, in particular for T cell suicide gene therapy, for which unmodified cells will escape suicide gene activation.

In study II, we have developed a new selection marker (OuaSelect) that could potentially fulfill the general criteria for clinical use, although further in depth studies are needed both *in vitro* and *in vivo* to certify its clinical applicability. It was possible to transduce primary human lymphocytes with this selection marker with transduction efficiencies about 30%. The cells were selected with ouabain to >98% pure populations of genetically modified cells with 48 hours of selection.

Immunogenicity is a major concern in clinical gene therapy. Immune responses elicited against transgenes can result in rapid and effective elimination of gene modified cells that express the transgene, in particular with repeated infusions[121]. Such events can seriously affect the efficacy of the T cell suicide gene therapy approach, although some argue that the risk/benefit ratio can be maintained with appropriate study design despite the use of immunogenic transgenes [177]. The OuaSelect is the human alpha 1 subunit of the housekeeping Na^+, K^+ ATPase with two amino acid substitutions. The minimal perturbations made of the protein are unlikely to be immunogenic, although immune reactions against the altered amino acids cannot be ruled out.

Co-expression of the OuaSelect and the HSVtk was more complicated than anticipated. The study presented in paper III, was devoted to this problem and identified an efficient co-expression strategy. The optimized HSVtk-OuaSelect

chimeric fusion protein permitted ouabain selection with coupled HSVtk activity. The HSVtk-OuaSelect gene expression resulted in a recovery of 94% of transduced cells with a purity >98% and an elimination of, on average, 98.4% of genetically modified lymphocytes with 4 days of 1 g/ml GCV exposure. In previous studies, lymphocytes transduced with tCD34/HSVtk suicide gene vectors were not eliminated to the same efficiency (95% and 85-90% respectively) when exposed to 1 g/ml GCV [157, 203]. An HSVtk suicide gene vector with the Thy-1 surface marker reached 96,4% killing efficiency at exposure to 2.5 g/ml, 2.5 times higher GCV concentration[159]. Although, killing efficiency depends on proliferation rate and a direct comparison cannot be made, the HSVtk-OuaSelect selection/suicide gene vector can offer an attractive selection regimen in combination with high killing efficiency. As such, is warrants further studies in view of existing alternative selection marker/suicide gene vectors.

The HSVtk/GCV suicide gene machinery is capable of controlling GvHD in patients. Despite the potential immunogenicity of HSVtk, it remains an attractive suicide gene candidate. It has been in use since the late 1980's and has been shown to be most effective in situations of rapidly dividing cells, high expression levels of HSVtk, and relatively high concentrations of GCV. Alloreactive lymphocytes causing GvHD are therefore an attractive target, but high expression levels have been difficult to reach, and GCV is immuno-supressive in administered doses. Increased GCV specificity of engineered HSVtk proteins have been demonstrated. Mutated HSVtk proteins include the SR39 mutant,[204] and the A168H single amino acid substitution.[129] In paper IV, we observed a significant improvement in the rate of GCV induced cell death *in vitro*, in addition to improved expression levels with the A168H HSVtk mutant in transduced hematopoietic cells. Moreover, a higher GCV sensitivity and bystander effect was observed of the A168H mutant in cancer cell lines. Further studies are needed to investigate if these promising observation could be confirmed *in vivo*, and more importantly, if they could translate into clinical use.

5 CONCLUSIONS

It seems to be the inability to completely eradicate residual tumor cells with chemotherapy, rather than the re-infusion of autologous grafts contaminated with tumor cells that constitute the source of malignant cells causing relapse in multiple myeloma patients.

A mutated human Na, K ATPase alpha 1 subunit with two amino acid substitutions (Q118R and N129D) can increase the sensitivity to ouabain induced cell death in gene-modified target cells by two orders of magnitude.

The Na, K ATPase alpha $1_{Q118R/N129D}$ (OuaSelect) can be used as a selection marker allowing efficient positive cell selection within 48 hours of exposure to ouabain.

Highly correlated functional expression of OuaSelect and the herpes simplex-1 thymidine kinase (HSVtk) conditional suicide gene can be achieved with coding sequence fusion in a retrovirus vector construct.

The codon-optimized $HSVtk_{A168H}$ (TK.007) increases the sensitivity to ganciclovir in transduced solid tumor cell lines and demonstrated an enhanced *in vitro* bystander effect to a ten-fold improvement as a suicide gene.

The HSVtk variant TK.007 allows for high transgene expression levels from a retrovirus vector and significantly increased rate of cell death induction with ganciclovir compared to wild-type HSVtk in hematopoietic cells.

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7 REFERENCES

1. Little, M.T. and R. Storb, *History of haematopoietic stem-cell transplantation.* Nat Rev Cancer, 2002. **2**(3): p. 231-8.
2. Storb, R., *Allogeneic hematopoietic stem cell transplantation--yesterday, today, and tomorrow.* Exp Hematol, 2003. **31**(1): p. 1-10.
3. Thomas, E.D., *The Nobel Lectures in Immunology. The Nobel Prize for Physiology or Medicine, 1990. Bone marrow transplantation--past, present and future.* Scand J Immunol, 1994. **39**(4): p. 339-45.
4. Jacobson, L.O., et al., *Effect of spleen protection on mortality following x-irradiation.* J Lab Clin Med, 1949. **34**: p. 1538-1543.
5. Lorenz, E., et al., *Modification of irradiation injury in mice and guinea pigs by bone marrow injections.* J Natl Cancer Inst, 1951. **12**(1): p. 197-201.
6. Main, J.M. and R.T. Prehn, *Successful skin homografts after the administration of high dosage X radiation and homologous bone marrow.* J Natl Cancer Inst, 1955. **15**(4): p. 1023-9.
7. Ford, C.E., et al., *Cytological identification of radiation-chimaeras.* Nature, 1956. **177**(4506): p. 452-4.
8. Nowell, P.C., et al., *Growth and continued function of rat marrow cells in x-radiated mice.* Cancer Res, 1956. **16**(3): p. 258-61.
9. Lindsley, D.L., T.T. Odell, Jr., and F.G. Tausche, *Implantation of functional erythropoietic elements following total-body irradiation.* Proc Soc Exp Biol Med, 1955. **90**(2): p. 512-5.
10. Thomas, E.D., et al., *Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy.* N Engl J Med, 1957. **257**(11): p. 491-6.
11. Bortin, M.M., *A compendium of reported human bone marrow transplants.* Transplantation, 1970. **9**(6): p. 571-87.
12. Billingham, R.E., *Reactions of grafts against their hosts.* Science, 1959. **130**: p. 947-53.
13. Billingham, R.E., *The biology of graft-versus-host reactions.* Harvey Lect, 1966. **62**: p. 21-78.
14. Epstein, R.B., et al., *Cytotoxic typing antisera for marrow grafting in littermate dogs.* Transplantation, 1968. **6**(1): p. 45-58.
15. Miescher, P. and M. Fauconnet, *[Antigenic components of the polynuclear leukocyte and their clinical importance.].* Schweiz Med Wochenschr, 1954. **84**(36): p. 1036-8.
16. Dausset, J., *[Iso-leuko-antibodies.].* Acta Haematol, 1958. **20**(1-4): p. 156-66.
17. Van Rood, J.J., J.G. Eernisse, and A. Van Leeuwen, *Leucocyte antibodies in sera from pregnant women.* Nature, 1958. **181**(4625): p. 1735-6.
18. Storb, R., et al., *Allogeneic canine bone marrow transplantation following cyclophosphamide.* Transplantation, 1969. **7**(5): p. 378-86.
19. Storb, R., et al., *Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow grafts.* Transplantation, 1970. **9**(3): p. 240-6.
20. Thomas, E., et al., *Bone-marrow transplantation (first of two parts).* N Engl J Med, 1975. **292**(16): p. 832-43.
21. Thomas, E.D., et al., *One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation.* Blood, 1977. **49**(4): p. 511-33.
22. Santos, G.W. and A.H. Owens, Jr., *Allogeneic marrow transplants in cyclophosphamide treated mice.* Transplant Proc, 1969. **1**(1): p. 44-6.
23. Clift, R.A., et al., *Allogeneic marrow transplantation in patients with acute myeloid leukemia in first remission: a randomized trial of two irradiation regimens.* Blood, 1990. **76**(9): p. 1867-71.
24. Clift, R.A., et al., *Allogeneic marrow transplantation in patients with chronic myeloid leukemia in the chronic phase: a randomized trial of two irradiation regimens.* Blood, 1991. **77**(8): p. 1660-5.

25. Lynch, M.H., et al., *Phase II study of busulfan, cyclophosphamide and fractionated total body irradiation as a preparatory regimen for allogeneic bone marrow transplantation in patients with advanced myeloid malignancies.* Bone Marrow Transplant, 1995. **15**(1): p. 59-64.
26. Storb, R., et al., *Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia.* N Engl J Med, 1986. **314**(12): p. 729-35.
27. Quellmann, S., et al., *Corticosteroids for preventing graft-versus-host disease after allogeneic myeloablative stem cell transplantation (Review)* The Cochrane Library 2009(1).
28. Ringden, O. and B. Nilsson, *Death by graft-versus-host disease associated with HLA mismatch, high recipient age, low marrow cell dose, and splenectomy.* Transplantation, 1985. **40**(1): p. 39-44.
29. Deeg, H.J., *How I treat refractory acute GVHD.* Blood, 2007. **109**(10): p. 4119-26.
30. Aversa, F., et al., *Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype.* N Engl J Med, 1998. **339**(17): p. 1186-93.
31. Ringden, O., et al., *Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease.* Transplantation, 2006. **81**(10): p. 1390-7.
32. Le Blanc, K., et al., *Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study.* Lancet, 2008. **371**(9624): p. 1579-86.
33. Boeckh, M., *Current antiviral strategies for controlling cytomegalovirus in hematopoietic stem cell transplant recipients: prevention and therapy.* Transpl Infect Dis, 1999. **1**(3): p. 165-78.
34. Burchenal, J.H., et al., *Effect of total-body irradiation on the transplantability of mouse leukemias.* Cancer Res, 1960. **20**: p. 425-30.
35. Barnes, D.W., et al., *Treatment of murine leukaemia with X rays and homologous bone marrow; preliminary communication.* Br Med J, 1956. **2**(4993): p. 626-7.
36. Mathe, G., et al., *Adoptive immunotherapy of acute leukemia: experimental and clinical results.* Cancer Res, 1965. **25**(9): p. 1525-31.
37. Weiden, P.L., et al., *Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts.* N Engl J Med, 1979. **300**(19): p. 1068-73.
38. Weiden, P.L., et al., *Antileukemic effect of chronic graft-versus-host disease: contribution to improved survival after allogeneic marrow transplantation.* N Engl J Med, 1981. **304**(25): p. 1529-33.
39. Horowitz, M.M., et al., *Graft-versus-leukemia reactions after bone marrow transplantation.* Blood, 1990. **75**(3): p. 555-62.
40. Kolb, H.J., et al., *Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients.* Blood, 1990. **76**(12): p. 2462-5.
41. Porter, D.L., et al., *Induction of graft-versus-host disease as immunotherapy for relapsed chronic myeloid leukemia.* N Engl J Med, 1994. **330**(2): p. 100-6.
42. Porter, D.L., et al., *Adoptive immunotherapy with donor mononuclear cell infusions to treat relapse of acute leukemia or myelodysplasia after allogeneic bone marrow transplantation.* Bone Marrow Transplant, 1996. **18**(5): p. 975-80.
43. Collins, R.H., Jr., et al., *Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation.* J Clin Oncol, 1997. **15**(2): p. 433-44.
44. Bortin, M.M., et al., *Graft-versus-leukaemia reactivity induced by alloimmunisation without augmentation of graft-versus-host reactivity.* Nature, 1979. **281**(5731): p. 490-1.
45. Weiss, L., et al., *Suppression and elimination of BCL1 leukemia by allogeneic bone marrow transplantation.* J Immunol, 1983. **130**(5): p. 2452-5.
46. Storb, R.F., et al., *Non-myeloablative transplants for malignant disease.* Hematology Am Soc Hematol Educ Program, 2001: p. 375-91.

47. Champlin, R., et al., *Harnessing graft-versus-malignancy: non-myeloablative preparative regimens for allogeneic haematopoietic transplantation, an evolving strategy for adoptive immunotherapy.* Br J Haematol, 2000. **111**(1): p. 18-29.
48. Schetelig, J., et al., *Evidence of a graft-versus-leukemia effect in chronic lymphocytic leukemia after reduced-intensity conditioning and allogeneic stem-cell transplantation: the Cooperative German Transplant Study Group.* J Clin Oncol, 2003. **21**(14): p. 2747-53.
49. Kroger, N., et al., *Autologous stem cell transplantation followed by a dose-reduced allograft induces high complete remission rate in multiple myeloma.* Blood, 2002. **100**(3): p. 755-60.
50. Maris, M.B., et al., *HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative conditioning for patients with hematologic malignancies.* Blood, 2003. **102**(6): p. 2021-30.
51. Bensinger, W.I., *The current status of reduced-intensity allogeneic hematopoietic stem cell transplantation for multiple myeloma.* Leukemia, 2006. **20**(10): p. 1683-9.
52. Schetelig, J., et al., *T cell-mediated graft-versus-leukemia reactions after allogeneic stem cell transplantation.* Cancer Immunol Immunother, 2005. **54**(11): p. 1043-58.
53. Voutsadakis, I.A., *NK cells in allogeneic bone marrow transplantation.* Cancer Immunol Immunother, 2003. **52**(9): p. 525-34.
54. Velardi, A., et al., *Clinical impact of natural killer cell reconstitution after allogeneic hematopoietic transplantation.* Semin Immunopathol, 2008. **30**(4): p. 489-503.
55. Law, T.M., et al., *Phase III randomized trial of interleukin-2 with or without lymphokine-activated killer cells in the treatment of patients with advanced renal cell carcinoma.* Cancer, 1995. **76**(5): p. 824-32.
56. Rosenberg, S.A., et al., *Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer.* N Engl J Med, 1985. **313**(23): p. 1485-92.
57. Alici, E., et al., *Autologous antitumor activity by NK cells expanded from myeloma patients using GMP-compliant components.* Blood, 2008. **111**(6): p. 3155-62.
58. Kershaw, M.H., et al., *Supernatural T cells: genetic modification of T cells for cancer therapy.* Nat Rev Immunol, 2005. **5**(12): p. 928-40.
59. Bonini, C., et al., *HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia.* Science, 1997. **276**(5319): p. 1719-24.
60. Link, C.J., Jr., et al., *Adoptive immunotherapy for leukemia: donor lymphocytes transduced with the herpes simplex thymidine kinase gene for remission induction. HGTRI 0103.* Hum Gene Ther, 1998. **9**(1): p. 115-34.
61. Champlin, R., et al., *Phase I/II study of thymidine kinase (TK)-transduced donor lymphocyte infusions (DLI) in patients with hematologic malignancies.* **Blood**, 1999. **94**(Suppl. 1): p. 324a.
62. Loren, A.W. and D.L. Porter, *Donor leukocyte infusions for the treatment of relapsed acute leukemia after allogeneic stem cell transplantation.* Bone Marrow Transplant, 2008. **41**(5): p. 483-93.
63. Ho, V.T. and R.J. Soiffer, *The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation.* Blood, 2001. **98**(12): p. 3192-204.
64. Link, C.J., Jr., et al., *Adoptive immunotherapy for leukemia: donor lymphocytes transduced with the herpes simplex thymidine kinase gene.* Cancer Treat Res, 1999. **101**: p. 369-75.
65. Tiberghien, P., et al., *Use of donor T-lymphocytes expressing herpes-simplex thymidine kinase in allogeneic bone marrow transplantation: a phase I-II study.* Hum. Gene Ther., 1997. **8**(5): p. 615-24.
66. Tiberghien, P., et al., *Administration of herpes simplex-thymidine kinase-expressing donor T cells with a T-cell-depleted allogeneic marrow graft.* Blood, 2001. **97**(1): p. 63-72.

67. Giralt, S., et al., *Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy.* Blood, 1997. **89**(12): p. 4531-6.
68. Drobyski, W.R., et al., *Ex vivo anti-CD3 antibody-activated donor T cells have a reduced ability to cause lethal murine graft-versus-host disease but retain their ability to facilitate alloengraftment.* J Immunol, 1998. **161**(5): p. 2610-9.
69. Sauce, D., et al., *Retrovirus-mediated gene transfer in primary T lymphocytes impairs their anti-Epstein-Barr virus potential through both culture-dependent and selection process-dependent mechanisms.* Blood, 2002. **99**(4): p. 1165-73.
70. Sauce, D., et al., *Influence of ex vivo expansion and retrovirus-mediated gene transfer on primary T lymphocyte phenotype and functions.* J Hematother Stem Cell Res, 2002. **11**(6): p. 929-40.
71. Weijtens, M., et al., *Reduced graft-versus-host disease-inducing capacity of T cells after activation, culturing, and magnetic cell sorting selection in an allogeneic bone marrow transplantation model in rats.* Hum Gene Ther, 2002. **13**(2): p. 187-98.
72. Marktel, S., et al., *Immunologic potential of donor lymphocytes expressing a suicide gene for early immune reconstitution after hematopoietic T-cell-depleted stem cell transplantation.* Blood, 2003. **101**(4): p. 1290-8.
73. Qasim, W., et al., *The impact of retroviral suicide gene transduction procedures on T cells.* Br J Haematol, 2003. **123**(4): p. 712-9.
74. Ferrand, C., et al., *Retrovirus-mediated gene transfer in primary T lymphocytes: influence of the transduction/selection process and of ex vivo expansion on the T cell receptor beta chain hypervariable region repertoire.* Hum Gene Ther, 2000. **11**(8): p. 1151-64.
75. Gorski, J., et al., *Circulating T cell repertoire complexity in normal individuals and bone marrow recipients analyzed by CDR3 size spectratyping. Correlation with immune status.* J Immunol, 1994. **152**(10): p. 5109-19.
76. Lange, C., et al., *CD34 modulates the trafficking behavior of hematopoietic cells in vivo.* Stem Cells Dev, 2007. **16**(2): p. 297-304.
77. Contassot, E., et al., *In vivo alloreactive potential of ex vivo-expanded primary T lymphocytes.* Transplantation, 1998. **65**(10): p. 1365-70.
78. Kornblau, S.M., et al., *Control of graft-versus-host disease with maintenance of the graft-versus-leukemia effect in a murine allogeneic transplant model using retrovirally transduced murine suicidal lymphocytes.* Exp Hematol, 2007. **35**(5): p. 842-53.
79. Pages, J.C. and T. Bru, *Toolbox for retrovectorologists.* J Gene Med, 2004. **6 Suppl 1**: p. S67-82.
80. Burt, R.K., et al., *Herpes simplex thymidine kinase gene-transduced donor lymphocyte infusions.* Exp Hematol, 2003. **31**(10): p. 903-10.
81. Ciceri, F., et al., *Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I–II study.* Lancet, 2009.
82. Ankersmit, H.J., et al., *Activation-induced T cell death, and aberrant T cell activation via TNFR1 and CD95-CD95 ligand pathway in stable cardiac transplant recipients.* Clin Exp Immunol, 2002. **128**(1): p. 175-80.
83. Yu, X.Z., P.J. Martin, and C. Anasetti, *CD28 signal enhances apoptosis of CD8 T cells after strong TCR ligation.* J Immunol, 2003. **170**(6): p. 3002-6.
84. Noel, P.J., et al., *CD28 costimulation prevents cell death during primary T cell activation.* J Immunol, 1996. **157**(2): p. 636-42.
85. Collette, Y., et al., *Distinct regulation of T-cell death by CD28 depending on both its aggregation and T-cell receptor triggering: a role for Fas-FasL.* Blood, 1998. **92**(4): p. 1350-63.
86. Tiberghien, P., *Use of suicide gene-expressing donor T-cells to control alloreactivity after haematopoietic stem cell transplantation.* J Intern Med, 2001. **249**(4): p. 369-77.
87. Hedfors, I.A. and J.E. Brinchmann, *Polyclonal T-cell activation protocol stimulates preferential expansion of EBV-specific T-cell clones in vitro.* Cancer Immunol Immunother, 2004. **53**(5): p. 439-44.

88. Sauce, D., et al., *Preferential retroviral-mediated transduction of EBV- and CMV-specific T cells after polyclonal T-cell activation.* Gene Ther, 2004. **11**(12): p. 1019-22.
89. Whitmire, J.K. and R. Ahmed, *Costimulation in antiviral immunity: differential requirements for CD4(+) and CD8(+) T cell responses.* Curr Opin Immunol, 2000. **12**(4): p. 448-55.
90. Movassagh, M., et al., *Retrovirus-mediated gene transfer into T cells: 95% transduction efficiency without further in vitro selection.* Hum Gene Ther, 2000. **11**(8): p. 1189-200.
91. Levine, B.L., et al., *Effects of CD28 costimulation on long-term proliferation of CD4+ T cells in the absence of exogenous feeder cells.* J Immunol, 1997. **159**(12): p. 5921-30.
92. Asano, Y., T. Komuro, and T. Tada, *Synergistic T-T cell interaction present in alloreactivity: determination of 'MLR helper' T cell subsets.* Int Immunol, 1990. **2**(12): p. 1203-11.
93. Hollatz, G., et al., *T cells for suicide gene therapy: activation, functionality and clinical relevance.* J Immunol Methods, 2008. **331**(1-2): p. 69-81.
94. Cavalieri, S., et al., *Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence.* Blood, 2003. **102**(2): p. 497-505.
95. Qasim, W., et al., *Lentiviral Vectors for T-cell Suicide Gene Therapy: Preservation of T-cell Effector Function After Cytokine-mediated Transduction.* Mol Ther, 2007. **15**(2): p. 355-60.
96. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.
97. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.
98. Sakaguchi, S., et al., *Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease.* Immunol Rev, 2006. **212**: p. 8-27.
99. Yong, Z., et al., *Role and mechanisms of CD4+CD25+ regulatory T cells in the induction and maintenance of transplantation tolerance.* Transpl Immunol, 2007. **17**(2): p. 120-9.
100. Hoffmann, P., et al., *Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation.* J Exp Med, 2002. **196**(3): p. 389-99.
101. Taylor, P.A., C.J. Lees, and B.R. Blazar, *The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality.* Blood, 2002. **99**(10): p. 3493-9.
102. Stanzani, M., et al., *CD25 expression on donor CD4+ or CD8+ T cells is associated with an increased risk for graft-versus-host disease after HLA-identical stem cell transplantation in humans.* Blood, 2004. **103**(3): p. 1140-6.
103. Mutis, T., T. Aarts-Riemens, and L.F. Verdonck, *The association of CD25 expression on donor CD8+ and CD4+ T cells with graft-versus-host disease after donor lymphocyte infusions.* Haematologica, 2005. **90**(10): p. 1389-95.
104. Wang, J., et al., *Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells.* Eur J Immunol, 2007. **37**(1): p. 129-38.
105. Guillot-Delost, M., et al., *Clinical-grade preparation of human natural regulatory T-cells encoding the thymidine kinase suicide gene as a safety gene.* J Gene Med, 2008. **10**(8): p. 834-46.
106. Montcuquet, N., et al., *Regulatory T-cell expansion and function do not account for the impaired alloreactivity of ex vivo-expanded T cells.* Immunology, 2008.
107. Mahic, M., et al., *Differentiation of naive CD4+ T cells into CD4+CD25+FOXP3+ regulatory T cells by continuous antigen stimulation.* J Leukoc Biol, 2008. **83**(5): p. 1111-7.
108. Pillai, V., et al., *Transient regulatory T-cells: a state attained by all activated human T-cells.* Clin Immunol, 2007. **123**(1): p. 18-29.

109. Coito, S., et al., *Retrovirus-mediated gene transfer in human primary T lymphocytes induces an activation- and transduction/selection-dependent TCR-B variable chain repertoire skewing of gene-modified cells.* Stem Cells Dev, 2004. **13**(1): p. 71-81.
110. Mesel-Lemoine, M., et al., *Initial depletion of regulatory T cells: the missing solution to preserve the immune functions of T lymphocytes designed for cell therapy.* Blood, 2006. **107**(1): p. 381-8.
111. Duarte, R.F., et al., *Functional impairment of human T-lymphocytes following PHA-induced expansion and retroviral transduction: implications for gene therapy.* Gene Ther, 2002. **9**(20): p. 1359-68.
112. Mercier-Letondal, P., et al., *Alloreactivity of ex vivo-expanded T cells is correlated with expansion and CD4/CD8 ratio.* Cytotherapy, 2008. **10**(3): p. 275-88.
113. Moolten, F.L., *Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy.* Cancer Res, 1986. **46**(10): p. 5276-81.
114. Haynes, P., T.R. Lambert, and I.D. Mitchell, *Comparative in-vivo genotoxicity of antiviral nucleoside analogues; penciclovir, acyclovir, ganciclovir and the xanthine analogue, caffeine, in the mouse bone marrow micronucleus assay.* Mutat Res, 1996. **369**(1-2): p. 65-74.
115. Ilsley, D.D., et al., *Acyclic guanosine analogs inhibit DNA polymerases alpha, delta, and epsilon with very different potencies and have unique mechanisms of action.* Biochemistry, 1995. **34**(8): p. 2504-10.
116. Fillat, C., et al., *Suicide gene therapy mediated by the Herpes Simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application.* Curr Gene Ther, 2003. **3**(1): p. 13-26.
117. Portsmouth, D., J. Hlavaty, and M. Renner, *Suicide genes for cancer therapy.* Mol Aspects Med, 2007. **28**(1): p. 4-41.
118. Baum, C., et al., *Novel retroviral vectors for efficient expression of the multidrug resistance (mdr-1) gene in early hematopoietic cells.* J Virol, 1995. **69**(12): p. 7541-7.
119. Riddell, S.R., et al., *T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients.* Nat Med, 1996. **2**(2): p. 216-23.
120. Bordignon, C., et al., *Transfer of the HSV-tk gene into donor peripheral blood lymphocytes for in vivo modulation of donor anti-tumor immunity after allogeneic bone marrow transplantation.* Hum Gene Ther, 1995. **6**(6): p. 813-9.
121. Fehse, B., et al., *Evidence for increased risk of secondary graft failure after in vivo depletion of suicide gene-modified T lymphocytes transplanted in conjunction with CD34+-enriched blood stem cells.* Blood, 2004. **104**(10): p. 3408-9.
122. Ciceri, F., et al., *Antitumor effects of HSV-TK-engineered donor lymphocytes after allogeneic stem-cell transplantation.* Blood, 2007. **109**(11): p. 4698-707.
123. Bonini, C., et al., *The suicide gene therapy challenge: how to improve a successful gene therapy approach.* Mol Ther, 2007. **15**(7): p. 1248-52.
124. Winston, D.J., et al., *Randomized comparison of oral valacyclovir and intravenous ganciclovir for prevention of cytomegalovirus disease after allogeneic bone marrow transplantation.* Clin Infect Dis, 2003. **36**(6): p. 749-58.
125. Caruso, M., *Gene therapy against cancer and HIV infection using the gene encoding herpes simplex virus thymidine kinase.* Mol Med Today, 1996. **2**(5): p. 212-7.
126. Biron, K.K., *Antiviral drugs for cytomegalovirus diseases.* Antiviral Res, 2006. **71**(2-3): p. 154-63.
127. Black, M.E., et al., *Creation of drug-specific herpes simplex virus type 1 thymidine kinase mutants for gene therapy.* Proc Natl Acad Sci U S A, 1996. **93**(8): p. 3525-9.
128. Kokoris, M.S., et al., *Enhancement of tumor ablation by a selected HSV-1 thymidine kinase mutant.* Gene Ther, 1999. **6**(8): p. 1415-26.

129. Balzarini, J., et al., *Engineering of a single conserved amino acid residue of herpes simplex virus type 1 thymidine kinase allows a predominant shift from pyrimidine to purine nucleoside phosphorylation.* J Biol Chem, 2006. **281**(28): p. 19273-9.
130. Balzarini, J., et al., *Comparative cytostatic activity of different antiherpetic drugs against herpes simplex virus thymidine kinase gene-transfected tumor cells.* Mol Pharmacol, 1994. **45**(6): p. 1253-8.
131. Balzarini, J., et al., *Superior cytostatic activity of the ganciclovir elaidic acid ester due to the prolonged intracellular retention of ganciclovir anabolites in herpes simplex virus type 1 thymidine kinase gene-transfected tumor cells.* Gene Ther, 1998. **5**(3): p. 419-26.
132. Degreve, B., E. De Clercq, and J. Balzarini, *Bystander effect of purine nucleoside analogues in HSV-1 tk suicide gene therapy is superior to that of pyrimidine nucleoside analogues.* Gene Ther, 1999. **6**(2): p. 162-70.
133. Mullen, C.A., M. Kilstrup, and R.M. Blaese, *Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system.* Proc Natl Acad Sci U S A, 1992. **89**(1): p. 33-7.
134. O'Brien, T.A., et al., *Coexpression of the uracil phosphoribosyltransferase gene with a chimeric human nerve growth factor receptor/cytosine deaminase fusion gene, using a single retroviral vector, augments cytotoxicity of transduced human T cells exposed to 5-fluorocytosine.* Hum Gene Ther, 2006. **17**(5): p. 518-30.
135. Greco, O. and G.U. Dachs, *Gene directed enzyme/prodrug therapy of cancer: historical appraisal and future prospectives.* J Cell Physiol, 2001. **187**(1): p. 22-36.
136. Ostermann, N., et al., *Potentiating AZT activation: structures of wild-type and mutant human thymidylate kinase suggest reasons for the mutants' improved kinetics with the HIV prodrug metabolite AZTMP.* J Mol Biol, 2000. **304**(1): p. 43-53.
137. Sato, T., et al., *Engineered Human tmpk/AZT As a Novel Enzyme/Prodrug Axis for Suicide Gene Therapy.* Mol Ther, 2007. **15**(5): p. 962-70.
138. Spencer, D.M., et al., *Controlling signal transduction with synthetic ligands.* Science, 1993. **262**(5136): p. 1019-24.
139. Thomis, D.C., et al., *A Fas-based suicide switch in human T cells for the treatment of graft-versus-host disease.* Blood, 2001. **97**(5): p. 1249-57.
140. Fan, L., et al., *Improved artificial death switches based on caspases and FADD.* Hum Gene Ther, 1999. **10**(14): p. 2273-85.
141. Grayson, J.M., et al., *Cutting edge: increased expression of Bcl-2 in antigen-specific memory CD8+ T cells.* J Immunol, 2000. **164**(8): p. 3950-4.
142. Straathof, K.C., et al., *An inducible caspase 9 safety switch for T-cell therapy.* Blood, 2005.
143. Tey, S.K., et al., *Inducible caspase 9 suicide gene to improve the safety of allodepleted T cells after haploidentical stem cell transplantation.* Biol Blood Marrow Transplant, 2007. **13**(8): p. 913-24.
144. Introna, M., et al., *Genetic modification of human T cells with CD20: a strategy to purify and lyse transduced cells with anti-CD20 antibodies.* Hum Gene Ther, 2000. **11**(4): p. 611-20.
145. Venditti, G., et al., *NeoR-based transduced T lymphocytes detected by real-time quantitative polymerase chain reaction.* J Hematother Stem Cell Res, 2003. **12**(1): p. 83-91.
146. Verzeletti, S., et al., *Herpes simplex virus thymidine kinase gene transfer for controlled graft-versus-host disease and graft-versus-leukemia: clinical follow-up and improved new vectors.* Hum Gene Ther, 1998. **9**(15): p. 2243-51.
147. Mavilio, F., et al., *Peripheral blood lymphocytes as target cells of retroviral vector-mediated gene transfer.* Blood, 1994. **83**(7): p. 1988-97.
148. Bonini, C., et al., *Safety of retroviral gene marking with a truncated NGF receptor.* Nat Med, 2003. **9**(4): p. 367-9.

149. Hantzopoulos, P.A., et al., *The low affinity NGF receptor, p75, can collaborate with each of the Trks to potentiate functional responses to the neurotrophins.* Neuron, 1994. **13**(1): p. 187-201.
150. Li, Z., et al., *Murine leukemia induced by retroviral gene marking.* Science, 2002. **296**(5567): p. 497.
151. Baum, C., et al., *Side effects of retroviral gene transfer into hematopoietic stem cells.* Blood, 2003. **101**(6): p. 2099-114.
152. Ashkenazi, A. and V.M. Dixit, *Apoptosis control by death and decoy receptors.* Curr Opin Cell Biol, 1999. **11**(2): p. 255-60.
153. Sadelain, M. and I. Riviere, *Sturm und drang over suicidal lymphocytes.* Mol Ther, 2002. **5**(6): p. 655-7.
154. Fehse, B., et al., *CD34 splice variant: an attractive marker for selection of gene-modified cells.* Mol Ther, 2000. **1**(5 Pt 1): p. 448-56.
155. Krause, D.S., et al., *CD34: structure, biology, and clinical utility.* Blood, 1996. **87**(1): p. 1-13.
156. Fackler, M.J., et al., *Full-length but not truncated CD34 inhibits hematopoietic cell differentiation of M1 cells.* Blood, 1995. **85**(11): p. 3040-7.
157. Fehse, B., et al., *A novel 'sort-suicide' fusion gene vector for T cell manipulation.* Gene Ther, 2002. **9**(23): p. 1633-8.
158. Craig, W., et al., *Expression of Thy-1 on human hematopoietic progenitor cells.* J Exp Med, 1993. **177**(5): p. 1331-42.
159. Lemoine, F.M., et al., *Efficient transduction and selection of human T-lymphocytes with bicistronic Thy1/HSV1-TK retroviral vector produced by a human packaging cell line.* J Gene Med, 2004. **6**(4): p. 374-86.
160. Dupuy, F.P., et al., *Lentiviral transduction of human hematopoietic cells by HIV-1- and SIV-based vectors containing a bicistronic cassette driven by various internal promoters.* J Gene Med, 2005. **7**(9): p. 1158-71.
161. Poloso, N.J., et al., *GPI-anchoring of GM-CSF results in active membrane-bound and partially shed cytokine.* Mol Immunol, 2002. **38**(11): p. 803-16.
162. Serafini, M., et al., *Characterization of CD20-transduced T lymphocytes as an alternative suicide gene therapy approach for the treatment of graft-versus-host disease.* Hum Gene Ther, 2004. **15**(1): p. 63-76.
163. Hultin, L.E., et al., *CD20 (pan-B cell) antigen is expressed at a low level on a subpopulation of human T lymphocytes.* Cytometry, 1993. **14**(2): p. 196-204.
164. Del Nagro, C.J., et al., *CD19 function in central and peripheral B-cell development.* Immunol Res, 2005. **31**(2): p. 119-31.
165. Hur, D.Y., et al., *CD19 signalling improves the Epstein-Barr virus-induced immortalization of human B cell.* Cell Prolif, 2005. **38**(1): p. 35-45.
166. Belusa, R., et al., *Changes in Na(+)-K(+)-ATPase activity influence cell attachment to fibronectin.* Am J Physiol Cell Physiol, 2002. **282**(2): p. C302-9.
167. Aints, A., et al., *Enhanced ouabain resistance gene as a eukaryotic selection marker.* Hum Gene Ther, 2002. **13**(8): p. 969-77.
168. Jorgensen, P.L., K.O. Hakansson, and S.J. Karlish, *Structure and mechanism of Na,K-ATPase: functional sites and their interactions.* Annu Rev Physiol, 2003. **65**: p. 817-49.
169. Geering, K., *Functional roles of Na,K-ATPase subunits.* Curr Opin Nephrol Hypertens, 2008. **17**(5): p. 526-32.
170. Blanco, G., *Na,K-ATPase subunit heterogeneity as a mechanism for tissue-specific ion regulation.* Semin Nephrol, 2005. **25**(5): p. 292-303.
171. Xie, Z. and T. Cai, *Na+-K+--ATPase-mediated signal transduction: from protein interaction to cellular function.* Mol Interv, 2003. **3**(3): p. 157-68.
172. Nesher, M., et al., *The digitalis-like steroid hormones: new mechanisms of action and biological significance.* Life Sci, 2007. **80**(23): p. 2093-107.
173. Akimova, O.A., et al., *Search for intermediates of Na+,K+-ATPase-mediated [Na+]i/[K+]i-independent death signaling triggered by cardiotonic steroids.* Pathophysiology, 2005. **12**(2): p. 125-35.
174. Huang, Y.T., et al., *Investigation of ouabain-induced anticancer effect in human androgen-independent prostate cancer PC-3 cells.* Biochem Pharmacol, 2004. **67**(4): p. 727-33.

175. Treschow, A., et al., *OuaSelect, a novel ouabain-resistant human marker gene that allows efficient cell selection within 48 h.* Gene Ther, 2007. **14**(22): p. 1564-72.
176. Berger, C., et al., *Analysis of transgene-specific immune responses that limit the in vivo persistence of adoptively transferred HSV-TK-modified donor T cells after allogeneic hematopoietic cell transplantation.* Blood, 2006. **107**(6): p. 2294-302.
177. Traversari, C., et al., *The potential immunogenicity of the TK suicide gene does not prevent full clinical benefit associated with the use of TK-transduced donor lymphocytes in HSCT for hematologic malignancies.* Blood, 2007.
178. Rosenzweig, M., et al., *Induction of cytotoxic T lymphocyte and antibody responses to enhanced green fluorescent protein following transplantation of transduced CD34(+) hematopoietic cells.* Blood, 2001. **97**(7): p. 1951-9.
179. Deans, T.L., C.R. Cantor, and J.J. Collins, *A tunable genetic switch based on RNAi and repressor proteins for regulating gene expression in mammalian cells.* Cell, 2007. **130**(2): p. 363-72.
180. Murphy, G.J., et al., *Exogenous control of mammalian gene expression via modulation of translational termination.* Nat Med, 2006. **12**(9): p. 1093-9.
181. Yamaizumi, M., et al., *One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell.* Cell, 1978. **15**(1): p. 245-50.
182. van Deutekom, J.C., et al., *Local dystrophin restoration with antisense oligonucleotide PRO051.* N Engl J Med, 2007. **357**(26): p. 2677-86.
183. Walther, W. and U. Stein, *Viral vectors for gene transfer: a review of their use in the treatment of human diseases.* Drugs, 2000. **60**(2): p. 249-71.
184. Cavazzana-Calvo, M., et al., *Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease.* Science, 2000. **288**(5466): p. 669-72.
185. Gaspar, H.B., et al., *Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning.* Mol Ther, 2006. **14**(4): p. 505-13.
186. Baum, C. and B. Fehse, *Mutagenesis by retroviral transgene insertion: risk assessment and potential alternatives.* Curr Opin Mol Ther, 2003. **5**(5): p. 458-62.
187. Baum, C., et al., *Mutagenesis and Oncogenesis by Chromosomal Insertion of Gene Transfer Vectors.* Hum Gene Ther, 2006.
188. Newrzela, S., et al., *Resistance of mature T cells to oncogene transformation.* Blood, 2008. **112**(6): p. 2278-86.
189. Naldini, L., et al., *In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector.* Science, 1996. **272**(5259): p. 263-7.
190. Hildinger, M., et al., *Bicistronic retroviral vectors for combining myeloprotection with cell-surface marking.* Gene Ther, 1999. **6**(7): p. 1222-30.
191. Amendola, M., et al., *Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters.* Nat Biotechnol, 2005. **23**(1): p. 108-16.
192. Donnelly, M.L., et al., *Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'.* J Gen Virol, 2001. **82**(Pt 5): p. 1013-25.
193. Staton, J.M., A.M. Thomson, and P.J. Leedman, *Hormonal regulation of mRNA stability and RNA-protein interactions in the pituitary.* J Mol Endocrinol, 2000. **25**(1): p. 17-34.
194. Wilusz, C.J., M. Wormington, and S.W. Peltz, *The cap-to-tail guide to mRNA turnover.* Nat Rev Mol Cell Biol, 2001. **2**(4): p. 237-46.
195. Garin, M.I., et al., *Molecular mechanism for ganciclovir resistance in human T lymphocytes transduced with retroviral vectors carrying the herpes simplex virus thymidine kinase gene.* Blood, 2001. **97**(1): p. 122-9.
196. Chalmers, D., et al., *Elimination of the truncated message from the herpes simplex virus thymidine kinase suicide gene.* Mol Ther, 2001. **4**(2): p. 146-8.
197. Moreno-Carranza, B., et al., *Transgene optimization significantly improves SIN vector titers, gp91phox expression and reconstitution of superoxide production in X-CGD cells.* Gene Ther, 2009. **16**(1): p. 111-8.

198. Barlogie, B., et al., *Treatment of multiple myeloma.* Blood, 2004. **103**(1): p. 20-32.
199. Bourhis, J.H., et al., *Relapse risk after autologous transplantation in patients with newly diagnosed myeloma is not related with infused tumor cell load and the outcome is not improved by CD34+ cell selection: long term follow-up of an EBMT phase III randomized study.* Haematologica, 2007. **92**(8): p. 1083-90.
200. Brenner, M.K., et al., *Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients.* Lancet, 1993. **342**(8880): p. 1134-7.
201. Brenner, M.K., *The contribution of marker gene studies to hemopoietic stem cell therapies.* Stem Cells, 1995. **13**(5): p. 453-61.
202. Dunbar, C.E., et al., *Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation.* Blood, 1995. **85**(11): p. 3048-57.
203. Junker, K., et al., *Kinetics of cell death in T lymphocytes genetically modified with two novel suicide fusion genes.* Gene Ther, 2003. **10**(14): p. 1189-97.
204. Kokoris, M.S. and M.E. Black, *Characterization of herpes simplex virus type 1 thymidine kinase mutants engineered for improved ganciclovir or acyclovir activity.* Protein Sci, 2002. **11**(9): p. 2267-72.