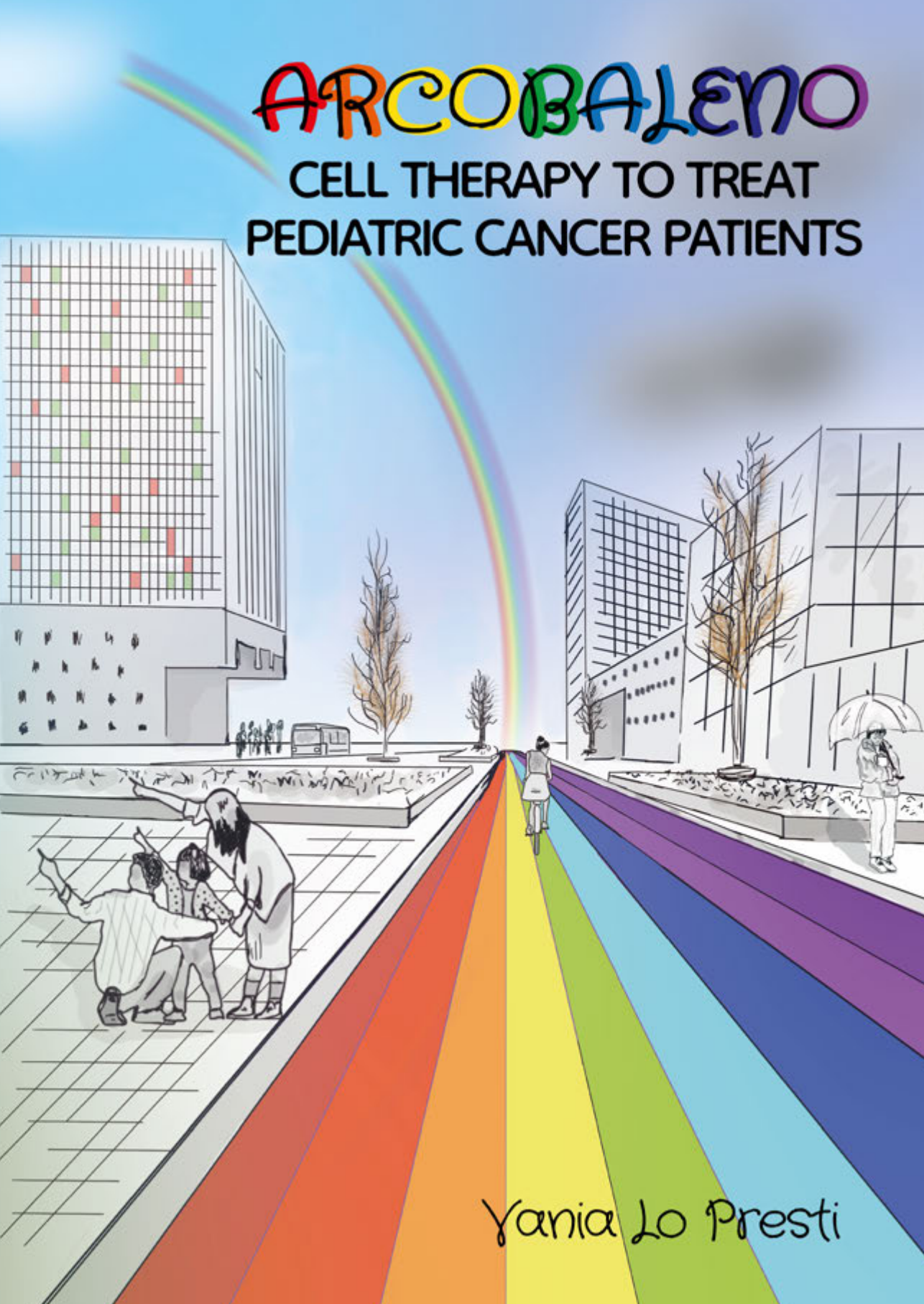
# ARCOBALENO

## CELL THERAPY TO TREAT PEDIATRIC CANCER PATIENTS



Yania Lo Presti

# ARCOBALENO CELL THERAPY
# TO TREAT PEDIATRIC CANCER PATIENTS

**VANIA LO PRESTI**

**ARCOBALENO CELL THERAPY TO TREAT PEDIATRIC CANCER PATIENTS**

Thesis with a summary in Dutch, English and Italian, Utrecht University

ISBN: 978-94-6458-141-6



The research in this thesis was performed at the Unviersity Medical Center Utrecht, Utrecht, the Netherlands. All studies were financially supported by KWF Kankerbestrijding. The copyrights of published articles have been transferred to the respective journals. 

Cover design: Luana Pennestrì and Vania Lo Presti

Layout Design: Vania Lo Presti

Printed by: Ridderprint | www.ridderprint.nl

Printing of this thesis was partially supported by: Infection & Immunity Grad School, GSLS, Utrecht University

# **A**dvancing **R**esearch on **CO**rd **B**lood **AL**logeneic, **EN**gineered and **O**ff-the-shelf cell therapy to treat pediatric cancer patients

**Vooruitgang in onderzoek naar allogene, gemanipuleerde en kant-en-klare celtherapie voor de behandeling van kinderkanker**

(met een samenvatting in het Nederlands)

**Progressi scientifici sull'utilizzo del cordone ombelicale per la produzione di terapie cellulari allogeniche, modificate geneticamente e pronte all'uso per pazienti oncologici pediatrici**

(con riassunto in lingua italiana)

## Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

donderdag 21 April 2022 des ochtends te 10.15 uur

door


## Vania Lo Presti

geboren op 17 mei 1993

te Reggio Calabria, Italië

**Promotoren:**

Prof. dr. J.H.E. Kuball

Dr. S. Nierkens

**Copromotor:**

Dr. N.P. van Til

**Beoordelingscommissie:**

Prof. dr. A.N. Bovenschen

Prof. dr. A.M.M. Eggermont

Prof. dr. J. Haanen

Prof. dr. M.C. Minnema

Prof. dr. L. Meyaard

**Paranimfen:**

Beatrice Lena

Annelisa M. Cornel

Ester Dünnebach

## CONTENT


# 1 GENERAL INTRODUCTION

# 1

Hey there interested reader, happy to see you! I am pretty sure that If you are reading this thesis you fall into one of those categories:

1) You are very curious to know what is the meaning of this long and strange (spoiler: Italian) word that is written in capital letters on the cover

*You will only have to keep reading until the end of this chapter and you will find your answer. However, I hope that, since you are already glancing through this booklet, I will convince you to keep reading about this fantastic topic that has fulfilled my life in the last four and a half years.*

2) You are genuinely interested in the progresses achieved in the field of cancer cell therapy using umbilical cord blood (CB) derived immune cells for the treatment of pediatric cancer patients

*I hope you will have fun, gain some knowledge and will get more and more interested on the great possibilities of using CB as a source for cell therapies, and, more importantly, you will keep moving the field forward.*

3) You are one of the members of my PhD reading/defense committee and you are preparing some interesting questions for the defense day

*I look forward to our discussion!*

4) You are part of my family and closest friends but totally unrelated to the field of biology

*Thank you for taking the time to go through this thesis even though it might be extremely difficult to understand. There are summaries in English, Dutch and Italian starting from page 220 (Chapter &) and the aknoledgment section (ringraziamenti) right after. I hope you will enjoy (at least) those two sections!*

Now, let's see how many people I am able to convince to keep reading!

## FROM CHEMOTHERAPY TO CELL THERAPY: A CENTURY OF DISCOVERIES

When people think about cancer treatment, they most often think of chemotherapy. The mechanism of action of chemotherapy drugs generally relies on highly toxic but non-specific effects on the cell cycle or the DNA. Those type of treatments have been life-savings for many cancer patients since the 1900s, but, even though they drastically improved, they remain non-specific, induce heavy side-effects, and are not always curative; illustrating the need for novel and better treatments[1]. For the treatment of hematological tumors, in 1950s, a revolutionary idea was introduced as part of standard clinical practice: hematopoietic cell transplantation (HCT). HCT relies on using hematopoietic stem and progenitor cells, from a healthy donor, with the aim of replacing the patients' diseased bone marrow with healthy and functional stem cells[2].

HCT became a treatment option for pediatric patients in 1968 when the first patient, diagnosed with a sex-linked lymphopenic immunological deficiency, was successfully treated[3]. Nowadays HCT remains a curative option for many malignant and non-malignant indications, where refractory/relapsed Acute Myeloid Leukemia (AML) and Acute Lymphoblastic Leukemia (ALL) remain leading cause to perform an HCT[4]. However, in both AML and ALL cases, the risk of relapse after HCT occurs in 20-40% of the treated pediatric patients and is considered the most common cause of treatment failure [5–7].

Therefore, there is an unmet clinical need to improve the treatment of relapsed pediatric patients, with the aim to finally increase the event free survival to 100%. Advances in research and understanding the biology of the HCT allowed to improve the efficacy of this therapy and to consider the multifaceted aspects of its success, including the graft versus leukemia (GvL) effect mediated by the immune cells[8]. It started to become clear that the immune system had a central role in recognizing and and eliminating the tumor. A successful anticancer immune response is characterized by a series of steps often referred as the cancer-immunity cycle. The main characters of this cycle are tumor associated antigens (TAA), dendritic cells (DCs) and T cells. More in detail, TAAs get released in the tumor environment, processed and presented by DCs, which in turn activate and prime T cells. The final stage of the cycle occurs when more TAA specific cytotoxic T cells are attracted to the tumor, bind their cognate antigen presented on an HLA-molecule on the surface of tumor cells via the T cell receptor (TCR), and initiate a signal cascade to eradicate the target tumor cells[9].

The goal of cancer immunotherapy strategies is to enforce and potentiate this cycle, to tip the balance towards the immune system.

It has not certainly been an easy journey and researchers have encountered many "inhibitory checkpoints" along the way; but the results achieved in the last decade are changing the way we think of cancer treatment. Acting on the cancer-immunity cycle comprises several approaches, such as the use of peptide-based vaccines, monoclonal antibodies blocking inhibitory pathways or immune cells specifically redirected to the tumor. Among many proposed options some have already been approved by the regulatory agency. Starting in 2011 with the approval of the first monoclonal antibody, *Ipilimumab*, targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) for the treatment of late-stage melanoma[10]; monoclonal antibodies are the frontline new treatment option for many tumors.

However, even more exceptional was the approval, for the first time in history in 2017, of a Chimeric Antigen Receptor (CAR) T cells based product named tisagenlecleucel, commercially known as *KYMRIAH*, for the treatment of refractory/ relapsed pediatric patients with B-cell precursor ALL[11]. This approval signed the beginning of "living" drugs moving towards the clinic. Immunotherapy accounts for almost endless possibilities on how to boost the immune system; of which TCR- T cell therapy and DCs vaccination strategies are the focus of this thesis.

## USE OF CORD BLOOD AS A SOURCE OF IMMUNE CELLS FOR CELL THERAPY APPLICATIONS

Adoptive cell therapy approaches rely on the use of autologous cells as shown in **figure 1A**. However, especially in some cancer types, like leukemia, the number and the fitness of the immune cells might be hampered by the disease itself or by first line standard treatment strategies (e.g. chemotherapy). Moreover, manufacturing of autologous cell therapy products requires complex logistics and therefore a long timeline, often detrimental for patients at the late stage of disease. The option of using an allogeneic source might be a good alternative opportunity for some patients, as shown in **figure 1B** [12,13]. The use of CB as a source of cells for the HCT has for long been in place, especially when patients lack a matched related or unrelated donor,accounting for about 30 to 50% of tumor patients in need of a transplantation[14]. Particularly pediatric patients benefit from the use of CB as an allogeneic source of cells, achieving comparable or, in some studies, better results than bone marrow derived transplantations[15]. CB is known to be highly rich of

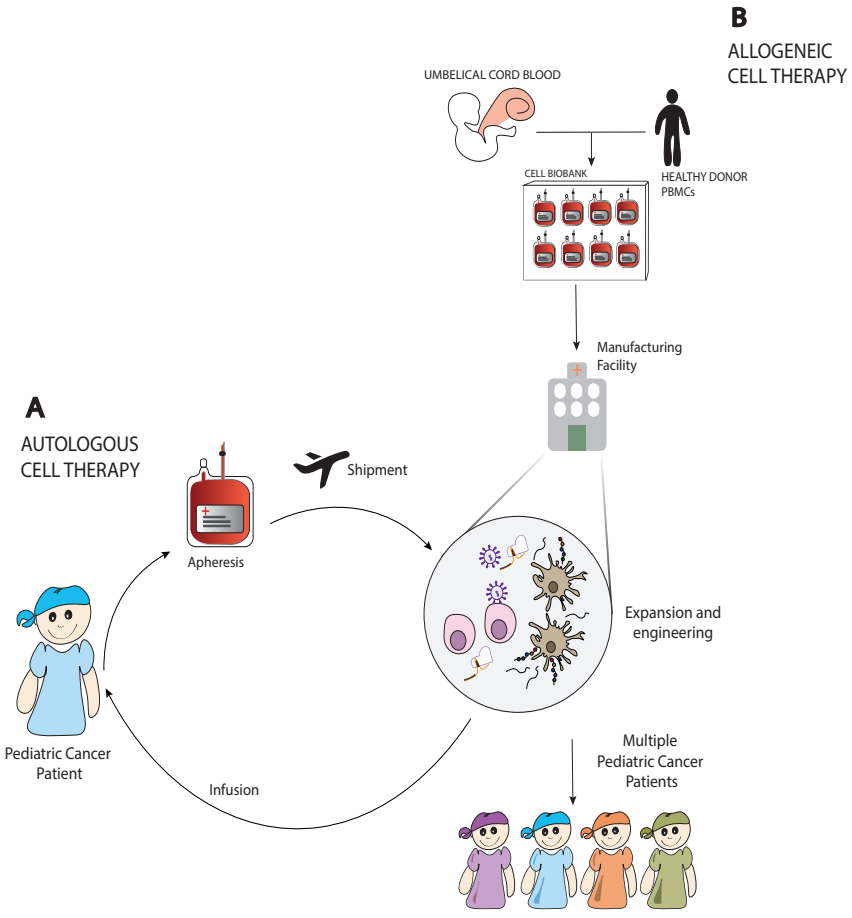



**Figure 1- Figurative explanation of autologous (A) versus allogeneic (B) cell therapy production**

hematopoietic stem cells and naïve immune cells; and it can be stored in biobanks to preserve its cellular composition[16]. Due to its cellular composition CB can also be considered for the generation of adoptive cell therapies. Previously in our group we described a clinically translatable method to generate CB-DCs from CB-CD34+ cells able to stimulate Wilms Tumor 1 specific T cells to prevent relapse after HCT[17].

Moreover, CB is known to be characterized by the presence of high quantity of naïve T cells that have been shown to have an enhanced tumor response compared to PB T cells, in a mouse model of B-cell lymphoma[18]. Adoptive cell therapies approaches derived from naïve peripheral blood T cells have proven to be superior in pre-clinical and clinical studies when compared to peripheral blood T cells, accentuating the possibilities to use CB as a source for cell therapy [19,20]. Even though advantages are clear, very limited studies have used CB derived cells for the generation of cell therapy products for cancer. In addition, unmodified immune cells are not always sufficient to eradicate the tumor, therefore the use of gene-engineering and gene-editing techniques are handy new tools in molecular biology to improve treatment options.

## GENE-AUGMENTATION AND GENE EDITING TO IMPROVE SAFETY AND EFFICACY OF ADOPTIVE CELL THERAPY

Genetic engineering techniques allow integration of exogenous genetic information into the genome of target cells. Gamma-retroviral (RV) and lentiviral (LV) vectors are the most used methods for gene transfer strategies. In both cases the potential of integration of the original virus is maintained, but their replication capacity is completely abrogated; making them suitable for clinical applications[21]. Major concern of using retroviral vectors is the semi-random insertion into the targeted genome of the cells, that could possibly disrupt pivotal genes or have unpredictable insertional mutagenesis consequences; however this problem diminishes when lentiviral vectors are used compared to gamma-retroviral vectors[22]. Retroviral vectors have been of great advantage for the development of T cell based advanced therapies, such as CAR-T cells and TCR-T cells, transferring the genetic information to redirect T cells against the tumor.

More recently, the development of gene-editing techniques, such as CRISPR-Cas9, has further amplified the therapeutic potential of adoptive cell therapy [23]. Gene editing techniques can specifically target the genome of cells and induce a double strand break (DSB) when a specific protospacer adjacent motif (PAM) sequence is present. The DSB is resolved by joining the disrupted genomic DNA using either

random insertion of nucleotide or deletion of bigger parts of genome (commonly known as indels) *via* the non-homologous end joining mechanism (NHEJ) or, when a donor template is present, *via* the homologous directed repair mechanism (HDR). The indels disrupt the gene's frame and therefore abrogate the productive translation of the targeted gene, causing non-sense mediated RNA decay and the so called protein knock-out (KO)[24]. Eliminating the expression of specific proteins can increase both the efficacy and safety of the final products.

The elimination of the endogenous TCR and/or PD-1 has recently been tested in clinical trials with promising results, paving the road to further implementations in the field[25,26]. On the other hand, the presence of an exogenous donor template can stimulate the cells to initiate the HDR mechanism. In fact, instead of using the sister chromatid, it is possible to deliver, together with the CRISPR/Cas9 system, an exogenous DNA molecule. Directly introducing a recombinant sequence in a specific locus has the advantage to increase the safety and the physiological expression of the gene of interest. This method has been lately investigated for generating CAR-T cells and TCR-T cells [27,28.] The most successful approach has shown a 12% of T cells precisely gene-edited to express a tumor specific TCR[29]; suggesting that further improvements are necessary to achieve clinically relevant numbers of cells.

Both gene-augmentation and gene editing techniques have been extensively used during this project both on CB derived T cells and DCs, with the aim to generate the next generation cell therapies.

## SCOPE AND OUTLINE OF THE THESIS

The aim of this thesis is to demonstrate how the use of CB for the generation of allogeneic cell therapy approaches can be an alternative option for pediatric patients. Specifically, the focus will be on the use of CB cytotoxic CD8+ T cells and a CB-CD34+ derived DC vaccine for the treatment of relapsed AML. And moreover, how new gene-editing techniques can further improve the safety and efficiency of the proposed cell therapies.

The first part of the thesis focuses on the possibilities and advantages of using CB as a source for cell therapy approaches. A literature review summarizes in **Chapter 2** the milestone achieved and the future perspective in the field of T cell therapy derived from CB cells when the project started. Back in 2017 the field of cell therapy was overall in its infancy, and CB derived strategies were minimally investigated. Therefore, we highlighted the potential of using CB starting from the HCT settings towards the generation of more advanced cell therapy products, such as CAR-T cells and TCR-T cells. The review ends with a "five-year view" that perfectly well summarizes what we have been experimentally investigated during my PhD trajectory.

**Chapter 3** focuses on how to achieve an optimal transduction efficiency in CB-CD8+ cells for cell therapy applications. More in detail, the paper shows how different methods during transduction have a strong impact on the percentage of transduced cells; and how a new type of transduction enhancer drastically improves the transduction efficiency without affecting viability, proliferation or killing capacity of CB-CD8+ T cells transduced with a tumor specific recombinant TCR (rTCR).

Moving to the other main character of an immunological synapse, **Chapter 4** describes the optimization and validation of good manufacturing practice (GMP)-grade production of a CB-DCs vaccine to prevent relapse after HCT in AML pediatric patients. Importantly, we demonstrate how GMP produced CB-DCs are able to stimulate the proliferation of CB derived WT1-specific T cells, which further have cytotoxic capacity in co-culture with AML cell lines and primary cells.

The second part of the thesis sheds a light on the use of recent gene-editing techniques to enhance safety and efficiency of CB-DCs vaccines and CB- CD8+ T cells.

**Chapter 5** focuses on the generation of a superior CB derived Wilm's Tumor 1 (WT1)-TCR T cell therapy. WT1-TCR engineered T cells have shown promising results to prevent relapse in adult AML patients. Using CB derived cells this manuscript proposes the generation of a WT1-TCR T cell therapy applicable to pediatric patients after CB-HCT. To decrease the possibility of mispairing and competition with the main TCR co-receptors, the manuscript evaluates the consequences of eliminating the expression of the endogenous TCR (eTCR) using CRISPR/Cas9.

Combining the disruption of the eTCR with the expression of a rTCR in a one-step procedure, HDR dependent, would be optimal for the generation of cell therapy products. Therefore, multiple strategies have been explored to optimally deliver a gene template and favor the HDR system after a CRISPR/Cas9 mediated DSB. In **Chapter 6** we describe a proof of concept on how to use integration deficiency lentiviral vectors for this purpose in CB-CD8+ T cells.

TCR engineering strategies have shown promising results for hematological tumors, the field of solid tumors is still struggling with achieve the same results. To further increase potency of CB derived cell therapy products, in **Chapter 7**, we propose an efficient method to eliminate the expression of checkpoint receptors using CRISPR/Cas9, both in CB-DCs and CB-CD8+ T cells.

**Chapter 8** highlights what the current possibilities are to further improve TCR-engineered approaches using gene augmentation and gene editing to better target solid tumors, still considered very difficult targets for immunotherapy.

Finally **Chapter 9** summarize the finding of this thesis, discusses the current developments of the aforementioned advanced therapies and highlights future perspective in the field.

1

# TITLE AND COVER EXPLANATION

As promised, for the people falling in Group 1 that are just waiting to know the meaning of ARCOBALENO (and that in the meantime they decided to read this chapter and not google it). Here a quote to give you more clues:

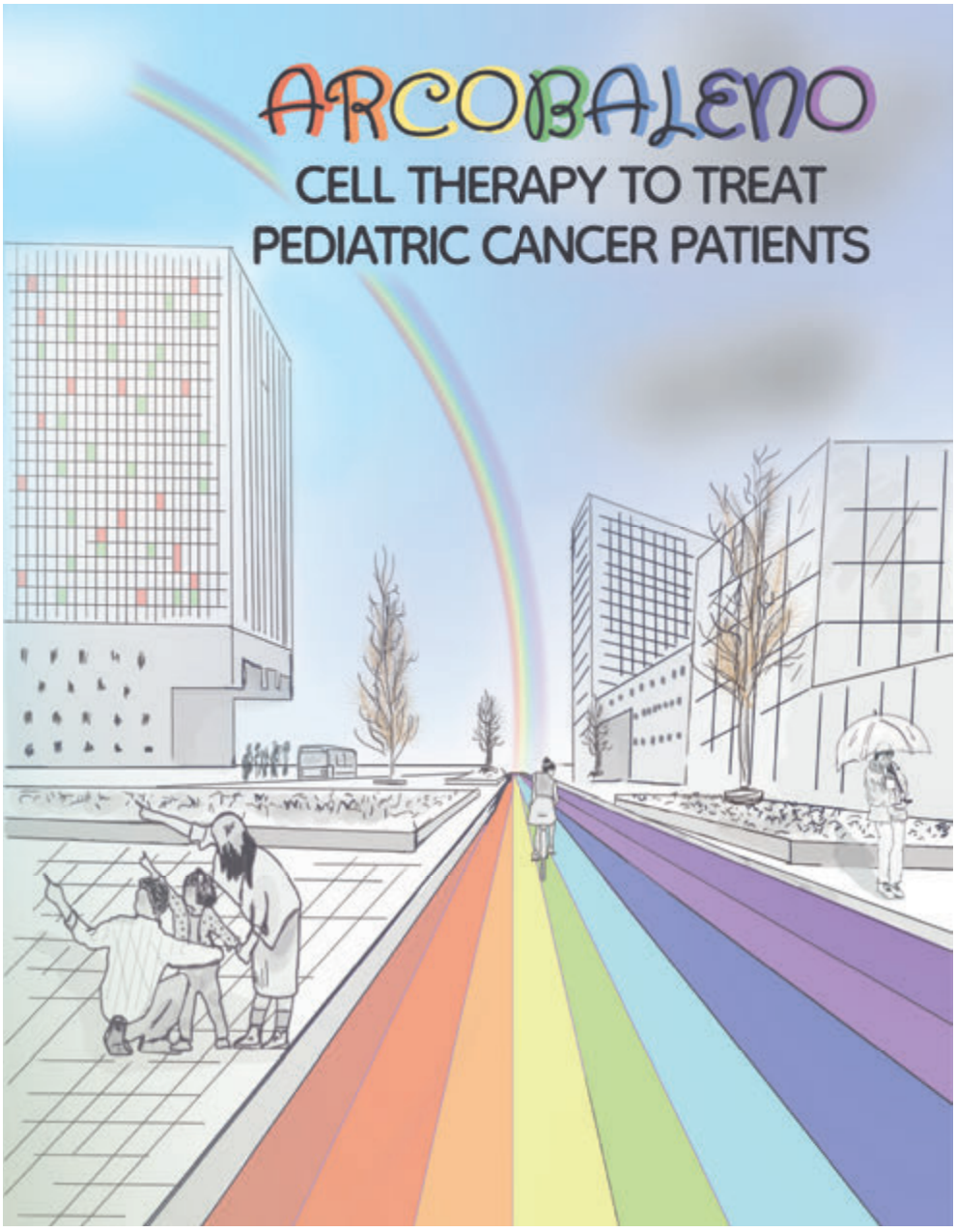
> *"But in the first days of spring, Yuko's writing changed. Little by little his poems became to take on color. [..] His haiku were no longer desperately white. They contained all the colors of the rainbow."*
>
> *Maxence Fermine, Snow*

I am sure you now have enough information to guess that ARCOBALENO in Italian means RAINBOW. And in this thesis it stands for "Advancing Research on COrd Blood Allogeneic, ENgineered and Off-the-shelf" cell therapy to treat pediatric cancer patients.

Every time someone asked the topic of my PhD, there were always some mixed feelings about my answer: everyone acknowledged the noble scope to do research for a better treatment; but a veil of sadness remained, because no one can stop thinking of pediatric cancer patients. That's the reason why, while deciding the title of the thesis, I was looking for an acronym that would give hope more than sadness. A word rich of color. Somehow the Italian word arcobaleno was just perfectly matching my idea and summarizing everything I have done during the PhD. And of course, in this particular case, the pot at the end of the rainbow is filled of a special type of gold: CB derived immune cells!

The funny thing is that once you start thinking of the rainbow, you will see it everywhere, as it happened to me in these last months. I am sure that at least everyone that has recently been at the **Utrecht Science Park** has noticed the rainbow colored bike lane that I have used for my cover, one of the recent symbols of Utrecht Unviersity and its effort towards inclusivity and equality. For every purpose and in every language the use and the idea of a rainbow brings joy and hope. And not to forget, now more than ever, that those are the colors of peace.

I hope that, one day, there will be a wonderful rainbow for EVERY pediatric cancer patient.


ARCOBALENO
CELL THERAPY TO TREAT
PEDIATRIC CANCER PATIENTS

## BIBLIOGRAPHY


1. DeVita, V. T. & Chu, E. A history of cancer chemotherapy. Cancer Res. 68, 8643–8653 (2008).
2. Storb, R. HSCT: Historical Perspective. EBMT Handb. Hematop. Stem Cell Transplant. Cell. Ther. 1967, 3–9 (2019).
3. Gatti, R. A., Meuwissen, H. J., Allen, H. D., Hong, R. & Good, R. A. Immunological reconstitution of sex-linked lymphopenic immunological deficiency. Lancet 2, 1366–1369 (1968).
4. Passweg, J. R. et al. Hematopoietic cell transplantation and cellular therapy survey of the EBMT: monitoring of activities and trends over 30 years. Bone Marrow Transplant. 56, 1651–1664 (2021).
5. Bajwa, R. et al. Outcome of children who experience disease relapse following allogeneic hematopoietic SCT for hematologic malignancies. Bone Marrow Transplant. 48, 661–665 (2013).
6. Rocha, V. et al. Comparison of outcomes of unrelated bone marrow and umbilical cord blood transplants in children with acute leukemia. Blood 97, 2962–2971 (2001).
7. Spadea, M. et al. Multivariate Analysis of Immune Reconstitution and Relapse Risk Scoring in Children Receiving Allogeneic Stem Cell Transplantation for Acute Leukemias. Transplant. Direct 7, e774 (2021).
8. Dickinson, A. M. et al. Graft-versus-leukemia effect following hematopoietic stem cell transplantation for leukemia. Front. Immunol. 8, (2017).
9. Chen, D. S. & Mellman, I. Oncology meets immunology: The cancer-immunity cycle. Immunity 39, 1–10 (2013).
10. Lipson, E. J. & Drake, C. G. Ipilimumab: An anti-CTLA-4 antibody for metastatic melanoma. Clin. Cancer Res. 17, 6958–6962 (2011).
11. O'Leary, M. C. et al. FDA Approval summary: Tisagenlecleucel for treatment of patients with relapsed or refractory b-cell precursor acute lymphoblastic leukemia. Clin. Cancer Res. 25, 1142–1146 (2019).
12. Graham, C., Jozwik, A., Pepper, A. & Benjamin, R. Allogeneic CAR-T Cells: More than Ease of Access? Cells 1–11 (2018) doi:10.3390/cells7100155.
13. Caldwell, K. J., Gottschalk, S. & Talleur, A. C. Allogeneic CAR Cell Therapy—More Than a Pipe Dream. Front. Immunol. 11, 1–12 (2021).
14. Broxmeyer, H. E. et al. Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. Proc. Natl. Acad. Sci. U. S. A. 86, 3828–32 (1989).
15. Eapen, M. et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. Lancet 369, 1947–1954 (2007).
16. Paloczi, K. Immunophenotypic and functional characterization of human umbilical cord blood mononuclear cells. Leukemia 13, 87–89 (1999).
17. de Haar, C. et al. Generation of a cord blood-derived Wilms Tumor 1 dendritic cell vaccine for AML patients treated with allogeneic cord blood transplantation. Oncoimmunology 4, e1023973 (2015).
18. Hiwarkar, P. et al. Cord blood T cells mediate enhanced antitumor effects compared with adult peripheral blood T cells. Blood 126, 2882–2891 (2015).

19. Hinrichs, C. S. et al. Human effector CD8 + T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy. Gene Th 117, 808–814 (2011).
20. Cieri, N. et al. IL-7 and IL-15 instruct the generation of human memory stemTcells from naive precursors. Blood 121, 573–585 (2013).
21. Morgan, R. A. & Boyerinas, B. Genetic modification of T cells. Biomedicines 4, (2016).
22. Gabriel, R., Schmidt, M. & von Kalle, C. Integration of retroviral vectors. Curr. Opin. Immunol. 24, 592–597 (2012).
23. Yin, H., Xue, W. & Anderson, D. G. CRISPR–Cas: a tool for cancer research and therapeutics. Nat. Rev. Clin. Oncol. 16, 281–295 (2019).
24. Hsu, P. D., Lander, E. S. & Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. Cell 157, 1262–1278 (2014).
25. Stadtmauer, E. A. et al. CRISPR-engineered T cells in patients with refractory cancer. Science (80-. ). 367, 1–64 (2020).
26. Lu, Y. et al. Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. Nat. Med. 26, 732–740 (2020).
27. Eyquem, J. et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nat. Publ. Gr. 543, 113–117 (2017).
28. Schober, K. et al. Orthotopic replacement of T-cell receptor α- and β-chains with preservation of near-physiological T-cell function. Nat. Biomed. Eng. (2019) doi:10.1038/s41551-019-0409-0.
29. Roth, T. L. et al. Reprogramming human T cell function and specificity with non- viral genome targeting. (2017) doi:https://doi.org/10.1101/183418.

2

# USE OF CORD BLOOD DERIVED T-CELLS IN CANCER IMMUNOTHERAPY: MILESTONES ACHIEVED AND FUTURE PERSPECTIVES

Vania Lo Presti[a], Stefan Nierkens[a], Jaap Jan Boelens [a,b] and Niek P. Van Til[a]

[a]Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, the Netherlands; [b]Pediatric Blood and Marrow Transplantation Program, University Medical Center Utrecht, Utrecht, the Netherlands

*Expert Review of Hematology, 2018*

## ABSTRACT

**Introduction**: Hematopoietic cell transplantation (HCT) is a potentially lifesaving procedure for patients with hematological malignancies who are refractory to conventional chemotherapy and/or irradiation treatment. Umbilical cord blood (UCB) transplantation, as a hematopoietic stem cell and progenitor (HSPC) source, has several advantages over bone marrow transplantation (BMT) with respect to matching and prompt availability for transplantation. Additionally, UCB has some inherent features, such as rapid expansion of T cells, lower prevalence of graft-versus-host disease and higher graft versus tumor efficacy that make this HSPC cell source more favorable over other HSPC sources.

**Areas covered:** Here, we summarize the current CB and CB derived T cell applications aiming to better disease control for hematological malignancies and future directions to more effective therapies.

**Expert commentary:** UCB transplantation could be used as a platform to extract cord blood derived T cells for ex vivo expansion and/or gene moification to improve cellular immunotherapies. In addition, combining cord blood gene-engineered T cell products with vaccination strategies, such as cord blood derived dendritic cell based vaccines may provide synergistic immunotherapies with enhanced anti-tumor effects.



**Keywords**

Chimeric antigen receptor

Dendritic cells

Hematological malignancies

Immunotherapy

T cell receptor

Umbilical cord blood T

## 1. INTRODUCTION



Despite major improvements in the treatment and care of cancer patients in the last decades, cancer is still a disease with a critical unmet need for more efficient therapies. But for many malignancies, chemo- and radiotherapy are still the main first line treatment, which are often not sufficient to cure the patient, e.g. in aggressive forms of hematological malignancies, where the second option for curative treatment is hematopoietic cell transplantation (HCT)[1]. HCT transplantation allows the replacement of patient hematopoietic system by long-term reconstitution of a donor-derived hematopoietic system. Successful HSPC transplantation requires a human leukocyte antigen (HLA) matched donor, in first instance a sibling, which is only available for ~30% of patients[2]. Because matched donor cells are often not available, unrelated or haplo-identical donor cells are often selected, which can be retrieved from mobilized apheresis peripheral blood, bone marrow or cord blood.

Although allogeneic transplantation provides higher survival chances for chemo-resistant high risk hematological disorders, overall survival rate remains suboptimal (20-70% depending on disease/status)[3]. Hence, there remains an unmet need to further improve the survival chances. The availability of immune therapies has increased impressively in the last few years, which include adoptive cellular immunotherapy. Chimeric antigen receptor (CAR)-T cell therapy has entered clinical care in an increasing number of centers and some trials explore the potency of tumor-infiltrating lymphocytes in an autologous setting. Acquiring and expanding T cells from patients is technically demanding and clearly has its limitations. Alternatively, the use of ex vivo gene-modified T cells is a promising strategy, especially for treatment of hematological malignancies. Applications based on cord blood as a source to develop effective T cell immunotherapy are particularly interesting as it provides the use of adoptive T cell therapy in the context of cord blood HCT where the T cell product is being generated from the same graft used for transplantation. We here describe the possibilities of this approach.

## 2. USE OF CORD BLOOD AS A SOURCE FOR HEMATOPOIETIC STEM AND T CELL TRANSPLANTATION FOR HEMATOLOGICAL MALIGNANCIES AND IMMUNOTHERAPY



Cord blood (CB) is derived from the placenta and umbilical cord after childbirth. It has a different composition than peripheral blood and bone marrow (BM)[4], with more enriched primitive HSPCs[5] and a comparable proliferative capacity to HSPC-BM [6,7]. CB contains mainly naïve T cells[4], that can undergo rapid peripheral expansion with memory-effector differentiation within 4 weeks[8].

CB units have been stored in public or private UCB banks [9], and are promptly available for transplantation, and it allows to use HLA mismatched unrelated donor cells up to 4/6 (high resolution (HR) typing) instead of 10/10 for bone marrow transplants, with reduced risk to develop graft versus host disease (GVHD)[10]. In the early days of UCBT, one of the main issues was the slower and delayed hematopoietic recovery and immune reconstitution that increased the possibility of infection-related mortality [11]. However, it has been shown that individualized dosing and timing of chemo- and/or serotherapy, such as anti-thymocyte globuline (ATG), can prevent rejection as well as GVHD and improves immune reconstitution. Low dose exposure of ATG improves T cell reconstitution, in particular CD4+ T cell recovery[12,13], which has been reported as a predictor for viral reactivations[14,15]. The CD4+-biased T-cell immune reconstitution, may be dictated by the distinct transcription profile similar to fetal CD4+ T cells[16], which are different from peripheral blood T cells. In an another study, CB CD4+ T cell characteristics were distinct from PB T cells in CD26 expression, and could produce higher interferon-ɣ and interleukin-5 expression after T helper (Th) type 1 and Th2 induced skewing[17]. These phenotypically distinct profiles may contribute to less viral infections even in the presence of low doses of ATG. Furthermore, the probability of relapse was found to be lower (40 vs 20%) after CB transplantation compared to the other groups[18].

However, although overall T-cell immune reconstitution in children is comparable after BMT or CB transplantation, CD8+ T-cell reconstitution is faster after BMT, whereas regulatory T cells (Tregs) and CD4+ T-cell recovery are faster after CB transplantation[3,19]. CD8+ T-cells have been shown to exhibit stronger proliferation and function after antigen-specific stimulation[20].

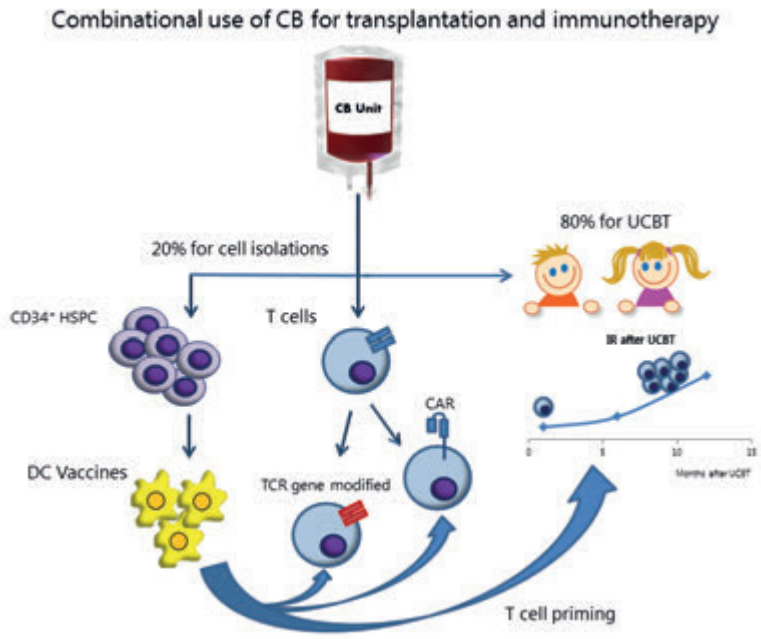



**Figure 1 - Combinational cellular immunotherapy based on umbilical cord blood.**

Schematic representation to combine cord blood transplantation (CBT) with immunotherapy products derived from the same cord blood unit. The remaining 20% of a CB unit that is not transplanted can be used to develop CD34+ derived dendritic cells (DCs) or TCR gene-engineered T-cells. These strategies can also be used as mono-therapy or combined to enhance the immunotherapeutic effect.

Even though CB can clearly provide advantageous responses in cancer treatment by inducing robust graft-versus-leukemia (GVT) responses[21] low numbers of tumor reactive T cells, especially during the early stages of immune reconstitution, still contribute to a considerable risk to develop relapses and reduced overall survival. To reduce these risks and provide lifelong curative therapies, the use of gene-modified T cells from CB is a promising strategy especially for treatment of hematological malignancies.

Because a residual 20% of a cord blood is not infused into the patient this can be used to generate a matched product by extracting cells for genetic modification to target tumor cells. T cell priming of reconstituting cord blood derived T cells or gene-engineered T cells can also be targeted in vivo by ex vivo engineered antigen-presenting cells from the same CB unit. This all underlines the potency to use of CB as a platform to develop adoptive T cell therapies in a CB transplantation setting or as off-the-shelf gene therapy products, which could also be used to treat patients with solid tumors (Figure 1).

# 3. GENE-ENGINEERED T CELLS

## 3.1 CORD BLOOD DERIVED CAR-T CELL IMMUNOTHERAPY

Chimeric antigen receptor (CAR)-T cells are engineered T cells containing antibody domains coupled to co-stimulatory T cell specific co-stimulatory domains through a transmembrane domain[22]. Those cells are able to recognize surface antigens in a major histocompatibility complex class I (MHCI)-independent manner[23]. The production of CAR-T cells has been largely improved over the recent years. The first generation CAR-T cells were characterized by the presence of the antibody domain linked to the CD3-ζ transmembrane domain of TCR receptors. Second generation CAR-T cells are equipped with additional co-stimulatory motifs for T cell signaling, such as CD28 or 4-1BB[24]. However, the presence of 4-1BB seems superior, due to its ability to induce a central memory phenotype to T cells that persist longer (over two years) than solely incorporating a CD28 domain (~30 days). The latest generation is called third generation CAR-T cells, combining more than one co-stimulatory domain to the CD3-ζ transmembrane motif (ref. Sadelaine- The basic principles of CAR design). This could be of particular interest for cord blood T cells, because 4-1BB and CD28 signaling play a synergistic role in redirecting UCB T cells against for instance B-cell malignancies[25].

The use of CAR-T cells is a promising strategy, that has recently resulted in significant successes in clinical trials targeting CD19 in chemo-refractory and relapsed B cell malignancies, particularly for acute lymphoid leukemia (ALL)[26,27]. CD19 is a B-cell specific surface antigen, highly expressed by the majority of B cell malignancies (80% of ALL, 88% of B cell lymphomas and 100% of B cell leukemias). CD19 it is also expressed on healthy B cells, which will be eliminated as well[28]. This could also play a role in the effectiveness of the therapy, because the CD19 CAR-T cells are strongly triggered due to the presence of high number of B cells, creating a strong immune response, also against the CD19 positive lymphoblasts. The loss of functional B cells can be counteracted by life-long immunoglobulin infusions. The FDA has recently approved the commercial use of Kymriah (Tisagenlecleucel), the first cell and gene-therapy based drug ever approved for the treatment of acute lymphoblastic leukemia (ALL)[29].

To generate CAR T cells, CB T cells have advantages over the use of PBMCs, because they can be rapidly expanded and genetic modification of CB derived T cells could therefore be an effective approach. CB-derived T cells are amenable for transduction by lentiviral vectors to introduce CAR or recombinant TCRs. Several

groups have produced impressive data regarding the efficacy of CD19-specific CAR CB derived T cells proving the cytolytic effect of CB derived and modified T cells both in vitro and in vivo in models of B-lineage acute lymphoblastic leukemia (B-ALL)[30,31]. Furthermore, in an ongoing clinical trial conducted by MD Anderson Cancer Center in Houston, Texas, gene-modified CB derived T cells containing a CAR against CD19 were infused in patients with B-lineage lymphoid malignancies after umbilical cord blood transplantation (NCT01362452), providing evidence of the potential of CB T cells for cancer immunotherapy and especially in combination with UCBT.



## 3.1 POTENTIAL USE OF T CELL RECEPTOR ENGINEERING FOR CORD BLOOD DERIVED T CELL IMMUNOTHERAPY

T cells use their T cell receptor (TCR) to recognize antigens of intracellular or extracellular proteins presented by MHCI or II on target cells. The first required signal for an effective T cell response is TCR activation followed by a series of co-stimulatory signals essential for the activation of intracellular pathways towards sustained responses. Tumor associated antigens (TAAs) that are selectively and/or overexpressed on malignancies can be used as target by engineered T cells with high affinity TCRs.

Hiwarkar *et al.* [16] demonstrated in a mouse model of B-cell lymphoma, the advantage of using CB derived T cells to increase graft versus tumor (GVT) effects significantly. Mice receiving CB T cells showed a reduction in the tumor growth that eventually resulted in complete regression. On the contrary, tumor growth continued in mice treated with PB T cells and untreated mice. No signs of GVHD were mentioned for up to 60 days in CB T cell transplanted mice, but in PB T cell transplanted mice severe signs of GVHD were observed three weeks after transplantation[16]. Effective therapy of CB T cells were also achieved by Lee *et al.*[32] in two mouse models of cervical and lung tumors. In those animal models, mice injected simultaneously with tumor cells and CB T cells did not develop any tumors as compared to control mice without CB T cells that showed normal tumor progression. Both these studies strongly supported that CB-derived T cells can eliminate cancer cells efficiently after infusion in tumor animal models.

The first clinical trial using TCR engineered T cells was published by Morgan *et al.*[33] in 2006 to treat melanoma patients, followed by trials for other types of solid tumors, such as esophageal, synovial sarcoma and colorectal cancer[33]. However, efficacy and safety of these therapies is often still suboptimal. There are intrinsic problems to some TCRs itself, such as low affinity towards the TAA, which could be

increased by modification in the amino acid composition of the crucial TCR-antigen-binding region[34]. Another problem has been that gene-modified TCR lose efficiency by mispairing with endogenous TCR α and β chains, whereby surface expression of the introduced TCR is decreased, and unwanted TCR reactivity against self-antigens can occur. This has been shown to risk lethal graft-versus-host disease in mouse models[35], which can be reduced by modifications that affect pairing between recombinant and endogenous TCRs, such inclusion of cysteine residues in the constant region of α and β chain[36] or by replacing human with mouse aminoacids in the constant domains [37]. The inherent nature of cord blood T cells to have reduced GVHD risk may also improve side-effects induced by mispairing of the introduced TCR with the endogenous TCRs. TAA-specific cytotoxic T cells (CTLs) were generated against multi-leukemia antigens, e.g. Wilms tumor 1 (WT1), human neutrophil elastase (NE) and melanoma-associated antigen A3[38].

It would be interesting to investigate if these TAA-specific CTLs could also be generated from cord blood T cells, because certain frequencies of TAA-specific T cells, in this case against PR1, are higher in cord blood than in adult blood[39]. Furthermore, engineered TCRs can also be used to target virus specific antigen. Indeed, virus reactivation is one of the cause of death after HSPCT [14]. CB derived T cells against cytomegalovirus (CMV), Epstein Barr virus (EBV) and adenoviruses could be expanded in culture and reinfused to protect against reactivation of viruses. Several groups have shown that CB T cells can be robustly expanded ex vivo by using CD3/CD28 beads and cytokines, such as IL-2, IL-7 and IL-15 [40]. By using IL-7 and IL-15 naïve T cells could be directed towards long-living memory stem T cells (Tscm), that have the ability to self-renew and ability to turn into potent T cell effectors [41], which could be an excellent source to generate gene-engineered T cells. Hanley *et al*. demonstrated that in vitro priming of CB-T cells with APCs provided recognition of all three above mentioned virus antigens, but CB-T cells showed to have a larger pool of unconventional CMV epitopes compared to adult derived T-cells [42]. This strategy has been used recently in a clinical trial (NCT00078533) to increase the immunity against virus reactivation and the overall survival of patients. The CB-T cells were reinfused 30 days after the HSPC transplantation. Results have not been published yet. Cord blood T cells lines could also be generated that recognized multiple common viruses and at the same time provide antileukemic activity through the expression of a CAR targeting CD19, without causing GVHD[43].

Since most of the preclinical work based on recombinant TCR or CAR technology has been performed in PBMCs, more comparative work is required to test whether enhanced effects may be incurred by cord blood derived gene therapy products.

### 3.3 GENERATION OF GENE MODIFIED T CELLS FROM CORD BLOOD CD34+ PROGENITOR CELLS

Another clinical approach of interest has been to obtain tumor-reactive T cells through in vitro generation of T cells, but this method has long been very inefficient. Culturing cord blood hematopoietic precursors on OP9 stromal cells that expresses Notch human ligand Delta-like1 showed that functional T cells with controlled antigen specificity in an human leukocyte antigen (HLA) restricted manner could be obtained after transduction with TCR specific retroviral vector [44]. In another similar study, these cells also acquired additional natural killer cell-like killing of tumor cell lines. Furthermore, the in vitro propagation of retroviral TCR transduced CD34+ cord blood derived T cells also displayed little endogenous TCR expression with the tumor-reactive TCR highly expressed on the surface [45].

More recent reports show that human cord blood T cells could also be efficiently generated from HSPCs specific for cytomegalovirus (CMV) or Influenza-A virus epitopes without direct stromal co-culture or retroviral TCR transduction [46], which was also a tool to generate tumor-specific T cells against the antigen dopachrome tautomerase (hTRP-2)[47]. Using gammaretroviral vectors to introduce CAR or TCRs in CB CD34+ progenitor cells, van Caeneghem *et al.*[48], demonstrated that endogenous TCR expression could be eliminated in most of the CD34 progenitor derived T cells, which could make this an excellent method to generate universal T cell products. Since cord blood HSPCs are highly amenable to lentiviral transduction[49] , this may increase overall production of gene-modified tumor-specific T cells generated by this method.

## 4. IN VIVO PRIMING OF CB T CELLS: COMBINATIONAL THERAPY STRATEGIES

Priming of CB T cells could also be done directly in vivo by dendritic cells (DCs), which are an important determinant in the initiation of adaptive immune responses. These cells internalize antigens and present them to T cells, priming their activation[50] and mount CD4+ and CD8+ specific responses that can be used for cellular vaccines[51]. In fact, peripheral blood monocyte derived, or primary DCs are used in several clinical trials and results suggest that patients are tolerating the infusion without showing severe side effects and with a good rate of short-term antitumor response. Most of the clinical trials are well summarized by Benteyn *et al.*[52].

However, this strategy requires a relatively high number of TAA-specific T cells present in the cancer patients to be efficient, which is usually not the case in hematological cancer patients after conditioning and subsequent HSPC transplantation. It takes time after a cord blood transplant for sufficient peripheral T cell expansion to occur and even longer to acquire sufficient numbers of T cells through thymic development. It can take months to obtain normal numbers of peripheral T cells. However, considering that DC vaccines can be used to stimulate CB derived and engineered T cells, this could be an interesting strategy for a combinational therapy to boost proliferation towards more tumor-reactive CB T cells.

This is even more interesting, because DCs vaccines can be generated from CB CD34+ progenitors. These cells have particularly shown efficient expansion properties and effective maturation into TAA specific DCs. De Haar *et al.*[53] developed a CB derived DC vaccine targeting Wilms' tumor 1 (WT1) positive acute myeloid leukemia (AML)-blasts, which is an antigen overexpressed in the majority of AML patients. The CB derived DCs were able to efficiently migrate and activate WT1 specific T cells. We can speculate that this cellular vaccine should be able to boost both naturally occurring WT-specific T cells derived from cord blood as well as WT-specific engineered T cells. This strategy assures a perfectly matched product utilizing the same CB unit for both the transplanted donor cells, the produced DC vaccine and the CB engineered T cells.

CB derived T cells can be primed against TAAs. Decker et al. demonstrated that chronic lymphocytic leukemia (CLL) patients-derived antigen presenting cells expressing CD40L are sufficient to prime CB T cells ex vivo, turning them into efficient cytotoxic lymphocytes (CTL) able to kill CLL cells in vivo[54].

This shows the possibility to combine T cell and DC vaccine approaches base on a CB platform to mount superior immune responses against tumor antigens.

## 5. STRATEGIES TO IMPROVING THE EFFICIENCY OF CB-T CELL THERAPY

To obtain a highly efficient immunotherapy product based on cord blood T cells, fine tuning of the specific molecular modifications related to tumor antigen recognition are required but not sufficient. Experience in the field of T-cell immunotherapy demonstrated that some of the most promising in vitro products were not able to achieve the same efficiency in vivo.

The interaction between TCR and MHCI/peptide complex is by itself not enough to activate the downstream pathways to efficiently eradicate cancer cells and provide long-term memory. This requires sufficient co- stimulatory activation and no deleterious co-inhibitory effects. The expression of inhibitory signals has been shown to induce T cell exhaustion, senescence and anergy. One of the most studied inhibitory pathway involves the interaction between programmed cell death protein 1 (PD-1), expressed on T cells, and programmed cell death protein ligand 1 (PD-L1), usually expressed by APC or DCs, but often overexpressed on cancer cells[55]. The expression of PD-L1 has been described as a key factor limiting in the efficacy of adoptive cell therapies. Of note, the frequency of PD-1+ CD8+ T cells was significantly higher in patients transplanted with UCB and that experienced leukemic relapse, so there is a rationale to target this specific checkpoint inhibitor[56]. Another checkpoint inhibitor is cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), which is another negative regulator of T cell immune function[57]. Therapies based on the use of blockade antibodies against those proteins lead to the suppression of the inhibitory pathway, increasing the survival and efficacy of CTL against the tumor[58]. In a specific cord blood humanized mouse model of Epstein-Barr virus (EBV)-induced lymphoma growth PD-1/CTLA-4 blockade markedly increased EBV-specific T cell responses, and enhanced tumor infiltration by CD4+ and CD8+ T cells[59].

In other mouse models using anti-Her-2 CAR T cells PD-1 blockade by anti-PD-1 antibodies boosted immune tumor-specific immune responses [60]. Instead of providing multiple checkpoint inhibitor antibody infusions, which also risk associated toxicities, T cells could be directly modified by gene-editing co-inhibitory genes. Transcription activator-like effector nuclease (TALEN)-mediated elimination of PD-1 expression on melanoma-reactive CD8+ T cells and in fibrosarcoma polyclonal T cells increased the persistence of T cells at the tumor site and increased tumor control[61]. In another study, CRISPR/Cas9 was used to eliminate PD-1 expression in CD19 CAR-T cells, which enhanced CAR T cell function significantly shows the potential of these applications[62], which could also be applied on cord blood T cells. Other checkpoint inhibitors may also be of interest depending on its expression on tumor or gene-modified T cells. Although more proof-of-principle studies are reported on the use of gene-editing systems, such as CRISPR/Cas9, since it is relatively novel in this field, identifying off-target cleavage events by genome-wide non-biased screening is important [63], as well as developing efficient Cas9 nucleases with less off-target cleavage sites[64]. This could particularly pose a risk in multiplexing gene-modified T cells.

Another approach to generate a universal off-the-shelf immunotherapy products is to eliminate endogenous TCRs, independent of MHC expression in the host, has been tested a few years ago by using zinc-fingers to target the α and β constant domains of TCRs[65]. These initial methods using zinc-fingers had relatively low efficiency. Another approach that followed was the use of TALEN to engineer CD19 T cells[66] and to eliminate expression of TCRα to make to product universal and delete CD52 to protect the cells from alemtuzumab treatment. These non–human leukocyte antigen–matched donor peripheral blood mononuclear cells (PBMCs) were infused into two infants with relapsed refractory CD19+ B cell acute lymphoblastic leukemia that received lymphodepleting chemotherapy and alemtuzumab serotherapy to successfully bridge to allogeneic stem cell transplantation[67].More recently, effective CRISPR/Cas9 mediated insertion of a CD19-specific CAR into the TCRα constant (TRAC) locus resulted in uniform expression and also enhanced T cell potency compared to conventionally generated CAR T cells, showing promising prospects for gene-edited CAR T cells products [68].

Of note, the production of universal TALEN gene-edited CAR T cells by CliniMACS magnetic bead selection for TCRαβ T cells still resulted in approximately 1% TCRαβ positive T cells that were infused in two B-ALL patients [67]. In one of these patients grade 2 skin GVHD was confirmed, derived from the small infused TCRαβ positive T cell population. The use of CB derived T cells as a source to make universal CAR T cells may further lower the risk of GVHD.

## 6. CORD BLOOD T CELL IMMUNOTHERAPY: OPPORTUNITIES AND LIMITATIONS

Although immunotherapy is showing promising results, there are still several problems that have not been addressed. In primis, the success achieved with those strategies in hematological malignancies has not been reproduced for solid tumors. TCR engineered T cells can be strongly affected by the downregulation of MHC class I, a strategy often adopted by tumor cells in order to avoid immune surveillance[69]. Martini *et al*. also demonstrated that MHCI expression can be increased by use of IFNɣ in a mouse model of prostate cancer[70], but the translation can be risky, considering that IFNɣ can be toxic. On the other side, since CAR-T cells are HLA independent, this could be an advantage for the treatment of solid tumors. Lately, clinical trials using CARs are also focusing on other malignancies, such as prostate and pancreatic cancer, glioma, neuroblastoma and sarcoma[33,71,72].

However, up to date, the two most positive trials reported are one targeting neuroblastoma, with three of eleven patients with complete remissions[73], and sarcoma, with four of seventeen patients showing stable disease[74]. Those limited results are mostly due to the immunosuppressive microenvironment, which is difficult to reach and attack by T cells[72]. Two other major problems are on-target[75] and off-target toxicity[76] with a risk of severe multi-organ failure. Optimized dosing after infusion of those cellular gene-modified products for predictable pharmacokinetics and pharmacodynamics is still largely unknown, but will be required to obtain maximal responses, but also to reduce the observed side effects and toxicity[77]. The most serious side-effect observed in CAR-T cell therapy was cytokine release syndrome (CRS), experienced by almost all the B-ALL patients treated[78]. Fortunately, CRS can be controlled with anti-IL6R antibodies to reverse the symptomatology, but the patients need to be strictly monitored[79].

Another problem is the manufacturing process of peripheral blood TCR or CAR T cells, which is generally slow and expensive but also results in batch to batch variability. Currently, a lot of effort is invested in optimizing the manufacturing process by automating the enrichment, transduction and expansion of gene-modified T cells to improve quality and reduce costs[80]. Automated manufacturing of immunotherapy products in closed systems should widen applicability also for cord blood off-the-shelf products[81], especially considering the high availability of CB unit stored in the CB biobanks.

Cord blood units could be potentially selected containing high numbers of T cells or specific subsets to be prepared for off-the-shelf products. The number of T cells in a cord blood unit may be limiting, and ex vivo expansion to create larger numbers may aid in efficacy of treating cancer. Additionally, storage of small numbers of T cells needs to be assessed to provide high quality recovery of for infusion into patients. Efficient recovery of functional HSPCs from long stored cord blood units have been reported (21-23.5 years)[82]. In addition, Domogala et al. demonstrated that cryopreservation has no effect on natural killer cells expanded in vitro from CB-CD34+[83]. Moreover, functional CB derived T cells can be recovered, but these frozen products contain apoptotic cells due to the freeze/thaw procedure. It has been demonstrated the overall percentage of viable T cells can be increased in cultures containing IL-7 during expansion[84]. In a transplantation setting, T cells can be derived from the 20% portion and gene-modified and expanded. For off-the-shelf purposes, a whole CB unit can be used to obtain a larger pool of CB derived T cells.

In order to improve the efficiency of T cell immunotherapy for the treatment of solid tumors it may also be important to increase homing to the tumor niche. In the first steps of this process, T cells may be hampered to efficiently home to the tumor environment due to interference with attractive chemokines, i.e. mainly chemokine (C-X-C motif) ligand (CXCL) 9,10 and 11[85] and/or production of vascular endothelial growth factor (VEGF) [86,87]. An example to improve trafficking and infiltration is supplementation of attractive chemokines or use VEGF inhibitors[88,89]. Additionally, lack of MHC I, as mentioned before, can be crucial in determining the efficiency of those therapies. For example, when the downregulation of MHC I is due to epigenetic modification resulting in DNA hypermethylation, the expression of the protein is suggested to be reversed using histone deacetylase (HDAC) inhibitors, such as Vorinostat[69].Consequently, it is also important to modify the immunosuppressive tumor microenvironment, characterized by inhibitory cells or soluble factors released by the tumor, able to decrease the efficiency of T cells immunotherapy products [72,90,91].

For future implementations better understanding of the environmental tumor biology is important to identify adjuvant strategies for the most optimal effect of CB-T cell immunotherapeutic products. CB-derived T cell therapies can be used in the HCT transplantation setting for treatment of hematological malignancies in the minimal residual disease stage to provide immunological memory to fight future relapses. In this case the T cells can be generated from the graft to ensure physiological memory formation. Alternatively, T cells can be used as an add-on cellular therapy to treat patients with progressed or established relapsed disease. For the latter, off-the-shelf cell therapies are an attractive possibility.

## 7. CONCLUSION

We discussed the developments of state-of-the-art T cell immunotherapy using CB cells, which are relevant to cord blood T cell applications aiming for better disease control in patient with malignant indication. Especially for gene modification of T cells, there is a clear need for improvement before developing it into a standard therapeutic practice. We focused on the advantages of using CB derived T cells above other cell sources as target cell for genetic modification for the future cell therapy products. The unlimited availability, making manufacturing of off the shelf products easier, the highly proliferative capacity and naïve phenotype makes these cells of special interest above other cell sources. In addition, it makes it easier to create standardized products.

Primarily it is of particular interest for hematological malignancies in combination with HCT transplantation, but also manufacturing of off the shelf products is of interest. Optimization of automated protocols should decrease batch to batch variability and improve the quality of the gene-therapy products. The presence of large CB biobanks provides an opportunity to also create effective "off-the-shelf" immunotherapeutic products, ready to be rapidly infused when required, and which can also be used for non-hematological related malignancies. To obtain improved immunotherapy products, homogeneity between hospital centers, cohorts, and especially manufacturing strategies needs to be optimized.



Combining immunotherapeutic gene-modified products can increase the chance of overall survival by consequently decreasing relapse rate and infection related mortality. However, more comparative studies are required, as well as further confirmation and optimization of its potential applications, with the aim to develop superior "off the shelf" CB derived immunotherapy products.

## 8. EXPERT COMMENTARY

Optimal immune reconstitution is required to prevent relapses and improve overall survival of patients with hematological malignancies after HST. This underlines the importance of personalized dosing of conditioning drugs and cellular immunotherapy products to provide optimal immune reconstitution. Optimal tuning of immunomonitoring should assist in the development of therapies that target leukemic cells, in particular combinational therapies which are required for effective anticancer treatments to prevent minimal residual disease, relapses and completely eradicate all tumor cells. This goal could be reached with the use of immunotherapy products consisting of engineered T cells derived from the peripheral blood of patients. The gene-therapy field has made considerable advancements in the last decade, which has resulted in gene-vehicles that can efficiently deliver CARs and TCRs to T cells to provide specific antitumor responses. This strategy has already been investigated especially for eradicating CD19+ ALL. However, the manufacturing process for producing personalized cellular drugs is still far from optimized and has not given standardized results yet.

The use of cord blood T cells as targets for gene-modified immunotherapy products have advantages, because of their potential expansion and reduced risk of graft versus host disease. This may provide safer gene-therapy products when recombinant TAA-specific TCRs are introduced. It is of particular interest that the research field has advanced in the generation of T cells derived from cord blood

CD34+ progenitor cells. Since these cells maintain their TCRαβ germline loci after introduction of a recombinant TCR, this also significantly reduces TCR mispairing, hence improving safety by lowering the risk of autoimmune reactivities. To use this as a broad application in cancer patients, it is important to expand the available antitumor specific TCRs to cover more HLA-types, hence more of these TCRs need to be retrieved from cancer patients or from in vitro TCR selection methods. Furthermore, multiple TAA-specific TCRs are also required to limit the risk of tumor-evasion of T cells, which may be limited through combining multiple TAA-specific T cells in a single cellular therapy. CD34+ progenitor cord blood derived T cells could be especially useful for preparation of off-the-shelf products, because very low numbers of cells are necessary to create billions of TAA-specific T cells through in vitro expansion.

More recently, especially with the development of TALEN and CRISPR/Cas9 mediated gene-editing, improved efficiencies to modulate or eliminate gene expression has created possibilities to bypass tumor strategies to evade immune surveillance and thereby to enhance antitumor responses. Since these gene-editing tools have been recently developed thorough safety assessment is required to predict potential side-effects of applying these tools for cellular anticancer gene therapy products. The potential effects of off-target gene-editing are still largely unknown and unpredictable, especially in an approach to target multiple genes at once. Whole-genome sequencing is therefore important to accurately assess the off-target effects, and to develop algorithms to predict single and multiplex gene-editing off-target cleavage sites. Additionally, the elimination of checkpoint inhibitors may also potentially lead to autoreactivity due to uncontrolled inhibition after TCR stimulation.

The applications of these tools to cord blood derived cells, in particular to cord blood T cells in a setting after UCB transplantation should be investigated for its feasibility and safety, and whether combining these strategies with other products derived from cord blood, such as dendritic cell vaccines, should lead to superior next-generation treatments of hematological malignancies.

## 9. FIVE-YEAR VIEW

UCB transplantation is a commonly accepted practice for patients with hematological malignancies who are refractory to conventional chemotherapy and/or irradiation treatment. More knowledge will be gathered over the next few years to predict the required personalized dosing to provide optimal immune reconstitution.

In the recent years, CB derived dendritic cell vaccines have been developed to boost T cell responses after transplantation. Because cord blood T cells can be retrieved from cord blood banks, personalized and/or universal products can be developed, that are gene-modified to specifically target tumor-antigens. Modifications on the genomic level through gene-editing also provide opportunities to modulate inhibitory signals to enhance antitumor effects. Initial results on PBMC T cells have shown that gene-engineering and multiple genomic modifications are feasible, and it is expected that this will lead to more multiplexed genome-edited anticancer cellular products. These modifications applied on PBMC T cells can be transferred to cord blood derived T cells. Ex vivo expansion and/or gene modification of these cord blood T cells can potentially lead to improved cellular immunotherapies. Recent improvements in the development of CD34+ derived cord blood T cells should lead to GMP protocols and possible translation to clinical applications. Combining these cord blood gene-engineered T cell products with cellular vaccinations, such as cord blood derived dendritic cell based vaccines is anticipated to enhance immunotherapeutic anti-tumor effects.



## KEY ISSUES

- **Personalized dosing of conditioning** drugs in umbilical cord blood transplantation settings is required to provide optimal immune reconstitution.
- CB derived T cells mediate **enhanced antitumor effects** compared to adult peripheral blood T cells.
- CB derived T cells can be rapidly **expanded ex vivo and genetically modified**. Their **naïve phenotype** can be directed to long-living memory stem T cells, which may provide improved antitumor effects.
- Recently developed gene-editing techniques, such as TALEN or CRISPR/Cas9 can be exploited for **multiplex gene-editing** to improve T cell antitumor responses, which can also be applied to cord blood T cells.
- Umbilical cord blood units can serve as a platform to generate superior **"off-the-shelf"** CB derived immunotherapy products that can be used to potentially boost antitumor immune responses for more effective therapies. CD34+ progenitor cord blood derived T cells could specifically serve as target cells to create large numbers of TAA-specific T cells with reduced risk of autoreactivity providing an alternative source for generating off-the-shelf antitumor products.
- **Automated manufacturing** of immunotherapy products in closed systems, including enrichment, transduction and expansion of gene-modified T cells to improve quality and reduce costs, should widen applicability also for cord blood off-the-shelf products, especially considering the high availability of CB unit stored in the CB biobanks.

## FUNDING

This paper was supported by the Dutch Cancer Society (grant number: 10474).



## DECLARATION OF INTEREST

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

## ORCID

Jaap Jan Boelens http://orcid.org/0000-0003-2232-6952

## BIBLIOGRAPHY

1. Gratwohl A, Baldomero H, Passweg J, et al. Hematopoietic stem cell transplantation for hematological malignancies in Europe. Leukemia . 2003;17:941–959.
2. Appelbaum FR. Pursuing the Goal of a Donor for Everyone in Need. N. Engl. J. Med. 2012;367:1555–1556.
3. De Koning C, Plantinga M, Besseling P, et al. Immune Reconstitution after Allogeneic Hematopoietic Cell Transplantation in Children. Biol. Blood Marrow Transplant. . 2016;22:195–206.
4. López MC, Palmer BE, Lawrence DA. Naïve T cells, unconventional NK and NKT cells, and highly responsive monocyte-derived macrophages characterize human cord blood. Immunobiology . 2014;219:756–765.
5. Wang JC, Doedens M, Dick JE. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood . 1997;89:3919–3924.
6. Broxmeyer HE, Douglas GW, Hangoc G, et al. Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. Proc. Natl. Acad. Sci. U. S. A. . 1989;86:3828–3832.
7. Boelens JJ. The power of cord blood cells. Blood. 2016;127:3300–3302.
8. Chiesa R, Gilmour K, Qasim W, et al. Omission of in vivo T-cell depletion promotes rapid expansion of naive CD4 + cord blood lymphocytes and restores adaptive immunity within 2months after unrelated cord blood transplant. Br. J. Haematol. 2012;156:656–666.
9. Ballen KK, Gluckman E, Broxmeyer HE. Umbilical cord blood transplantation : the first 25

years and beyond. Blood. 2014;122:491–499.

10. Eapen M, Rubinstein P, Zhang M-J, et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. Lancet . 2007;369:1947–1954.
11. Rocha V, Cornish J, Sievers EL, et al. Comparison of outcomes of unrelated bone marrow and umbilical cord blood transplants in children with acute leukemia. Blood . 2001;97:2962–2971.
12. Admiraal R, van Kesteren C, Jol-van der Zijde CM, et al. Association between anti-thymocyte globulin exposure and CD4+ immune reconstitution in paediatric haemopoietic cell transplantation: a multicentre, retrospective pharmacodynamic cohort analysis. Lancet Haematol. . 2015;2:e194–e203.
13. Admiraal R, Lindemans C a., van Kesteren C, et al. Excellent T-cell reconstitution and survivaldepend on low ATG exposure after pediatric cord blood transplantation. Blood. 2016;128:2734–2742.
14. Hiwarkar P, Gaspar HB, Gilmour K, et al. Impact of viral reactivations in the era of pre-emptive antiviral drug therapy following allogeneic haematopoietic SCT in paediatric recipients. Bone Marrow Transplant. . 2013;48:803–808.
15. Admiraal R, de Koning CCH, Lindemans C a., et al. Viral reactivations and associated outcomes in the context of immune reconstitution after pediatric hematopoietic cell transplantation. J. Allergy Clin. Immunol. . 2017;140:1643–1650.e9.
16. Hiwarkar P, Qasim W, Ricciardelli I, et al. Cord blood T cells mediate enhanced antitumor effects compared with adult peripheral blood T cells. Blood. 2017;126:2882–2892.
17. Jacks RD, Keller TJ, Nelson A, et al. Cell intrinsic characteristics of human cord blood naïve CD4T cells. Immunol. Lett. . 2017;193:51–57.
18. Milano F, Gooley T, Wood B, et al. Cord-Blood Transplantation in Patients with Minimal Residual Disease. N. Engl. J. Med. . 2016;375:944–953.
19. De Koning C, Nierkens S, Boelens JJ. Strategies before, during, and after hematopoietic cell transplantation to improve T-cell immune reconstitution. Blood . 2016;128:2607–2615.
20. Kwoczek J, Riese SB, Tischer S, et al. Cord blood-derived T cells allow the generation of a more naïve tumor-reactive cytotoxic T-cell phenotype. Transfusion. 2017;58.
21. Hiwarkar P, Qasim W, Ricciardelli I, et al. Cord blood T cells mediate enhanced antitumor effects compared with adult peripheral blood T cells. Blood. 2015;126:2882–2891.
22. Eshhar Z, Waks T, Gross G, et al. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. Proc. Natl. Acad. Sci. . 1993;90:720–724.
23. Zhou G, Levitsky H. Towards curative cancer immunotherapy: Overcoming posttherapy tumor escape. Clin. Dev. Immunol. 2012;2012.
24. Holzinger A, Barden M, Abken H. The growing world of CAR T cell trials: a systematic review. Cancer Immunol. Immunother. 2016;65:1433–1450.
25. Porter DL, Hwang W-T, Frey N V., et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. Sci. Transl. Med. . 2015;7:303ra139–ra303ra139.

26. Maude SL, Frey N, Shaw P a., et al. Chimeric Antigen Receptor T Cells for Sustained Remissions in Leukemia. N. Engl. J. Med. . 2014;371:1507–1517.
27. Grupp SA, Kalos M, Barrett D, et al. Chimeric Antigen Receptor–Modified T Cells for Acute Lymphoid Leukemia. N. Engl. J. Med. . 2013;368:1509–1518.
28. Wang K, Wei G, Liu D. CD19: a biomarker for B cell development, lymphoma diagnosis and therapy. Exp. Hematol. Oncol. . 2012;1:36.
29. Maude SL, Teachey DT, Porter DL, et al. CD19-targeted chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. Blood. 2016;125:4017–4024.
30. Pegram HJ, Purdon TJ, van Leeuwen DG, et al. IL-12-secreting CD19-targeted cord blood-derived T cells for the immunotherapy of B-cell acute lymphoblastic leukemia. Leukemia . 2015;29:415–422.
31. Serrano LM, Pfeiffer T, Olivares S, et al. Differentiation of naive cord blood T cells into CD19-specific cytolytic effectors for post-transplant adoptive immunotherapy. Blood . 2005;107:2643–2653.
32. Lee Y-S, Kim T-S, Kim D-K. T lymphocytes derived from human cord blood provide effective antitumor immunotherapy against a human tumor. BMC Cancer . 2011;11:225.
33. Kershaw MH, Westwood J a., Darcy PK. Gene-engineered T cells for cancer therapy. Nat. Rev. Cancer . 2013;13:525–541.
34. Robbins PF, Li YF, El-Gamil M, et al. Single and Dual Amino Acid Substitutions in TCR CDRs Can Enhance Antigen-Specific T Cell Functions. J. Immunol. . 2008;180:6116–6131.
35. Bendle GM, Linnemann C, Hooijkaas AI, et al. Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. Nat. Med. . 2010;16:565–570.
36. Kuball J, Dossett ML, Wolfl M, et al. Facilitating matched pairing and expression of TCR chains introduced into human T cells. Blood. 2007;109:2331–2338.
37. Cohen CJ, Zhao Y, Zheng Z, et al. Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. Cancer Res. 2006;66:8878–8886.
38. Weber G, Gerdemann U, Caruana I, et al. Generation of multi-leukemia antigen-specific T cells to enhance the graft-versus-leukemia effect after allogeneic stem cell transplant. Leukemia . 2013;27:1538–1547.
39. St. John LS, Wan L, He H, et al. PR1-specific cytotoxic T lymphocytes are relatively frequent in umbilical cord blood and can be effectively expanded to target myeloid leukemia. Cytotherapy . 2016;18:995–1001.
40. Okas M, Gertow J, Uzunel M, et al. Clinical expansion of cord blood-derived T cells for use as donor lymphocyte infusion after cord blood transplantation. J Immunother . 2010;33:96–105.
41. Points K. IL-7 and IL-15 instruct the generation of human memory stemTcells from naive precursors. Blood. 2014;121:573–585.
42. Hanley PJ, Russell C, Cruz Y, et al. Functionally active virus-specific T cells that target CMV , adenovirus , and EBV can be expanded from naive T-cell populations in cord blood and will target a range of viral epitopes. Blood. 2009;114:1958–1968.
43. Micklethwaite KP, Savoldo B, Hanley PJ, et al. Derivation of human T lymphocytes from

cord blood and peripheral blood with antiviral and antileukemic specificity from a single culture as protection against infection and relapse after stem cell transplantation. Blood. 2010;115:2695–2703.

44. Lent AU van, Nagasawa M, Loenen MM van, et al. Functional Human Antigen-Specific T Cells Produced In Vitro Using Retroviral T Cell Receptor Transfer into Hematopoietic Progenitors. J. Immunol. . 2007;179:4959–4968.
45. Zhao Y, Parkhurst MR, Zheng Z, et al. Extrathymic generation of tumor-specific T cells from genetically engineered human hematopoietic stem cells via notch signaling. Cancer Res. 2007;67:2425–2429.
46. Fernandez I, Ooi TP, Roy K. Generation of functional, antigen-specific CD8+ human T cells from cord blood stem cells using exogenous Notch and tetramer-TCR signaling. Stem Cells . 2014;32:93–104.
47. Dervovic DD, Ciofani M, Kianizad K, et al. Comparative and Functional Evaluation of In Vitro Generated to Ex Vivo CD8 T Cells. J. Immunol. . 2012;189:3411–3420.
48. Van Caeneghem Y, De Munter S, Tieppo P, et al. Antigen receptor-redirected T cells derived from hematopoietic precursor cells lack expression of the endogenous TCR/CD3 receptor and exhibit specific antitumor capacities. Oncoimmunology . 2017;6:1–14.
49. Van Til NP, Wagemaker G. Lentiviral Gene Transduction of Mouse and Human Hematopoietic Stem Cells BT - Hematopoietic Stem Cell Protocols. In: Bunting KD, Qu C-K, editors. New York, NY: Springer New York; 2014. p. 311–319.
50. Trombetta ES, Mellman I. Cell Biology of Antigen Processing in Vitro and in Vivo. Annu. Rev. Immunol. . 2005;23:975–1028.
51. Bonini C, Lee SP, Riddell SR, et al. Targeting antigen in mature dendritic cells for simultaneous stimulation of CD4+ and CD8+ T cells. J. Immunol. . 2001;166:5250–5257.
52. Benteyn D, Heirman C, Bonehill A, et al. mRNA-based dendritic cell vaccines. Expert Rev. Vaccines . 2015;14:161–176.
53. De Haar C, Plantinga M, Blokland NJ, et al. Generation of a cord blood-derived Wilms Tumor 1 dendritic cell vaccine for AML patients treated with allogeneic cord blood transplantation. Oncoimmunology . 2015;4:e1023973.
54. Decker WK, Shah N, Xing D, et al. Generation of Functional CLL-Specific Cord Blood CTL Using CD40-Ligated CLL APC. PLoS One. 2012;7.
55. Ishida Y, Agata Y, Shibahara K, et al. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J. . 1992;11:3887–3895.
56. Merindol N, Champagne M a, Duval M, et al. CD8+ T-cell reconstitution in recipients of umbilical cord blood transplantation and characteristics associated with leukemic relapse. Blood. 2011;118:4480–4488.
57. Buchbinder EI, Desai A. CTLA-4 and PD-1 Pathways. Am. J. Clin. Oncol. . 2016;39:98–106.
58. Ohaegbulam K, Assal A, Lazar-Molnar E, et al. Human cancer immunotherapy with antibodies to the PD-1 and PD-L1 pathway. Trends Mol. Med. 2015;42:115–125.
59. Ma S-D, Xu X, Jones R, et al. PD-1/CTLA-4 Blockade Inhibits Epstein-Barr Virus-Induced Lymphoma Growth in a Cord Blood Humanized-Mouse Model. PLOS Pathog. .

2016;12:e1005642.

60. John LB, Kershaw MH, Darcy PK. Blockade of PD-1 immunosuppression boosts CAR T-cell therapy. Oncoimmunology . 2013;2:e26286.
61. Menger L, Sledzinska A, Bergerhoff K, et al. TALEN-Mediated Inactivation of PD-1 in Tumor-Reactive Lymphocytes Promotes Intratumoral T-cell Persistence and Rejection of Established Tumors. Cancer Res. 2016;76:2087–2093.
62. Rupp LJ, Schumann K, Roybal KT, et al. CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. Sci. Rep. . 2017;7:737.
63. Frock RL, Hu J, Meyers RM, et al. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. Nat. Biotechnol. . 2014;33:179–186.
64. Kleinstiver BP, Pattanayak V, Prew MS, et al. High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. Nature . 2016;529:490–495.
65. Torikai H, Reik A, Liu PQ, et al. A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. Blood. 2012;119:5697–5705.
66. Philip LPB, Schiffer-Mannioui C, Le Clerre D, et al. Multiplex genome-edited T-cell manufacturing platform for “off-the-shelf” adoptive T-cell immunotherapies. Cancer Res. 2015;75:3853–3864.
67. Qasim W, Zhan H, Samarasinghe S, et al. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. Sci. Transl. Med. . 2017;9:eaaj2013.
68. Eyquem J, Mansilla-soto J, Giavridis T, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nat. Publ. Gr. . 2017;543:113–117.
69. Haworth K, Leddon J, Chen C-Y, et al. Going Back to Class I: MHC and Immunotherapies for Childhood Cancer. Pediatr Blood Cancer. 2010;119:5124–5136.
70. Martini M, Testi MG, Pasetto M, et al. IFN-??-mediated upmodulation of MHC class I expression activates tumor-specific immune response in a mouse model of prostate cancer. Vaccine . 2010;28:3548–3557.
71. Jackson HJ, Rafiq S, Brentjens RJ. Driving CAR T-cells forward. Nat. Rev. Clin. Oncol. . 2016;13:370–383.
72. Newick K, Moon E, Albelda SM. Chimeric antigen receptor T-cell therapy for solid tumors. Mol. Ther. - Oncolytics . 2016;3:16006.
73. Louis CU, Savoldo B, Dotti G, et al. Antitumor activity and long-term fate of chimeric antigen receptor – positive T cells in patients with neuroblastoma. Blood . 2011;14:1324–1334.
74. Ahmed N, Brawley VS, Hegde M, et al. Human Epidermal Growth Factor Receptor 2 (HER2) – Specific Chimeric Antigen Receptor–Modified T Cells for the Immunotherapy of HER2-Positive Sarcoma. J. Clin. Oncol. . 2015;33:1688–1696.
75. Kochenderfer JN, Dudley ME, Feldman S a, et al. B-¬cell depletion and remissions of malignancy along with cytokine¬ associated toxicity in a clinical trial of anti¬CD19 chimeric¬antigen¬ receptor–transduced T cells. Blood . 2012;119:2709–2720.
76. Parkhurst MR, Yang JC, Langan RC, et al. T Cells Targeting Carcinoembryonic Antigen Can Mediate Regression of Metastatic Colorectal Cancer but Induce Severe Transient Colitis. Mol.

Ther. . 2011;19:620–626.

77. Norelli M, Casucci M, Bonini C, et al. Clinical pharmacology of CAR-T cells: Linking cellular pharmacodynamics to pharmacokinetics and antitumor effects. Biochim. Biophys. Acta - Rev. Cancer . 2016;1865:90–100.
78. Maude SL, Barrett D, Teachey DT, et al. Managing Cytokine Release Syndrome Associated with Novel T cell-Engaging Therapies. Cancer J. 2014;20:119–122.
79. Bonifant CL, Jackson HJ, Brentjens RJ, et al. Toxicity and management in CAR T-cell therapy. Mol. Ther. - Oncolytics . 2016;3:16011.
80. Kaiser a D, Assenmacher M, Schröder B, et al. Towards a commercial process for the manufacture of genetically modified T cells for therapy. Cancer Gene Ther. . 2015;22:72–78.
81. Mock U, Nickolay L, Philip B, et al. Automated manufacturing of chimeric antigen receptor T cells for adoptive immunotherapy using CliniMACS Prodigy. Cytotherapy . 2016;18:1002–1011.
82. Broxmeyer HE, Lee M, Hangoc G, et al. Hematopoietic stem / progenitor cells , generation of induced pluripotent stem cells , and isolation of endothelial progenitors from 21- to 23 . 5-year cryopreserved cord blood. Blood. 2011;117:4773–4777.
83. Domogala A, Alejandro Madrigal J, Saudemont A. Cryopreservation has no effect on function of natural killer cells differentiated in vitro from umbilical cord blood CD34+ cells. Cytotherapy . 2016;18:754–759.
84. Davis CC, Marti LC, Sempowski GD, et al. Interleukin-7 permits Th1/Tc1 maturation and promotes ex vivo expansion of cord blood T cells: A critical step toward adoptive immunotherapy after cord blood transplantation. Cancer Res. 2010;70:5249–5258.
85. Franciszkiewicz K, Boissonnas A, Boutet M, et al. Role of chemokines and chemokine receptors in shaping the effector phase of the antitumor immune response. Cancer Res. 2012;72:6325–6332.
86. Huang H, Langenkamp E, Georganaki M, et al. VEGF suppresses T-lymphocyte infiltration in the tumor microenvironment through inhibition of NF-κB-induced endothelial activation. FASEB J. 2015;29:227–238.
87. Voron T, Colussi O, Marcheteau E, et al. VEGF-A modulates expression of inhibitory checkpoints on CD8 + T cells in tumors. J. Exp. Med. . 2015;212:139–148.
88. Pan J, Burdick MD, Belperio JA, et al. CXCR3/CXCR3 ligand biological axis impairs RENCA tumor growth by a mechanism of immunoangiostasis. J. Immunol. . 2006;176:1456–1464.
89. Francis L, Guo ZS, Liu Z, et al. Modulation of chemokines in the tumor microenvironment enhances oncolytic virotherapy for colorectal cancer. Oncotarget. 2016;7:22174–22185.
90. Gabrilovich DI, Nagaraj S. Myeloid-derived-supressor cells as regulators of th eimmune system. Nat. Rev. Immunol. 2009;9:162–174.
91. Berraondo P, Umansky V, Melero I. Changing the tumor microenvironment: New strategies for immunotherapy. Cancer Res. 2012;72:5159–5164.

3

# EFFICIENT LENTIVIRAL TRANSDUCTION METHOD TO GENE MODIFY CORD BLOOD CD8+ T CELLS FOR CANCER THERAPY APPLICATIONS

Vania Lo Presti[1,2], Annelisa M. Cornel[*1,2], Maud Plantinga[*1,2], Ester Dünnebach[1,2], Jurgen Kuball[1,6], Jaap Jan Boelens[3], Stefan Nierkens*[1,2] and Niek P. van Til* [1,4,5]

* These authors equally contributed to this work

[1] Center for Translational Immunology, UMC Utrecht, Utrecht, The Netherlands;

[2] Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands;

[3] Stem Cell Transplant and Cellular Therapies, MSK Kids, Memorial Sloan-Kettering Cancer Center, New York, USA

[4] AVROBIO, Inc., Cambridge, MA, USA

[5] Child Neurology, Emma Children's Hospital, Amsterdam University Medical Centers, Vrije Universiteit and Amsterdam Neuroscience, Amsterdam, The Netherlands

[6] Departement of Hematology, UMC Utrecht, Utrecht, The Netherlands

*Molecular Therapy - Methods and Clincial Development, 2021*

## ABSTRACT

Adoptive T cell therapy utilizing tumor-specific autologous T cells has shown promising results for cancer treatment. However, the limited numbers of autologous tumor associated antigen (TAA)-specific T cells and the functional aberrancies, due to disease progression or treatment, remain factors that may significantly limit the success of the therapy. The use of allogeneic T cells, such as umbilical cord blood (CB) derived, overcomes these issues, but requires gene-modification to induce a robust and specific anti-tumor effect. CB T cells are readily available in cord blood banks, show low toxicity, high proliferation rates and increased anti-leukemic effect upon transfer. However, combining anti-tumor gene modification and preserving advantageous immunological traits of CB T cells represents a major challenge for the harmonized production of T cell therapy products.

In this manuscript we optimized a protocol for expansion and lentiviral vector (LV) transduction of CB CD8+ T cells, achieving a transduction efficiency up to 83%. Timing of LV treatment, selection of culture media, and the use of different promoters were optimized in the transduction protocol. LentiBOOST was confirmed as a non-toxic transduction enhancer of CB CD8+ T cells, with minor effects on the proliferation capacity and cell viability of the T cells. Positively, the use of LentiBOOST does not affect the functionality of the cells, in the context of tumor cell recognition. Finally, CB CD8+ T cells were more amenable to LV transduction than peripheral blood (PB) CD8+ T cells and maintained a more naïve phenotype.

In conclusion, we show an efficient method to genetically modify CB CD8+ T cells using LV, which is especially useful for off-the-shelf adoptive cell therapy products for cancer treatment.

## INTRODUCTION

Adoptive T cell therapy has developed into a promising treatment option for cancer patients in the last decade. The rationale towards efficient T cell therapies is to redirect the patients' T cells against tumor antigens, either by selection and ex vivo expansion of tumor-infiltrating lymphocytes (TIL)[1] or by gene-modified lymphocytes. TIL therapy1, combined with interleukin-2, has shown encouraging results in the treatment of metastatic melanoma patients with a remission rate up to 72%[2,3]. Treatment with gene- modified T cell products[4], such as chimeric antigen receptor (CAR) T cells[5] , have shown very promising results in several clinical trials[6] and Kymriah® became the first approved cell therapy by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for B cell acute lymphoblastic leukemia (ALL) patients[7,8]. An alternative gene modification strategy for the generation of a T cell product, is the transfer of a transgenic tumor associated antigen (TAA)-specific T cell receptor (TCR) recognizing a specific peptide in the context of major histocompatibility complex (MHC). Also this strategy has been explored in several early phase clinical trials, predominantly in melanoma, gastrointestinal cancer, and leukemia[9]. MART1-TCR engineered T cells persisted for at least 2 months after the infusion in peripheral blood of 15 melanoma patients. Two patients with high sustained levels of circulating MART1 specific T cells 1 year post-infusion exhibited regression of metastatic melanoma lesions[10]. NY-ESO-TCR engineered T cells were well tolerated and exhibited an encouraging clinical response in multiple myeloma[11]. Post-transplantation transfer of WT1-TCR engineered T cells prevented relapse in all AML treated patients in a median of 44 month post-infusion[12]. These recent results indicate the potential of TCR engineering therapy, even if the tumor mutational burden is low, as for example in many childhood cancers[13].

A commonly used method for gene-modifying T cells is the use of lentiviral vectors (LV) to express a gene of interest, which are highly efficient in transducing many cell types[14], including quiescent cells. More specifically, the transduction efficiency in T cells is not only dependent on the proliferation status but also on the activation status. The presence of sufficient numbers of functional patient derived cells with adequate activation potential might be hampered by disease biology, disease progression, and/or previous therapies applied (such as chemotherapy)[15]. It has been reported that during chemotherapy cycles, T cells drastically decrease in number[16], show signs of senescence, low proliferation capacity, activate apoptotic pathways[17], and require several months to return to physiological levels[18–20].

Together, the decline in numbers of naïve and stem central memory T cells, as well as the reduced proliferation and stimulation potential limits the use of autologous T cell for harmonized production of autologous T cell therapy products.

A solution to overcome this problem is to utilize a patient unrelated cell source, such as T cells derived from healthy donors. Umbilical cord blood (CB) derived T cells are of particular interest as they show a naïve phenotype with elevated proliferation potential and low expression of exhaustion markers compared to peripheral blood (PB) derived T cells[21,22]. Additionally, Hiwarkar et al. showed that CB CD8+ T cells have an increased killing effect on HLA-mismatched EBV-driven tumor cells compared to other sources[23]. The use of CB derived cells has been mostly limited to CD34+ hematopoietic stem and progenitor cells, which are amenable to high lentiviral transduction rates[24–27]. The feasibility of the approach to introduce CARs[28–30] or recombinant TCRs[31] into CB derived T cells has been shown mostly using non-viral gene transfer methods or retroviral vectors (RV). However, as shown by Cieri *et al.*, the use of LV is superior to RV, especially for the transduction of naïve and central memory T cells[32]. Up to date and to the best of our knowledge, the use of LV to gene modify CB derived T cells is very scarce, but showed promising results[33].

In the present study, we developed an efficient method, using a serum-free transduction system, to transduce CB CD8+ T cells with LV with limited effect on their naïve immunophenotype. Using this protocol, CB CD8+ T cells are more susceptible to LV transduction compared to PB CD8+ T cells. In addition, we demonstrate how to further increase the transduction efficiency using the transduction enhancer LentiBOOST without altering their functionality and maintaining ability to expand in vitro, especially when using a low MOI. Altogether, these results show efficient gene modification of CB CD8+ T cells using LV to potentially generate off-the-shelf T cell therapy products for cancer treatment.

## MATERIALS AND METHODS

### LENTIVIRAL VECTOR PRODUCTION AND TITRATION

HIV-derived self-inactivating third generation lentiviral vectors were constructed using the CMV promoter driving the expression of the viral transcript (pCCL plasmid backbone). Additionally to the packaging signal (ψ), the REV responsive element (RRE), and the central polypurine tract (cPPT), the transfer vector plasmid contains a modified Woodchuck posttranslational regulatory element (bPRE4)[57,58]. Lentiviral particles coding for enhanced green fluorescent protein (GFP) were produced by transient cotransfection of the lentiviral transfer vector plasmid (LV.eGFP) and the respective packaging plasmids (pRSV-Rev, pMDLg/pRRE and pMD2-VSV-G) to HEK293T cells (ATCC® CRL-3216™) using CalPhos™ Mammalian Transfection Kit (Clontech), as previously described[59]. Viral supernatants were filtered through 0.45 µm low-protein binding filters, concentrated by ultracentrifugation at 20,000g for 2 hours, resuspended in StemMACS HSC expansion media (Miltenyi Biotech) and stored at -80°C. We generated two lentiviral vectors expressing enhanced GFP (eGFP) under the control of the phosphoglycerate kinase promoter[60] (PGK.eGFP) or a a synthetic promoter that contains the U3 region of a modified MoMuLV LTR with myeloproliferative sarcoma virus enhancer[61,62] (MND.eGFP). Additionally, two extra lentiviral vectors were generated expressing a codon optimized human TCR alpha and beta chain sequence bridged by a T2A self-cleaving peptide targeting Wilm's Tumor 1 (WT1) antigen[63,64] under the control of the MND promoter (MND. WT1-TCR) (Fig. 1) and a Chimeric Antigen Receptor against CD19 (CAR19) under the control of the PGK promoter (PGK.CAR19) (Fig. S2). Vesicular stomatitis virus (VSV)-g protein pseudotyped lentiviral particles were titrated by serial dilution on Jurkat cells (Clone E6-1;ATCC® TIB-152™). At 72h post transduction, cells were harvested and analyzed by flow cytometry for GFP expression and viral titer was calculated.

### CD8+ T CELL ISOLATION AND EXPANSION

Fresh CB or PB was collected after informed consent was obtained according to the Declaration of Helsinki. The collection protocol was approved by the ethical committee of the University Medical Center Utrecht. CB or PB was processed to isolate CD8+ T cells using Ficoll (GE Healthcare Bio-Sciences AB) separation and CD8 positive magnetic bead separation according to the manufacturer's protocol (Miltenyi Biotech). CD8+ T cells were subsequently cultured in RPMI (Fisher Scientific) supplemented with 10% human serum, 1% penicillin/streptomycin (P/S) (cytotoxic

T lymphocytes media (CTL media)), with antiCD3/CD28 Dynabeads (GIBCO, Thermo Fisher Scientific) in a 1:3 ratio (beads:T cells); 50U of IL2/ml (UMC – Pharmacy) , 5ng/ml IL7, and 5ng/ml IL15 (Miltenyi Biotech). CD8+ T cells were kept in culture for 3 days, resulting in an average 4.5-fold expansion rate.

## T CELL TRANSDUCTION



Expanded and stimulated CD8+ T cells were transduced at MOI 1 and 10 with two different methods: spinoculation and overnight incubation. For the spinoculation, T cells were mixed and incubated with the lentiviral vector for 30 minutes at room temperature and centrifuged for 30 minutes at 800g at 32°C. After the centrifugation step transduction media was replaced with complete CTL-media. For the overnight transduction, T cells were mixed and incubated with the lentiviral vector for 14-16 hrs, after which transduction media was replaced with complete CTL-media. We used two different transduction media: X-Vivo15 (Lonza) and StemMACS HSC Expansion Media XF (Miltenyi Biotech) supplemented with 1% P/S; with or without supplementation of the transduction enhancer LentiBOOST (1:100 of the total volume; SIRION Biotech).

## VECTOR COPY NUMBER (VCN)

Genomic DNA was isolated from transduced cells using a Genomic DNA Purification kit (QIAGEN). qRT-PCR was performed with SyBR Green PCR master mix (Thermo Fisher) and primers targeting the U3 region (FW: CTGGAAGGGCTAATTCACTC) and Ψ (RV: GGTTTCCCTTTCGCTTTCAA). Amplification of the β-actin gene was used as a reference gene (forward primer: AGCGGGAAATCGTGCGTGAC; reverse primer: CAATGG TGATGACCTGGCCGT). Serial dilutions of a standard DNA plasmid containing one integrated copy of lentiviral vector sequence were used to plot a standard curve. Samples and standard serial dilutions were run in duplicate.

## CD8+ T CELL PROLIFERATION ASSAY

Untransduced and transduced CD8+ T cells were washed with PBS to remove serum that affects staining. Then, cells were suspended in PBS at a concentration of $2.4 \times 10^6$ cells/ml and labeled with CellTraceTM Violet Cell (CTV) Proliferation Kit (5 mM; ThermoFisher) at 37°C for 20 min. Subsequently, cells were washed with warm fetal calf's serum and resuspended in CTL-media. After 24hrs, cells were analyzed with flow cytometry to confirm CTV staining. Cells were stimulated with aCD3/CD28 Dynabeads, in a ratio 1:8 (Beads:T cells) or with a tetramer molecule loaded with

pWT1126. After 4 and 7 days, T cells were stained with Fixable Viability Dye and analyzed with flow cytometry to detect decrease of CTV signal.

## TETRAMER STAINING

A tetramer molecule was generated according to an established protocol [65], consisting of WT1(126) peptide stained with fluorescent labeled streptoavidin (PE and PE-Cy7) . Untransduced and transduced CD8+ T cells were treated with Dasatinib (VWR international) at a final concentration of 50nM. Cells were incubated at 37°C for 30 min to allow stabilization of the TCR on the cell surface. Cells were subsequently washed once with FACS buffer, after which the tetramer staining was initiated by adding approximately 0.1 µg per peptide:MHC complex and incubated 15 minutes at 37°C. Without washing, an antibody mix was added to the cells: CD8-APC (clone RPA-T8, ref. 555369, BD Pharmingen); CD3-Pacific Blue (clone UCHT1, ref. 558117, BD); Fixable Viability Dye eFluor™ 780 (ref. 65-0865-14, ThermoFisher); TCR Vβ21.3 (Clone IG125, ref. PN IM1483, Beckman Coulter). Cells were incubated with the antibodies mix for 15 minutes at 4°C, washed and analysed on a BD Fortessa using FACSDiva.

## KILLING ASSAY AND CYTOKINE ANALYSIS

T2 cells (ATCC® CRL-1992™), were stained with CTV (following the method explained in the above paragraph "CD8+ T cell proliferation assay") and subsequently pulsed with 1 µg/ml of $WT1_{(126)}$ peptide. T2 cells were co-cultured overnight with untransduced or transduced CB-CD8+ T cells, in a 1:1 or a 1:10 ratio (Effector:Target cells). After 14-16hrs cells were stained with 7-AAD (BD Pharmingen), after which flow cytometry analysis was performed on a BD LSRFortessa using FACSDiva, with acquisition of a fixed amount of total volume for each sample. Cytokines in supernatants were measured using LEGENDplex assay (BioLegend). Flow cytometry analysis was performed on a BD LSRFortessa using FACSDiva and post-acquisition analysis was performed with LEGENDplex Data Analysis Software.

## FLOW CYTOMETRY ANALYSIS

Transduced CD8+ T cells were stained for 15 minutes at 4°C with fluorescently labeled antibodies against CD3-Pacific Blue (clone UCHT1, ref. 558117, BD) and CD8-PE (clone RPA-T8, ref. 301008, Biolegend); Fixable Viability Dye eFluor™ 780 (ref. 65-0865-14, ThermoFisher) to evaluate transduction efficiency. Fresh, expanded, and transduced cells were stained with Fixable Viability Dye eFluor™ 780 (ref. 65-0865-14, ThermoFisher), CD62L-BV650 (clone DREG-56, ref. 2124160, Sony Biotechnology), CD25-PerCP Cy5.5 (clone BC96, ref. 2113130, Sony Biotechnology), TIM3-APC (Clone 34482, ref. FAB2365A, R&D), CD3-AF700 (clone UCHT1, ref.300424, Sony Biotechnology), CD45RA-BV421 (clone HI100, ref. 304118, Biolegend), CD45RO-BV711 (clone UCHL1, ref. 304236, Biolegend), LAG3-PE (ref. FAB2319P, R&D), and CD8-PeCy7 ( clone SK1, ref. 335822, BD), TCR Vβ21.3 (Clone IG125, ref. PN IM1483, Beckman Coulter), CD19 CAR Detection Reagent (ref. AB_2811310, Miltenyi Biotec). Flow cytometry analysis was performed on a BD LSRFortessa using FACSDiva, with acquisition of a fixed number of cells for each experiment. FACS data analysis was performed using FlowJo V10 . Data are presented as % of cells and as median fluorescent intensity (MFI).

## STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA;). Due to the small number of samples normality was assessed using QQ-plot visualization. One-way analysis of variance and Student's t test was used to determine differences between groups. The results are presented as average ± standard deviation (SD). P-values are considered statistically significant when $p \leq 0.05$ (*).

# RESULTS

## METHOD OPTIMIZATION FOR EFFICIENT LENTIVIRAL TRANSDUCTION OF CB CD8+ T CELLS

Two different methods were compared for transduction efficiency: spinoculation and overnight incubation. 3 days after expansion, CB CD8+ T cells were transduced with either PGK.eGFP or MND.eGFP (Fig. 1) at a multiplicity of infection (MOI) of 10 using X-Vivo15 media. Using spinoculation, 6 days after transduction, 24 ± 5% of CD8+ T cells were GFP+ with the PGK.eGFP vector and 31 ± 9% with the MND.eGFP vector, whereas overnight incubation showed 48 ± 11% GFP+ cells for PGK.eGFP and 68 ± 8% for MND.eGFP (Fig. 2A). Additionally, the use of two different types of media during the transduction strongly influenced the efficiency of transduction of CD8+ T cells. Overnight culture in StemMACS HSC Expansion Media XF (StemMACS) showed a significant increase in the percentage of transduced cells up to 78 ± 7.7% for PGK.eGFP and 83 ± 8.8% for MND.eGFP in contrast to X –Vivo15 (Fig. 2B and Fig. 2C). Hence, overnight incubation and StemMACS medium was used for further experiments (from hereafter referred to as STND protocol). No significant differences were observed in the percentage of transduced cells if the eGFP was under the control of the MND or the PGK promoter, however the protein expression, calculated as median fluorescence intensity (MFI), increased significantly using the MND promoter. GFP positive CB CD8+ T cells showed a 3-fold increase in MFI compared to the CB CD8+ T cells transduced with PGK.eGFP (Fig. 2D), also when normalized for vector copy number (VCN) (Fig. 2E).


**Figure 1. Schematic overview of lentiviral transfer vector plasmid maps**

Third generation self-inactivating (SIN) lentiviral vectors were constructed using the CMV promoter driving the expression of the viral transcript (pCCL). The transfer vector contains the packaging signal (ψ), the REV response element (RRE), central polypurine tract (cPPT), and a modified Woodchuck post-translational regulatory element (bPRE4). Expression of the reporter gene eGFP was under the control of either the PGK or MND promoter (arrows). The WT1-TCR was under the control of the MND promoter.

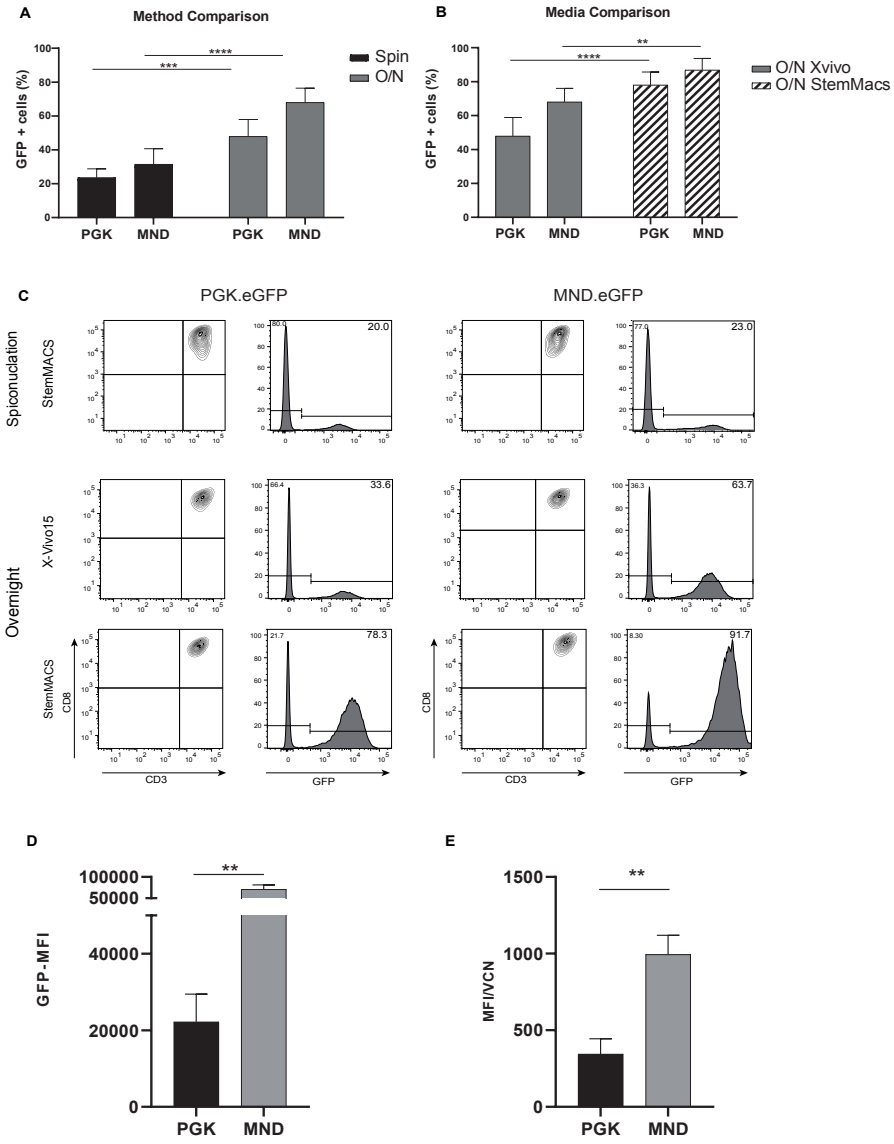



**Figure 2. Optimization of the lentiviral transduction method for CB CD8+ T cells**

(A) Transduction efficiency, presented as % GFP+ cells, comparing two methods for T cell transduction: spinoculation (black) and overnight incubation (gray) (B) Transduction efficiency, presented as % GFP+ cells, comparing the use of different culture media during overnight transduction: X-Vivo15 (gray) and StemMACS media (stripes). (C) Representative flow cytometry plots of methods and media tested using both transduction with PGK.eGFP and MND.eGFP. The density plots are showing the measured CD3 and CD8 expression (on the left) and GFP expression in CD3+ CD8+ T cells (on the right) in the left (PGK.eGFP) and right panel (MND.eGFP). (D) Protein expression, presented as MFI of eGFP signal, is statistically increased using the MND promoter . (E) qPCR analysis of the VCN positive correlates with the MFI results. The data corresponds to ≥ 3 independent experiments and are shown as average ± standard deviation; $p \leq 0.05$ (*); $p \leq 0.01$ (**) ; $p \leq 0.001$ (***); $p \leq 0.0001$ (****).

## CB CD8+ T CELLS ARE MORE EFFICIENTLY TRANSDUCED THAN PB CD8+ T CELLS WITH LV.MND.eGFP

Autologous PB T cells are often the main cell source for adoptive cell therapy to treat cancer. Here, we developed a method to transduce allogeneic CB CD8+ T cells and hence we compared the two T cell sources for efficient transduction by lentiviral vectors. PB CD8+ T cells were isolated from healthy donors, expanded and transduced following the same method developed for CB CD8+ T cells. As shown in Fig. 3A and Fig. 3B this method has an advantage to transduce CB CD8+ T cells more efficiently than PB CD8+ T cells, which was observed using MND.eGFP. The immunophenotype based on markers for naïve T-cells (CD45RO, CD62L, CD45RA) of freshly isolated (Day 0; fresh) T cells is substantially different between CB CD8+ T cells and PB CD8+ T cells. As shown in Fig. 3C and Fig. 3D, fresh CB CD8+ T cells are characterized by a homogenous population expressing classic naïve T cell markers (CD45RO- CD62L+ CD45RA+), while fresh PB CD8+ T cells consist of a more heterogeneous populations with different expression levels of both naïve and memory T cell markers, characteristic of memory T cell subsets (CD45RO+CD62L- effector memory T cells (Tem) and CD45RO+CD62L+ central memory T cells (Tcm)). The expansion phase, characterized by the presence of cytokines and anti-CD3/CD28 (aCD3/CD28) beads, confers to both CB and PB CD8+ T cells an increased proportion of population expressing CD45RO and CD62L (Tcm). At the moment of transduction (Day 3; expanded), expanded T cells from both sources were more similar, contrary to the initial differences shown in fresh cells (Day 0) (Fig. 3C-E). Moreover, both CB and PB CD8+ T cells showed similar high levels of the IL2 receptor (CD25), indicating an activated state at the moment of transduction (Fig. S1). Interestingly, 6 days after transduction (Day 10; transduced), CB CD8+ T cells reverted to the naïve T cell markers, with a specific loss of expression of CD45RO and increased expression of CD45RA, which did not occur in PB CD8+ T cells (Fig. 3E). Moreover, CB and PB CD8+ T cells showed differential expression of co-inhibitory receptor (TIM3 and LAG3) during all experimental steps (Fig. 3F and Fig. S1).

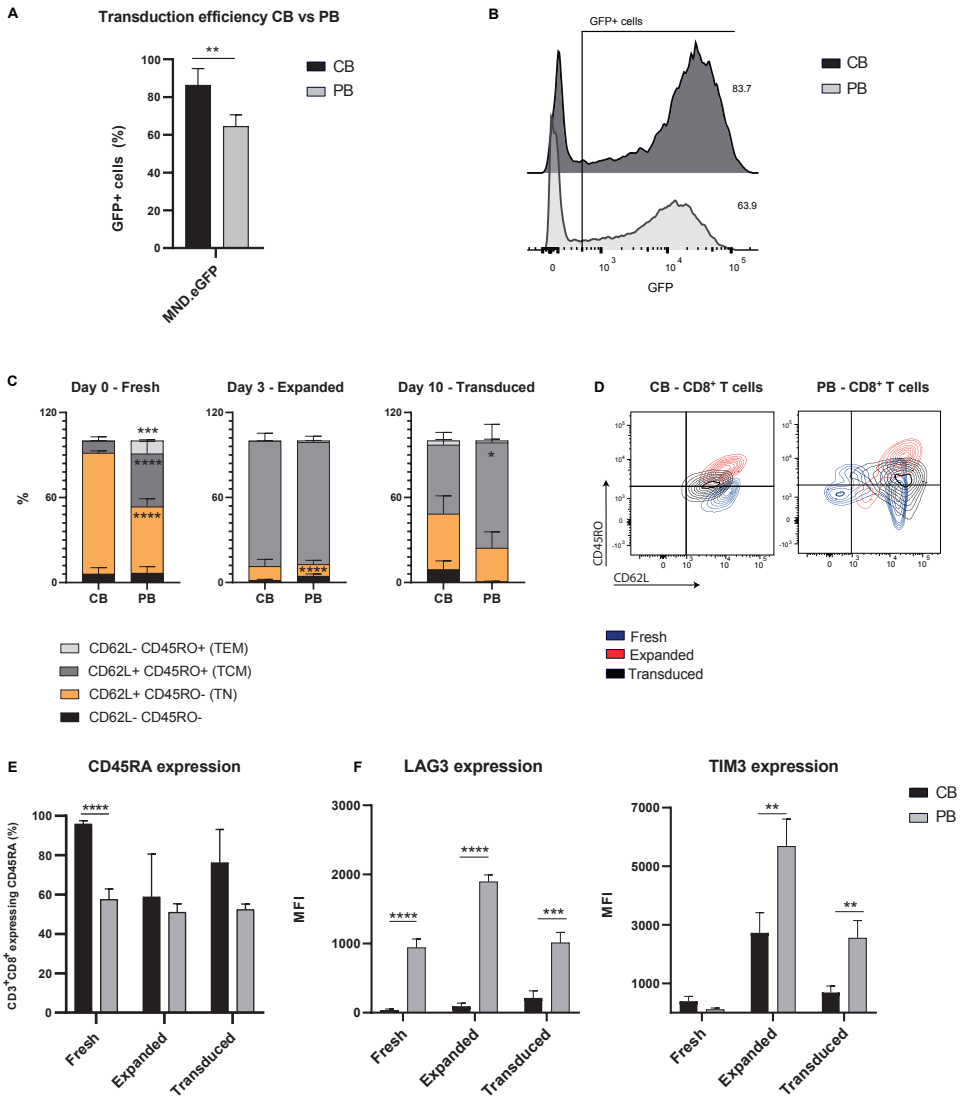



**Figure 3. CB and PB CD8+ T cells show different immune phenotype and transduction efficiency**

(A) Transduction efficiency, as % of GFP+ cells in CB CD8+ T cells (black) compared to PB CD8+ T cells (gray) using MND.eGFP. (B) Representative flow cytometry plot of CB and PB CD8+ T cells transduced with MND.eGFP. (C) Proportion of naïve T cells (Tn), effector memory T cells (Tem), and central memory T cells (Tcm) based on the expression of CD45RO and CD62L, in both CB and PB in CD8+ fresh, expanded and transduced T cells. (D) Representative flow cytometry plot of CD45RO-CD62L expressing cells during the three selected timepoints (i.e. fresh, expanded, transduced). (E) CD45RA expression in CB (black) and PB (gray) CD8+ fresh, expanded and transduced T cells. (F) Co-inhibitory receptor expression in CB (black) and PB (gray) CD8+ fresh, expanded and transduced T cells. The data corresponds to ≥ 3 independent experiments and are shown as average ± standard deviation; $p \leq 0.05$ (*); $p \leq 0.01$ (**) ; $p \leq 0.001$ (***); $p \leq 0.0001$ (****).

## THE TRANSDUCTION ENHANCER LENTIBOOST INCREASES TRANSDUCTION EFFICIENCY AND MAINTAIN PROLIFERATION CAPACITY OF CB CD8+ T CELLS IN VITRO

To further improve the transduction efficiency, we tested if the addition of the non-ionic, amphiphilic poloxamer synperonic F108 (LentiBOOST)[25,34] could further enhance the GFP expression in CB CD8+ T cells, while limiting its effect on viability and proliferation capacity of the CB CD8+ T cells and using MOIs in the range of MOI 1-20, commonly used to transduce PB T cells [35–37]. As shown in Fig. 4A and Fig. 4B, the use of LentiBOOST (LB) increased both the percentage of transduced T cells and the MFI of the signal. Using an MOI 10 to transduce CB CD8+ T cells, the LB resulted in 15% more GFP+ T cells, and with a lower MOI (MOI 1) the increase in the percentage of GFP+ T cells was even more pronounced, with an average increase of 25% compared to the STND. Lentiviral VCN analysis by qPCR confirmed that in both cases (MOI 10 and MOI 1), the use of LB significantly increased the VCN (Fig. 4C). The use of LB increased the transduction efficiency also when using an LV.rTCR (next paragraph) and an LV.CAR19 (Fig. S2). Six days post-transduction, CB CD8+ T cells were stained with CellTrace Violet (CTV) and followed up over 4 days to determine the proliferation capacity. CB CD8+ T cells transduced with an MOI 1, in the presence of LB, maintained their proliferation capacity after a strong stimulation with aCD3/CD28 beads, but this was lower than untransduced cells (Fig. 4E). To determine the direct influence of the LB on proliferation, untransduced cells were cultured overnight in the presence of LB. LB alone did not show any effect on the proliferation rate of untransduced CB CD8+ T cells (Fig. 4E). FlowJo analysis using the proliferation tool additionally showed a trend of impaired proliferation, division and expansion rate in CB CD8+ T cells transduced in the presence of LB, but not a significant difference (Fig. 4F). In addition, CB CD8+ T cells transduced with an MOI 1, in the presence of LB, did not show lower proliferation capacity (Fig. S3). The use of LB only slightly and not significantly decreased viability when compared to transduced T cells , in the absence of LB (Fig. 4G).

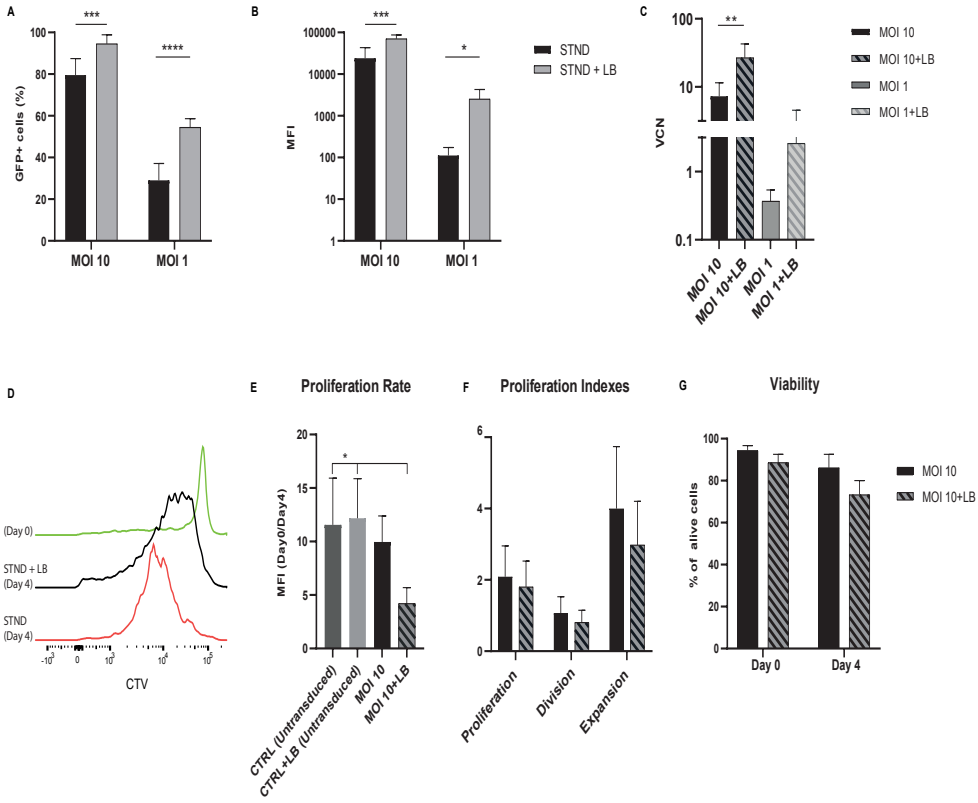
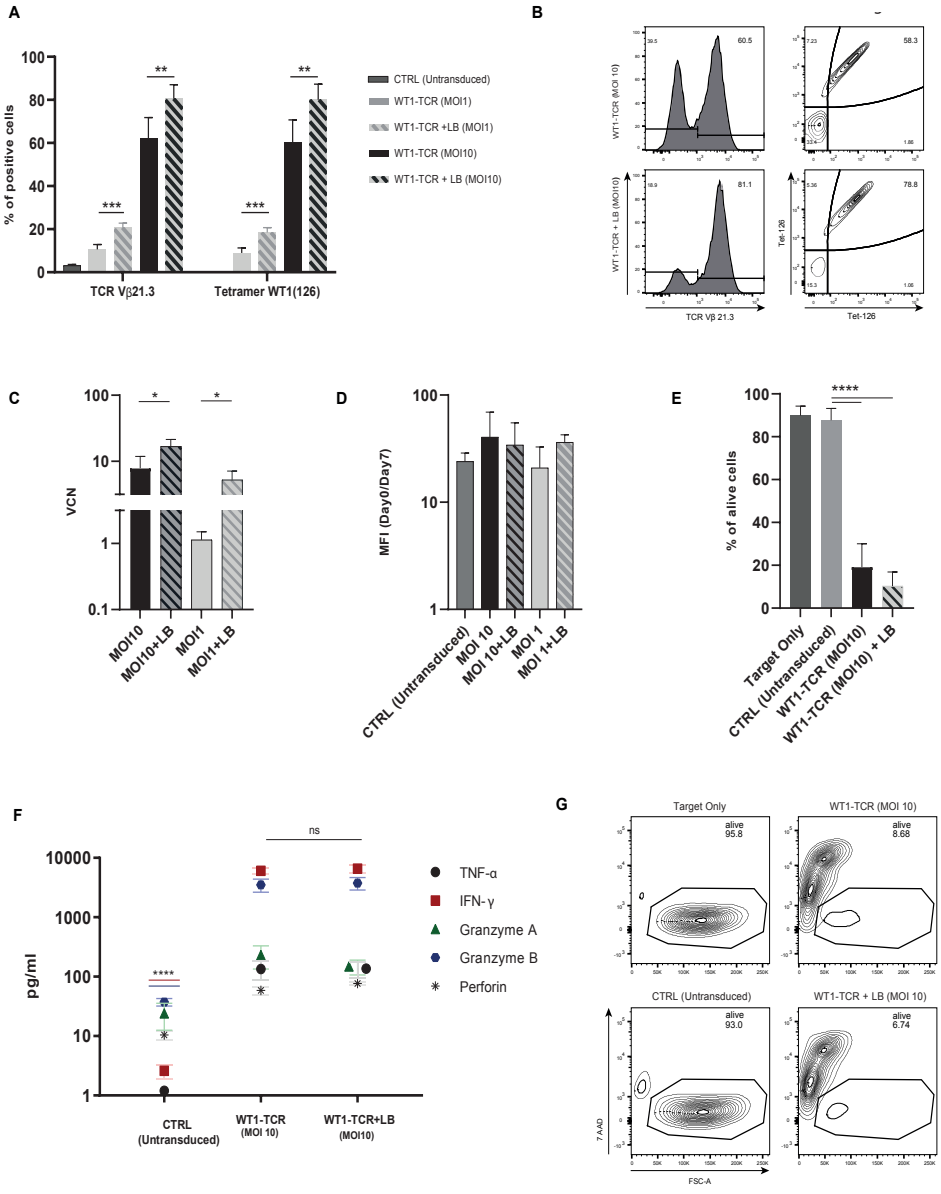



**Figure 4. LentiBOOST (LB) increases transduction efficiency of CB CD8+ transduced T cells and maintain proliferation in vitro**

(A) Transduction efficiency, presented as % GFP+ cells, is improved by the use of LB together with LV at MOI 10 (left) and MOI 1 (right). (B) Protein expression, calculated as MFI of eGFP signal, is increased by the use of LB together with both LV at MOI 10 (black) and MOI 1 (gray). (C) qPCR analysis of the VCN in CB-CD8+ T cells transduced with an MOI 10 in the absence (black) or presence of LB (black stripes); or transduced with an MOI 1 in the absence (gray) or presence of LB (gray stripes). (D) Representative flow cytometry plot of proliferation analysis using CellTrace Violet (CTV) on CB CD8+ T cells transduced with STND method or with STND + LB. (E) Proliferation rate (as CTV-MFI of Day0/Day4) in CB CD8+ T cells untransduced and transduced in the presence or absence of LB. (F) Proliferation indexes (calculated using proliferation model of FlowJoV10) define proliferation, division and expansion rate of CB CD8+ T cells transduced in the presence of LB (black stripes) compared to STND protocol (black). (G) Cell viability respectively at Day 0 and Day 4 of proliferation assay, in CB CD8+ T cells transduced in the presence of LB (black stripes) compared to STND protocol (black). The data corresponds to ≥ 3 independent experiments and are shown as average ± standard deviation; $p \leq 0.05$ (*); $p \leq 0.01$ (**) ; $p \leq 0.001$ (***); $p \leq 0.0001$ (****).

## CB CD8+ T CELLS TRANSDUCED WITH A TAA-SPECIFIC TCR, IN THE PRESENCE OR ABSENCE OF LB, SHOW COMPARABLE AND EFFICIENT ANTIGEN RECOGNITION, PROLIFERATION AND KILLING CAPACITY

To further test the effect of LB on CB CD8+ T cell proliferation and functionality in vitro, CB CD8+ T cells transduced with a LV expressing a WT1-specific TCR in the presence of LB, were tested for antigen recognition, antigen-specific proliferation and killing capacity. To test antigen recognition, transduced CB CD8+ T cells were incubated with fluorophores coupled tetramer molecules pulsed with the target WT1 derived peptide (pWT1126). As shown in Fig. 5A and B both T cells transduced, in the presence or absence of LB, showed recognition of pWT1126, with a percentage similar as the transduction efficiency, with an MOI 1 (STND: 8.9% ±2.3; STND+LB: 18.4% ±2.2) and an MOI 10 (STND:60.5% ±10; STDN+LB: 80% ±7.3). qPCR analysis of VCN shows higher number of integration, both when using an MOI 10 and an MOI 1, in the presence of LB (Fig. 5C). After 6 days from the transduction, CB CD8+ T cells were stained with CellTrace Violet (CTV) and followed up over the course of 7 days in the presence of pWT1126 tetramer stimulation. WT1-TCR CB CD8+ T cells, transduced in the presence or absence of LB, showed a similar proliferation capacity (Fig. 5D). The same cells were co-cultured overnight with T2 cells, loaded with pWT1126, in a 1:1 ratio between target and effector cells (T:E). The WT1-TCR engineered cells, transduced in the absence or presence of LB, were effectively killing, on average, 81% and 90% of target T2 cells respectively, which was statistically significant compared to untransduced cells, that only killed 2% of target cells (Fig. 5E and G). Cytotoxic cytokine levels (IFNɣ, TNFα, Granzyme A, Granzyme B and Perforin) were analyzed in the supernatant of the killing assays. WT1-TCR transduced cells produced an higher amount of cytotoxic cytokines compared to untransduced cells, statistically significant for IFNɣ and Granzyme B (p-value: <0,0001), whereas there were no differences between transduced T cells, in the presence or absence of LB (Fig. 5F). Additional experiments to support our findings were performed with a more limiting T:E ratio (10:1), and showed a slightly increased killing capacity of WT1-TCR transduced CB CD8+ T cells in the presence of LB, likely due to the presence of a larger proportion of WT1-TCR transduced T cells (Fig. S4).


A
% of positive cells
100
80
60
40
20
0
**
**
***
***
TCR Vβ21.3
Tetramer WT1(126)
CTRL (Untransduced)
WT1-TCR (MOI1)
WT1-TCR +LB (MOI1)
WT1-TCR (MOI10)
WT1-TCR + LB (MOI10)
B
WT1-TCR (MOI 10)
WT1-TCR + LB (MOI10)
60.5
58.3
81.1
78.8
TCR Vβ 21.3
Tet-126
C
VCN
100
10
1
0.1
*
*
MOI10
MOI10+LB
MOI1
MOI1+LB
D
MFI (Day0/Day7)
100
10
1
CTRL (Untransduced)
MOI 10
MOI 10+LB
MOI 1
MOI 1+LB
E
% of alive cells
100
80
60
40
20
0
****
Target Only
CTRL (Untransduced)
WT1-TCR (MOI10)
WT1-TCR (MOI10) + LB
F
pg/ml
10000
1000
100
10
1
ns
****
CTRL (Untransduced)
WT1-TCR (MOI 10)
WT1-TCR+LB (MOI10)
TNF-α
IFN-γ
Granzyme A
Granzyme B
Perforin
G
Target Only
WT1-TCR (MOI 10)
alive 95.8
alive 8.68
CTRL (Untransduced)
WT1-TCR + LB (MOI 10)
alive 93.0
alive 6.74
7-AAD
FSC-A

**◀ Figure 5. LentiBOOST (LB) does not affect functionality of WT1-TCR transduced CB CD8+ T cells**

(A) Transduction efficiency and antigen recognition, presented as % TCR Vb21.3+ T cells (left) and tetramer+ cells (right), improved by the use of LB at MOI 1 and MOI 10 compared to STND method. (B) Representative flow cytometry plot of CB CD8+ T cell transduction efficiency and pWT1126 tetramer staining. (C) qPCR analysis of the VCN in CB CD8+ T cells transduced with an MOI 10 in the absence (black) or presence of LB (black stripes); or transduced with an MOI 1 in the absence (gray) or presence of LB (gray stripes). (D) Proliferation rate (as CTV-MFI of Day0/Day7) of CB CD8+ T cells untransduced and transduced with WT1-TCR (MOI10), in the presence or absence of LB, and stimulated with tetramer loaded with pWT1126. (E) Killing capacity of WT1-TCR CB CD8+ T cells transduced in the presence or absence of LB, depicted as % of alive target cells in a co-culture experiment with target cells loaded with pWT1126. Co-culture experiments were performed in a ratio of 10:1 (Target:Effector). (F) Levels of cytotoxic cytokines in the supernatant of co-culture experiments produced by WT1-TCR transduced CB CD8+ T cells, in the presence or absence of LB, compared to untransduced cells . (G) Representative flow cytometry plot of % of alive target cells after overnight co-culture with WT1-TCR transduced CB CD8+ T cells. Co-culture experiments were performed in a ratio of 1:1 (Target:Effector). The data corresponds to ≥ 3 independent experiments and are shown as average ± standard deviation; $p \leq 0.05$ (*); $p \leq 0.01$ (**) ; $p \leq 0.001$ (***); $p \leq 0.0001$ (****).



## DISCUSSION

The possibility to gene-modify immune cells has opened a broad range of cell therapy applications for difficult to treat tumor types. However, it remains challenging to obtain enough functionally proficient autologous T cells from cancer patients and to efficiently gene-modify immune cells. In this manuscript we studied the use of allogeneic CB cells as a rich source to obtain CD8+ T cells that are amenable for lentiviral transduction. The use of CB derived cells is well established in clinical applications such as hematopoietic cell transplantation (HCT)[38–40]. CB T lymphocyte cultures can result in more than 100-fold magnitude of expansion, enabling feasible upscaling processes to generate sufficient cell numbers for therapeutic purposes[41,42]. Furthermore, CB lymphocytes are associated with favorable graft versus tumor effects, reaching sufficient numbers and efficiency for donor lymphocytes infusion post HCT[43,44].

Several clinical trials are testing the safety and the potential benefit of infusing CB derived, naturally occurring, lymphocytes[45,46], including T cells, natural killer (NK) cells and cytokine-induced killer cell (CIK), to avoid relapse (NCT01630564) or viral reactivation (NCT03594981) after HCT. Although the results on CB derived T cell infusions are promising, the generation of specific, gene modified CB T lymphocytes for therapeutic approaches is still in its infancy compared to using PB T cells. I

n this manuscript, we present an optimized method to obtain highly efficient lentiviral transduction of CB CD8+ T cells, which could potentially be translated for use in clinical settings. In fact, the use of serum-free StemMACS medium, showed a transduction efficiency of more than 80% of CB CD8+ T cells, using a relatively low MOI, favorable for future GMP production and safety concerns (Fig. 2). This method also confirmed the enhanced expression achieved with the use of a viral derived promoter (MND) compared to the constitutively expressed promoters (PGK).



Using this protocol, the total fraction of CB CD8+ T cells were more amenable to transduction compared to the total fraction of PB CD8+ T cells. We speculate that this protocol may be beneficial by exploiting the naïve characteristics of CB CD8+ T cells, in accordance to previous studies that showed an increased transduction efficiency in PB derived T naïve (Tn) cells and T memory stem cells (Tscm)[32,47]. Nevertheless, we may expect an increased potency of CB CD8+ T cells due to the differences in immune phenotype between the two cell sources[48,49]. Pre-clinical and clinical studies have shown the short persistence and low proliferation properties of engineered T cells derived from PB of adult patients[50]. Moreover, in vivo studies have shown the superiority of Tn over T memory cells (Tm) in adoptive immunotherapeutic applications[51,52].

The proposed method combines high transduction efficiency with a global maintenance of the least differentiated Tscm or Tn phenotype of the CB CD8+ T cells, confirmed by the downregulation of CD45RO and the upregulation of CD45RA (Fig 3).This method increases the feasibility to use CB CD8+ T cells in clinical applications, however its clinical significance will rely on the activation, proliferation and long-term survival of genetically modified cells in vivo. The naïve phenotype, together with the intermediate and the low expression of inhibitory receptors (TIM3, LAG3), which were maintained low in the current protocol, could be a valuable feature for improving proliferation, persistence and efficacy in future pre-clinical and clinical studies. The difference in transduction efficiency between the cell sources may also be influenced by the expression levels of the cognate glycoprotein receptors on the target cells, defining the probability of binding between viral particle and cell membrane[53]. This effect was previously shown by substituting the envelope from VSV-G to measles virus (MV)[37] or baboon retroviral envelope (BaEV)[53], which increased the transduction efficiency of the lentiviral vector; a strategy that can be further explored.

Further improvements in the transduction efficiency was achieved using the transduction enhancer LentiBOOST (LB). The active component of LB is Poloxamer

338/Pluronic F108 and belongs to the class of A-B-A type tri block copolymers composed of hydrophilic ethyleneoxide and hydrophobic propyleneoxide. This structure confers a capability of interacting with hydrophobic particles and biological membranes. The advantage of using this enhancer, and alternative poloxamers (Poloxamer 407), have already been proven in HSCs and mouse derived T cells[25,34,54] and its GMP grade formula has already been used in clinical trials. The use of LB clearly increased the transduction efficiency also in CB CD8+ T cells using different lentiviral vector contructs (LV.eGFP; LV.WT1-TCR; LV.CAR19), especially interesting for applications requiring low MOI, or when vector titers are limited. As previously shown[34,55] the use of LB very minimally affects T cell viability, as shown also by using our optimized protocol (Fig. 4G).



Using a tracer molecule, we observed that LB affected the proliferation rate of CB CD8+ T cells transduced with an MOI 10, after providing both the primary and co-stimulatory signals that are required for activation and expansion of T cells via aCD3/CD28 beads. The reduced proliferation rate was visible only when using LV.eGFP and an MOI 10, while untransduced cells treated with the LB molecule (Fig. 4E) and cells transduced with an MOI 1 (Fig. S3) did not show impaired proliferation upon aCD3/CD28 stimulation (Fig S4). The effect on proliferation coincided with an increase in VCN in the transduced T cells in the presence of LB, possibly caused by genotoxic stress in the CB-CD8+ T cells, supported by the observation that CB CD8+ T cells transduced at an MOI 1 did not show a decreased proliferation rate. As previously reported, self-inactivating LV have the tendency to integrate into transcriptionally active genes in T cells[56], potentially causing genotoxic effects at higher vector copy numbers. Interestingly, the decrease in proliferation was not visible when CB CD8+ T cells where transduced with a TAA-specific TCR in the presence of LB, and stimulated with antigen specific tetramers. Moreover, WT1-TCR CB CD8+ T cells, transduced in the presence of LB, did not show any impairment in terms of functionality as assessed by killing capacity and cytotoxic cytokines produced (Fig. 5 E-G).

In conclusion, we present an efficient expansion and transduction method for CB CD8+ T cells using lentiviral vectors that is GMP-compliant. Furthermore, this method shows better transduction of the total fraction CB CD8+ T cells compared to the total fraction of PB CD8+ T cells, while preserving the CB naïve phenotypic markers, increasing the chance to generate a cellular product that will persist and proliferate in vivo. Additionally, we confirmed the use of the LB as a valid transduction enhancer for CB CD8+ T cells in the field of gene and cell therapy. Overall, these results underline the possibilities and advantages of using gene-modified CB CD8+ T cells as a source for future application in cancer cell therapy.

## ACKNOWLEDGMENTS

The authors thank the staff of the Flow Core Facility (Center for Translational Immunology, UMCU, The Netherlands), especially Jeroen van Velzen for the support and help in the sample analysis. The authors also thank the women for donating the CB and the nurses (Wilhelmina's Children Hospital, UMCU, the Netherlands) for collecting it. The work done for the manuscript was supported by the Dutch Cancer Society (KWF - grant number: 10474).



## AUTHOR CONTRIBUTIONS

VLP designed and conducted the experiments, analyzed the data, and wrote the manuscript. AC and MP contributed to the execution of experiments and the writing of the manuscript. ED helped in performing experiments. JK and JJB scientifically contributed to the design of the project and the manuscript. NvT and SN designed and supervised the experimental phase and contributed to drafting the manuscript.

## CONFLICT OF INTEREST

NvT is employee of AVROBIO, Cambridge, MA, USA. The other authors have no potential conflicts of interest. JK is cofounder and shareholder of Gadeta. JK is inventor on multiple patents dealing with γδTCR topics as well as isolation of engineered immune cells. JK received research support from Miltenyi Biotech, Novartis and Gadeta

## BIBLIOGRAPHY


1. Dudley, M.E., Wunderlich, J.R., Shelton, T.E., Even, J., and Rosenberg, S.A. (2003). Generation of Tumor-Infiltrating Lymphocyte Cultures for Use in Adoptive Transfer Therapy for Melanoma Patients. J. Immunother. 26, 332–342.
2. Rosenberg, S. a., Yang, J.C., Sherry, R.M., Kammula, U.S., Hughes, M.S., Phan, G.Q., Citrin, D.E., Restifo, N.P., Robbins, P.F., Wunderlich, J.R., et al. (2011). Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin. Cancer Res. 17, 4550–4557.
3. Dudley, M.E., Yang, J.C., Sherry, R., Hughes, M.S., Royal, R., Kammula, U., Robbins, P.F., Huang, J., Citrin, D.E., Leitman, S.F., et al. (2008). Adoptive cell therapy for patients with metastatic melanoma: Evaluation of intensive myeloablative chemoradiation preparative regimens. J. Clin. Oncol. 26, 5233–5239.
4. Lim, W.A., and June, C.H. (2017). The Principles of Engineering Immune Cells to Treat Cancer. Cell 168, 724–740.
5. June, C.H. (2018). Chimeric Antigen Receptor Therapy. N. Engl. J. Med.
6. Grigor, E.J.M., Fergusson, D., Kekre, N., Montroy, J., Atkins, H., Seftel, M.D., Daugaard, M., Presseau, J., Thavorn, K., Hutton, B., et al. (2019). Risks and Benefits of Chimeric Antigen Receptor T-Cell (CAR-T) Therapy in Cancer: A Systematic Review and Meta-Analysis. Transfus. Med. Rev. 33, 98–110.
7. European Medicines Agency: CHMP (2018). Assessment Report - Kymriah.
8. Leary, M.O., Przepiorka, D., George, B., and Theoret, M. (2017). BLA Clinical Review Memorandum.
9. Zhang, J., and Wang, L. (2019). The emerging world of TCR-T cell trials against cancer: A systematic review. Technol. Cancer Res. Treat. 18, 1–13.
10. Morgan, R.A., Dudley, M.E., Wunderlich, J.R., Hughes, M.S., Yang, J.C., Sherry, R.M., Royal, R.E., Topalian, S.L., Kammula, U.S., Restifo, N.P., et al. (2006). Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 314, 126–9.
11. Rapoport, A.P., Stadtmauer, E.A., Binder-Scholl, G.K., Goloubeva, O., Vogl, D.T., Lacey, S.F., Badros, A.Z., Garfall, A., Weiss, B., Finklestein, J., et al. (2015). NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. Nat. Med. 21, 914–921.
12. Chapuis, A.G., Egan, D.N., Bar, M., Schmitt, T.M., McAfee, M.S., Paulson, K.G., Voillet, V., Gottardo, R., Ragnarsson, G.B., Bleakley, M., et al. (2019). T cell receptor gene therapy targeting WT1 prevents acute myeloid leukemia relapse post-transplant. Nat. Med.
13. Gröbner, S.N., Worst, B.C., Weischenfeldt, J., Buchhalter, I., Kleinheinz, K., Rudneva, V.A., Johann, P.D., Balasubramanian, G.P., Segura-Wang, M., Brabetz, S., et al. (2018). The landscape of genomic alterations across childhood cancers. Nature 555, 321–327.
14. Zufferey, R., Dull, T., Mandel, R.J., Bukovsky, a, Quiroz, D., Naldini, L., and Trono, D. (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J. Virol. 72, 9873–80.

15. Graham, C., Jozwik, A., Pepper, A., and Benjamin, R. (2018). Allogeneic CAR-T Cells : More than Ease of Access ? Cells, 1–11.

16. Mackall, C.L., Fleisher, T.A., Brown, M.R., Magrath, I.T., Shad, A.T., Horowitz, M.E., Wexler, L.H., Adde, M.A., McClure, L.L., and Gress, R.E. (1994). Lymphocyte depletion during treatment with intensive chemotherapy for cancer. Blood 84, 2221–2228.

17. Stahnke, K., Fulda, S., Friesen, C., Strauß, G., and Debatin, K.M. (2001). Activation of apoptosis pathways in peripheral blood lymphocytes by in vivo chemotherapy. Blood 98, 3066–3073.

18. Mackall, B.C.L., Fleisher, T.A., Brown, M.R., Andrich, M.P., Chen, C.C., Feuerstein, I.M., Magrath, I.T., Wexler, L.H., Dimitrov, D.S., and Gress, R.E. (1997). Distinctions Between CD8+ and CD4+ T-Cell Regenerative Pathways Result in Prolonged T-Cell Subset Imbalance After Intensive Chemotherapy. Blood.

19. Fagnoni, F.F., Lozza, L., Zibera, C., Zambelli, A., Gibelli, N., Oliviero, B., Pavesi, L., Gennari, R., Vescovini, R., Sansoni, P., et al. (2002). T-cell dynamics after high-dose chemotherapy in adults : elucidation of the elusive CD8 + subset reveals multiple homeostatic T-cell compartments with distinct implications for immune competence. Immunology.

20. Onyema, O.O., Decoster, L., Njemini, R., Forti, L.N., Bautmans, I., Waele, M.D.E., and Mets, T. (2015). Chemotherapy-induced Changes and Immunosenescence of CD8 + T-Cells in Patients with Breast Cancer. Anticancer Res. 1490, 1481–1489.

21. Paloczi, K. (1999). Immunophenotypic and functional characterization of human umbilical cord blood mononuclear cells. Leukemia 13, 87–89.

22. Lo Presti, V., Nierkens, S., Boelens, J.J., and Van Til, N.P. (2018). Use of cord blood derived T-cells in cancer immunotherapy: milestones achieved and future perspectives. Expert Rev. Hematol. 00, 1–10.

23. Hiwarkar, P., Qasim, W., Ricciardelli, I., Gilmour, K., Quezada, S., Saudemont, A., Amrolia, P., and Veys, P. (2017). Cord blood T cells mediate enhanced antitumor effects compared with adult peripheral blood T cells. Blood 126, 2882–2892.

24. Oki, M., Ando, K., Hagihara, M., Miyatake, H., Shimizu, T., Miyoshi, H., Nakamura, Y., Matsuzawa, H., Sato, T., Ueda, Y., et al. (2001). Efficient lentiviral transduction of human cord blood CD34+ cells followed by their expansion and differentiation into dendritic cells. Exp. Hematol. 29, 1210–1217.

25. Hauber, I., Beschorner, N., Schrödel, S., Chemnitz, J., Kröger, N., Hauber, J., and Thirion, C. (2018). Improving Lentiviral Transduction of CD34+ Hematopoietic Stem and Progenitor Cells. Hum. Gene Ther. Methods 29, hgtb.2017.085.

26. Liu, Y., Hangoc, G., Campbell, T.B., Goodman, M., Tao, W., Pollok, K., Srour, E.F., and Broxmeyer, H.E. (2008). Identification of paramenters required for efficient lentiviral vector transduction and engraftment of human cord blood CD34+ NOD/SCID repopulating cells. Exp. Hematol. 36, 947–956.

27. Evans, J.A.Y.T., Kelly, P.F., Neill, E.O., and Garcia, J.V. (1999). Human Cord Blood CD34+ CD38 - Cell Transduction via Lentivirus-Based Gene Transfer Vectors. Hum. Gene Ther. 1489, 1479–1489.

28. Serrano, L.M., Pfeiffer, T., Olivares, S., Numbenjapon, T., Bennitt, J., Kim, D., Smith, D.,

McNamara, G., Al-Khadimi, Z., Rosenthal, J., et al. (2005). Differentiation of naive cord blood T cells into CD19-specific cytolytic effectors for post-transplant adoptive immunotherapy. Blood 107, 2643–2653.

29. Micklethwaite, K.P., Savoldo, B., Hanley, P.J., Leen, A.M., Demmler-Harrison, G.J., Cooper, L.J.N., Liu, H., Gee, A.P., Shpall, E.J., Rooney, C.M., et al. (2010). Derivation of human T lymphocytes from cord blood and peripheral blood with antiviral and antileukemic specificity from a single culture as protection against infection and relapse after stem cell transplantation. Blood 115, 2695–2703.

30. Pegram, H.J., Purdon, T.J., van Leeuwen, D.G., Curran, K.J., Giralt, S. a, Barker, J.N., and Brentjens, R.J. (2015). IL-12-secreting CD19-targeted cord blood-derived T cells for the immunotherapy of B-cell acute lymphoblastic leukemia. Leukemia 29, 415–22.

31. Frumento, G., Zheng, Y., Aubert, G., Raeiszadeh, M., Lansdorp, P.M., Moss, P., Lee, S.P., Chen, F.E., and Lee, S.P. (2013). Cord Blood T Cells Retain Early Differentiation Phenotype Suitable for Immunotherapy After TCR Gene Transfer to Confer EBV Specificity. Am. J. Transplant. 13, 45–55.

32. Cieri, N., Camisa, B., Cocchiarella, F., FOrcato, M., Oliviera, G., Provasi, E., Bondanza, A., Bordignon, C., Peccatori, J., Ciceri, F., et al. (2013). IL-7 and IL-15 instruct the generation of human memory stemTcells from naive precursors. Blood 121, 573–585.

33. Tammana, S., Huang, X., Wong, M., Milone, M.C., Ma, L., Levine, B.L., June, C.H., Wagner, J.E., Blazar, B.R., and Zhou, X. (2010). 4-1BB and CD28 Signaling Plays a Synergistic Role in Redirecting Umbilical Cord Blood T Cells Against B-Cell Malignancies. Hum. Gene Ther. 21, 75–86.

34. Delville, M., Soheili, T., Bellier, F., Durand, A., Denis, A., Lagresle-peyrou, C., Cavazzana, M., Andre-schmutz, I., and Six, E. (2018). A Nontoxic Transduction Enhancer Enables Highly Efficient Lentiviral Transduction of Primary Murine T Cells and Hematopoietic Stem Cells. Mol. Ther. Methods Clin. Dev. 10, 341–347.

35. Cavalieri, S., Cazzaniga, S., Geuna, M., Magnani, Z., Bordignon, C., Naldini, L., and Bonini, C. (2003). Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. Blood 102, 497–505.

36. Jones, S., Peng, P.D., Yang, S., Hsu, C., Cohen, C.J., Zhao, Y., Abad, J., Zheng, Z., Rosenberg, S. a, and Morgan, R. a (2009). Lentiviral vector design for optimal T cell receptor gene expression in the transduction of peripheral blood lymphocytes and tumor-infiltrating lymphocytes. Hum. Gene Ther. 20, 630–640.

37. Frecha, C., Costa, C., Nègre, D., Gauthier, E., Russell, S.J., Cosset, F.L., and Verhoeyen, E. (2008). Stable transduction of quiescent T cells without induction of cycle progression by a novel lentiviral vector pseudotyped with measles virus glycoproteins. Blood 112, 4843–4852.

38. Ballen, K.K., Gluckman, E., and Broxmeyer, H.E. (2014). Umbilical cord blood transplantation : the first 25 years and beyond. Blood 122, 491–499.

39. Boelens, J.J. (2016). The power of cord blood cells. Blood 127, 3300–3302.

40. Barker, J.N., Davies, S.M., DeFor, T., Ramsay, N.K.C., Weisdorf, D.J., and Wagner, J.E. (2001). Survival after transplantation of unrelated donor umbilical cord blood is comparable to that

of human leukocyte antigen-matched unrelated donor bone marrow: Results of a matched-pair analysis. Blood 97, 2957–2961.

41. Azuma, H., Yamada, Y., Shibuya-Fujiwara, N., Yamaguchi, M., Murahashi, H., Fujihara, M., Sato, N., Fukazawa, K., Ikebuchi, K., and Ikeda, H. (2002). Functional evaluation of ex vivo expanded cord blood lymphocytes. Exp. Hematol. 30, 346–351.
42. Okas, M., Gertow, J., Uzunel, M., Karlsson, H., Westgren, M., Karre, K., Ringden, O., Mattsson, J., and Uhlin, M. (2010). Clinical expansion of cord blood-derived T cells for use as donor lymphocyte infusion after cord blood transplantation. J Immunother 33, 96–105.
43. Parmar, S., Robinson, S.N., Komanduri, K., John, L.S., Decker, W., Xing, D., Yang, H., Mcmannis, J., Champlin, R., Lima, M. De, et al. (2006). Ex vivo expanded umbilical cord blood T cells maintain naive phenotype and TCR diversity. Cytotherapy 8, 149–157.
44. Kwoczek, J., Riese, S.B., Tischer, S., Bak, S., Lahrberg, J., Oelke, M., Maul, H., Blasczyk, R., Sauer, M., and Eiz-Vesper, B. (2018). Cord blood–derived T cells allow the generation of a more naïve tumor-reactive cytotoxic T-cell phenotype. Transfusion 58, 88–99.
45. Berglund, S., Gertow, J., Uhlin, M., and Mattsson, J. (2014). Expanded umbilical cord blood T cells used as donor lymphocyte infusions after umbilical cord blood transplantation. J. Cytotherapy 16, 1528–1536.
46. Balassa, K., and Rocha, V. (2017). Anticancer cellular immunotherapies derived from umbilical cord blood. Expert Opin. Biol. Ther. 18, 1–14.
47. Sabatino, M., Hu, J., Sommariva, M., Gautam, S., Fellowes, V., Hocker, J.D., Dougherty, S., Qin, H., Klebanoff, C.A., Fry, T.J., et al. (2016). Generation of clinical-grade CD19-specific CAR-modified CD81 memory stem cells for the treatment of human B-cell malignancies. Blood 128, 519–528.
48. Gattinoni, L., Klebanoff, C.A., Palmer, D.C., Wrzesinski, C., Kerstann, K., Yu, Z., Finkelstein, S.E., Theoret, M.R., Rosenberg, S.A., and Restifo, N.P. (2005). Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. J. Clin. Invest. 115, 1616–1626.
49. Klebanoff, C.A., Gattinoni, L., Torabi-Parizi, P., Kerstann, K., Cardones, A.R., Finkelstein, S.E., Palmer, D.C., Antony, P.A., Hwang, S.T., Rosenberg, S.A., et al. (2005). Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. Proc. Natl. Acad. Sci. 102, 9571–9576.
50. Robbins, P.F., Dudley, M.E., Wunderlich, J., El-Gamil, M., Li, Y.F., Zhou, J., Huang, J., Powell, D.J., and Rosenberg, S.A. (2004). Cutting Edge: Persistence of Transferred Lymphocyte Clonotypes Correlates with Cancer Regression in Patients Receiving Cell Transfer Therapy. J. Immunol. 173, 7125–7130.
51. Hinrichs, C.S., Borman, Z. a, Gattinoni, L., Yu, Z., Burns, W.R., Klebanoff, C. a, Johnson, L. a, Kerkar, S.P., Yang, S., Muranski, P., et al. (2011). Human effector CD8 + T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy. Gene Th 117, 808–814.
52. Sommermeyer, D., Hudecek, M., Kosasih, P.L., Gogishvili, T., Maloney, D.G., Turtle, C.J., and Riddell, S.R. (2016). Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity in vivo. Leukemia 30, 492–500.

53. Costa, C., Hypolite, G., Bernadin, O., Lévy, C., Cosset, F.-L., Asnafi, V., Macintyre, E., Verhoeyen, E., and Tesio, M. (2017). Baboon envelope pseudotyped lentiviral vectors: a highly efficient new tool to genetically manipulate T-cell acute lymphoblastic leukaemia-initiating cells. Leukemia 31, 977–980.

54. Uchida, N., Nassehi, T., Drysdale, C.M., Gamer, J., Yapundich, M., Demirci, S., Haro-Mora, J.J., Leonard, A., Hsieh, M.M., and Tisdale, J.F. (2019). High-Efficiency Lentiviral Transduction of Human CD34 + Cells in High-Density Culture with Poloxamer and Prostaglandin E2. Mol. Ther. - Methods Clin. Dev. 13, 187–196.

55. Simon, B., Harrer, D.C., Thirion, C., Schuler-Thurner, B., Schuler, G., and Uslu, U. (2019). Enhancing lentiviral transduction to generate melanoma-specific human T cells for cancer immunotherapy. J. Immunol. Methods 472, 55–64.

56. Field, A.C., Vink, C., Gabriel, R., Al-Subki, R., Schmidt, M., Goulden, N., Stauss, H., Thrasher, A., Morris, E., and Qasim, W. (2013). Comparison of Lentiviral and Sleeping Beauty Mediated αβ T Cell Receptor Gene Transfer. PLoS One 8.

57. Schambach, A., Bohne, J., Baum, C., Hermann, F.G., Egerer, L., von Laer, D., and Giroglou, T. (2006). Woodchuck hepatitis virus post-transcriptional regulatory element deleted from X protein and promoter sequences enhances retroviral vector titer and expression. Gene Ther. 13, 641–645.

58. Van Til, N.P., Sarwari, R., Visser, T.P., Hauer, J., Lagresle-Peyrou, C., Van Der Velden, G., Malshetty, V., Cortes, P., Jollet, A., Danos, O., et al. (2014). Recombination-activating gene 1 (Rag1)-deficient mice with severe combined immunodeficiency treated with lentiviral gene therapy demonstrate autoimmune Omenn-like syndrome. J. Allergy Clin. Immunol. 133, 1116–1123.

59. Tiscornia, G., Singer, O., and Verma, I.M. (2006). Production and purification of lentiviral vectors. Nat. Protoc. 1, 241–245.

60. Singer-Sam, J., Keith, D.H., Tani, K., Simmer, R.L., Shively, L., Lindsay, S., Yoshida, A., and Riggs, A.D. (1984). Sequence of the promoter region of the gene for human X-linked 3-phosphoglycerate kinase. Gene 32, 409–417.

61. Li, H., and Ginzburg, Y.Z. (2010). Crosstalk between Iron Metabolism and Erythropoiesis. Adv. Hematol. 2010, 605435.

62. Logan, A.C., Nightingale, S.J., Haas, D.L., Cho, G.J., Pepper, K.A., and Kohn, D.B. (2004). Factors influencing the titer and infectivity of lentiviral vectors. Hum. Gene Ther. 15, 976–988.

63. Wolfl, M., Ho, W.Y., Nguyen, H., Manley, T.J., Bleakley, M., Greenberg, P.D., and Kuball, J. (2007). Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiring knowledge of epitope specificities. Blood 110, 201–210.

64. Kuball, J., Dossett, M.L., Wolfl, M., Ho, W.Y., Voss, R.H., Fowler, C., and Greenberg, P.D. (2007). Facilitating matched pairing and expression of TCR chains introduced into human T cells. Blood 109, 2331–2338.

65. Rodenko, B., Toebes, M., Hadrup, S.R., van Esch, W.J.E., Molenaar, A.M., Schumacher, T.N.M., and Ovaa, H. (2006). Generation of peptide–MHC class I complexes through UV-mediated ligand exchange. Nat. Protoc. 1, 1120–1132.

## SUPPLEMENTAL INFORMATION

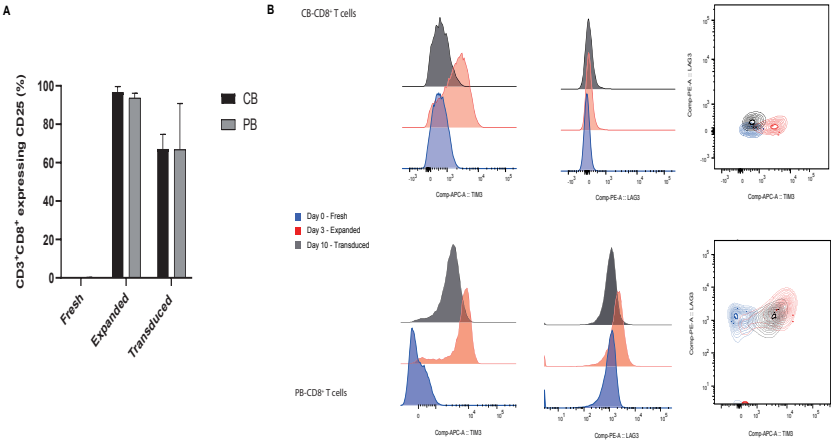



**Figure S1. Expression of CD25, TIM3 and LAG3 in CB and PB CD8+T cells**

(A) CD25 expression in CB (black) and PB (gray). (B) TIM3 and LAG3 expression histograms in both CB (top) and PB (bottom) CD3+CD8+ T cells, in the three different time points: Day 0 – Fresh (blue), Day 3 - Expanded (red), Day 10 – Transduced (black). (C) Co-staining of TIM-3 and LAG-3 representative plots for both CB and PB CD3+CD8+ T cells. The data corresponds to ≥ 3 independent experiments and are shown as average ± standard deviation.

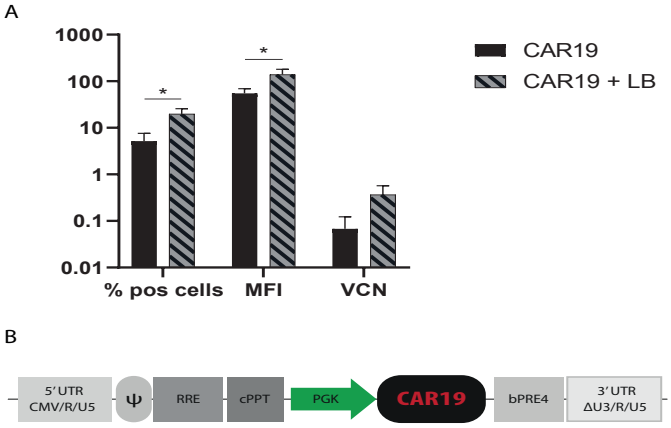



**Figure S2. LB increase transduction efficiency also with LV.CAR19 construct**

(A) CB CD8+ T cells transduced with LV.CAR19 (MOI1) showed 5.2% ± 2.5 transduction efficiency (black), while the use of LB increased the % of transduced cells to 19.9 ± 5.9 (black stripes). The same trend is also visible for the MFI and the VCN. (B) Schematic representation of plasmid LV.CAR19 vector design. The data corresponds to ≥ 3 independent experiments and are shown as average ± standard deviation; $p \leq 0.05$ (*); $p \leq 0.01$ (**) ; $p \leq 0.001$ (***); $p \leq 0.0001$ (****).

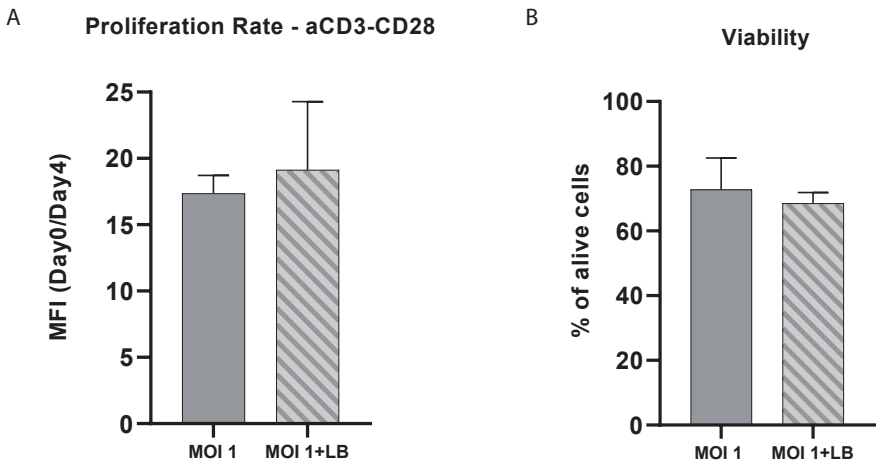





**Figure S3. LB effect on proliferation and cell viability on CB CD8+ T cells transduced with an MOI 1**

(A) Proliferation rate after 4 days of aCD3/CD28 beads stimulation in CB CD8+ T cells transduced with and MOI 1 in the presence of LB (gray stripes) compared to STND method (gray). (B) Cell viability analysis, 4 days after aCD3/CD28 beads stimulation, of CB CD8+ T cells transduced with and MOI 1 in the presence of LB (gray stripes) compared to STND method (gray). The data corresponds to ≥ 3 independent experiments and are shown as average ± standard deviation; p ≤ 0.05 (*); p ≤ 0.01 (**) ; p ≤ 0.001 (***); p ≤ 0.0001 (****).

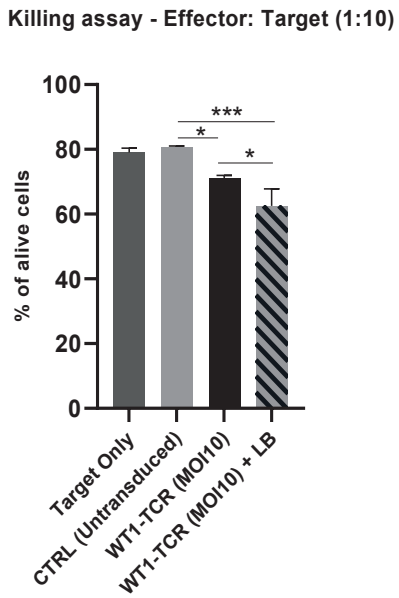



**Figure S4**

Killing capacity of WT1-TCR CB CD8+ T cells transduced, in the presence or absence of LB, depicted as % of alive cells in a co-culture experiment with target cells loaded with pWT1126. Co-culture experiments were performed in a ratio of 10:1 (Target:Effector). The data corresponds to ≥ 3 independent experiments and are shown as average ± standard deviation; p ≤ 0.05 (*); p ≤ 0.01 (**) ; p ≤ 0.001 (***); p ≤ 0.0001 (****).

# CLINICAL GRADE PRODUCTION OF WILMS' TUMOR-1 LOADED CORD BLOOD-DERIVED DENDRITIC CELLS TO PREVENT RELAPSE IN PEDIATRIC AML AFTER CORD BLOOD TRANSPLANTATION

4

Maud Plantinga[1], Vania Lo Presti[1], Colin G. de Haar[2], Ester Dünnebach[1], Alejandro Madrigal[3], Caroline A. Lindemans[4], Jaap Jan Boelens[5] and Stefan Nierkens[1,4]*

[1] Center for Translational Immunology, University Medical Center Utrecht, Utrecht, Netherlands

[2] Pharmacy Department, Cell Therapy Facility, University Medical Center Utrecht, Utrecht, Netherlands

[3] UCL Cancer Institute, Royal Free Hospital,London, United Kingdom

[4] Princess Maxima Center for Pediatric Oncology, Blood and Marrow Transplantation Program, Utrecht, Netherlands

[5] Stem Cell Transplant and Cellular Therapies, Department of Pediatrics, Memorial Sloan Kettering Cancer Center, New York, NY, United States

*Frontiers in Immunology, 2019*

## ABSTRACT

Hematopoietic cell transplantation (HCT) is a last resort, potentially curative treatment option for pediatric patients with refractory acute myeloid leukemia (AML). Cord blood transplantation (CBT) results in less relapses and less graft-versus-host disease when compared to other sources. Nevertheless, still more than half of the children die from relapses. We therefore designed a strategy to prevent relapses by inducing anti-AML immunity after CBT, using a CB-derived dendritic cell (CBDC) vaccine generated from CD34+ CB cells from the same graft. We here describe the optimization and validation of good manufacturing practice (GMP)-grade production of the CBDC vaccine. We show the feasibility of expanding low amounts of CD34+ cells in a closed bag system to sufficient DCs per patient for at least three rounds of vaccinations. The CBDCs showed upregulated costimulatory molecules after maturation and showed enhanced CCR7-dependent migration toward CCL19 in a trans-well migrations assay. CBDCs expressed Wilms' tumor 1 (WT1) protein after electroporation with WT1-mRNA, but were not as potent as CBDCs loaded with synthetic long peptides (peptivator). The WT1-peptivator loaded CBDCs were able to stimulate T-cells both in a mixed lymphocyte reaction as well as in an antigen-specific (autologous) setting. The autologous stimulated T-cells lysed not only the WT1+ cell line, but most importantly, also primary pediatric AML cells. Altogether, we provide a GMP-protocol of a highly mature CBDC vaccine, loaded with WT1 peptivator and able to stimulate autologous T-cells in an antigen-specific manner. Finally, these T cells lysed primary pediatric AML demonstrating the competence of the CBDC vaccine strategy.

**Keywords:** dendritic cells, vaccine, cord blood, immunotherapy, transplantation, good manufacturing practice

## INTRODUCTION

Acute myeloid leukemia (AML) is the second most prevalent leukemia (15%–20%) with a 5-year survival rate in children of ~50%–60% [1]. As a last treatment resort, patients with certain high-risk cytogenetic features (e.g. FLT3-ITD without concomitant NPM1)[2] and therapy-refractory patients or patients with relapsed AML who achieve CR2 after reinduction therapy are eligible for allogeneic hematopoietic cell transplantation (allo-HCT) [3]. Nevertheless, despite the better survival rates, a clear unmet need to further reduce the relapse rates and enhance survival post HCT remains for high risk AML patients. Maintenance, preemptive or salvage therapeutic strategies have been explored to prevent relapse post allo-HCT, e.g. FMS-like tyrosine kinase 3 (FLT3) inhibitors, Isocitrate Dehydrogenase (IDH) Inhibitors, Hypomethylating Agents and HDAC Inhibitors and more recently also cellular therapies[4]. A promising cellular therapeutic strategy to induce specific anti-AML immunity early after HCT is dendritic cell (DC) vaccination. In the past, mainly monocyte-derived (mo)DC based vaccines or peptides were tested in clinical trials[5]. However, the numbers of monocytes and antigen-presenting cells, in particular DC, are very low after transplant which very much limits the potential success of vaccination strategies at these early time points. The use of Cord blood (CB) as stem cell source for allo-HCT specifically in children, has important advantages over either bone marrow (BM) or mobilized peripheral blood (PB) stem cells, since CB produce a more powerful graft-versus-leukemia effect[6–9]; the T-cell reconstitution (in particular CD4+ cells) is excellent in absence or with personalized dosing of antithymocyte globulin (ATG) and contains mostly naïve T-cells that seem to transform rapidly into effector T-cells[10, 11]. The reduced risk of developing graft versus host disease (GvHD)[10, 12, 13] and viral reactivation after HCT without serotherapy have supported the use of CB as a cell source for transplantation[10, 12, 14, 15]. In addition, this provides an autologous source for stem cell-derived DC for vaccination after HCT.

We previously designed a protocol which enables to generate DC from the 20% fraction of a CB unit (the 80% fraction is used to transplant the patient). In short, enriched CD34+ CB-derived stem cells were expanded for 1 week and differentiated for an additional week, followed by maturation using an inflammatory cytokine mix and thereafter electroporated with Wilms' tumor 1 (WT1) mRNA[16]. The protocol generated CBDC in sufficient numbers as used in previous moDC vaccination studies (total dose in adults 0.1–20×10^6)[17–19]. We used WT1 as a tumor-specific target as it is overexpressed in the majority (>80-90%) of patients with AML, including cellcycle quiescent AML stem cells located in the BM[20]. In addition, younger subjects with AML

showed more frequent recurrent mutations in WT1 than adults[21].

In a recent study, Chapuis *et al.* inserted a WT1-specific TCR (C4) into Epstein-Bar virus-specific donor CD8+ T-cells from healthy donors and infused these cells prophylactically post-HCT into 12 adult AML patients with encouraging results[22].

This study supports the rationale to stimulate WT1-specific T-cell responses post-HCT to reduce relapse rates. We here describe the translation of a preclinical DC culture protocol to a good manufacturing practices (GMP)-setting using a closed bag culture system. We compared the use of peptivator, consisting of lyophilized long peptides covering the complete sequence of the human WT1 protein, with the introduction of a GMP-grade WT1-mRNA construct for electroporation to ensure the expression of the full length WT1 protein. The potency to select and stimulate WT1-specific T-cells from CB and subsequently lyse primary pediatric AML cells by these T-cells were studied as functional outcomes.

## MATERIALS AND METHODS

### CB COLLECTION AND CD34 ISOLATION

Umbilical cord bloods (CB) were obtained from the Anthony Nolan Cord Blood bank (UK) with inform consent in accordance with the Human Tissue Act 2004, the Human Tissue Regulations 2007 and the HTA's Code of Practice in the United Kingdom. The CB was shipped using a dry-shipper and stored in the vapor phase of a dedicated controlled and continuously monitored liquid nitrogen tank to the production site until thawing. CB was thawed using thaw-medium (CliniMACS® buffer with HSA, MgCL2 and Pulmozyme) in a transfer bag. Cells were collected in tubes and after washing twice, the cells were labeled with magnetic-anti CD34 beads, washed to remove unbound beads and thereafter the labeled cells were resuspended in a transfer bag and enriched using the CliniMACS device (Miltenyi Biotec).



### CBDC CULTURE

The two-step protocol consists of an expansion and differentiation phase and is performed in the GMP accredited Cell Therapy Facility of the University Medical Center Utrecht. For the first week of expansion $5\times10^4$ CD34+ cells/ml are cultured and the second week $1\times10^5$ CD34+ cells/ml are cultured in X-VIVO 15 (Lonza) supplemented with Flt3L (50 IU/ ml), SCF (50 IU/ml), IL-3 (80 IU/ml) and IL-6 (2,400 IU/ml). In the 3rd and 4th test run 5-10% human serum is added to increase cell yield. After washing, the cells are differentiated at $1\times10^5$ cells/ ml in X-VIVO 15 containing 5% human serum and supplemented with Flt3L (50 IU/ml), SCF (20 IU/ml), GMCSF (280 IU/ml) and IL-4 (240 IU/ml) for another week[23]. To induce maturation the cytokines used for differentiation plus an inflammatory cytokine mix, a combination of IL-1beta (700 IU/ ml), IL-6 (2,400 IU/ml), TNFa (350 IU/ml), and PGE2 (1 µg/ml) from Pfizer, was added to the culture for 24 h with or without WT1 peptivator (0,12 nmol/ml). The WT1 peptivator contains a pool of lyophilized overlapping oligopeptides, covering the complete sequence of the WT1 (Swiss-prot Acc.no.P19544). Overall, cells are thawed, enriched, expanded and differentiated in bags. Between different culture phases, e.g. expansion differentiation and maturation, cells were collected in 50 or 250 ml centrifuge tubes, spun down and resuspended in a small volume new X-VIVO 15 in a sterile flask, including the cytokines of either expansion, differentiation or maturation. Cell counting was performed using trypan blue. Medium was added

to obtain the correct concentration and the cell suspension was transferred to a MACS® Cell Differentiation Bag (CDB; Miltenyi) via a luer lock system. All GMP-grade recombinant human cytokines, growth factors and WT1 Peptivator® were obtained from Miltenyi Biotec. In total 10 CB donors were used, five for the optimization runs and five for the validation runs. At the end of the procedure WT1-loaded CBDCs were frozen per 10–15×$10^6$ cells/vial in freezing medium (70% X-VIVO 15, 20% human serum and 10% DMSO) at −196°C. For further use the vial was thawed in 50% human serum and 50% X-VIVO 15 medium at 37°C.

## ELECTROPORATION AND WT1 DETECTION



Mature CBDC were loaded with WT1-encoding mRNA (AmpTec GmbH) by electroporation (EP) as previously described[24], with minor modifications. Briefly, 5–10×$10^6$ cells in 200 µl OptiMEM medium were transferred to a 4-mm electroporation cuvette (Bio-Rad, Hercules, CA, USA) and electroporated with 9,5 ug RNA by a time constant pulse of 300 V for 7 ms using the Gene Pulser Xcell device (Bio-Rad). After EP the cells recovered for 4h in the medium used to mature the cells. Next, WT1 expression was determined. Cells were washed with FACS buffer (PBS containing 2% BSA (Sigma- Aldrich) and 0.1% sodium azide (NaN3, Sigma-Aldrich) prior to a 15 min incubation at room temperature (RT) in the dark with a fixable viability dye (Thermo fisher). Next, cells were washed and fixed/permeabilized (eBioscience) for 30 min at 4°C. After washing with permeabilization buffer, anti-WT1 (F6-H2;(Dako) or purified mouse IgG1k (MG1-45; Biolegend) as isotype control and FcR Blocking (Miltenyi Biotec) was added for 15 min at RT in de dark. The cells were washed again with permeabilization buffer before adding the F(ab')2 Anti-mouse IgG PE antibody (eBioscience) for 15 min at RT in the dark. Cells are resuspended in FACS buffer and analyzed using a FACS CantoII (BD) flow cytometer. Analysis was performed using FlowJo software (Tree Star, Inc.).

## QUALITY CONTROL: MICROBIOLOGY

The cells were carefully monitored by morphology during the entire culture period, and sterility testing of vaccines was performed. Mycoplasma was analyzed by the Nucleic acid amplification technique (NAT) by Polymerase Chain Reactions (PCR). Endotoxin was assessed by the kinetic chromogenic limulus amoebocyte lysate (LAL) test (Lonza).

## DC PHENOTYPE

To assess DC phenotype, cells were collected from the bags and washed twice with FACS buffer. Thereafter, the cells were incubated at 4°C and stained with the appropriate antibody combinations. Antibodies used for flow cytometry for DC phenotype and purity include: HLA-DR, CD45, CCR7 (from Biolegend), CD11b, CD15, CD16, CD33, CD56, 7-AAD, CD14, CD3, CD83, CD80, CD11c (all from BD), CD14, CD19, and CD117 (from Beckman & Coulter). For intracellular (IC) stainings, cells were washed with FACS buffer after surface staining and treated with Cytofix/Cytoperm (BD), according to the manufacturer's protocol, followed by 30 min of incubation at 4°C with the following Abs for the non-DC fraction: cytoplasmic Myeloperoxidase (MPO; Dako). Multiparameter analysis was performed on a FACS Canto II or LSR Fortessa II (BD) flow cytometer. Dead cells were excluded by scatter gating. Analysis was performed using DIVA (BD) or FlowJo software (Tree Star, Inc.).



## TRANSWELL MIGRATION ASSAY

In vitro migration assays were performed using 24 transwell (3µm pore size) plates (Greiner). In brief, 400.000 CBDCs in 200 ul culture medium (X-VIVO 15 with 5% human serum) were plated in the upper compartment. Culture medium, either alone or supplemented with 250ng/ml CCL19 (R&D systems), was added to the lower compartment. After 2 h, DCs were collected from the lower compartment. The cells are washed and stained for CD11c, HLA-DR and CD83 and analyzed using flow cytometry in a fixed volume. Counts measured by flow were used to validate migration.

## MIXED LEUKOCYTE REACTION

CD3 cells were purified from allogenic CD34- cells using anti- CD3 magnetic microbeads (Miltenyi). These responder CD3 cells ($1x10^6$/ml) were then labeled with cell trace violet (5 µM; Invitrogen), and cocultured with matured CBDCs ($2x10^5$/ml) as stimulator cells. Unstimulated cell trace violet-labeled cells served as negative control. After 4 or 5 days, cells were stained with CD3, CD8, CCR7 (Biolegend), CD4 (Ebioscience), CD45RO (BD), and CD69 (Sony) and analyzed using a FACS LSR Fortessa (BD). T-cell proliferation analysis was performed using the proliferation tool in flowjo (Tree Star,Inc.), providing the division index, the average number of cell divisions of one cell in the original population.

## WT1 ANTIGEN PRESENTATION

WT1 mRNA electroporated CBDCs in combination with WT1 peptivator, or peptivator loaded DCs alone or DC alone were co-cultured with $1\times10^6$/ml of our previously developed HLA- A2–restricted WT1-specific T-cell clone recognizing the $WT1_{37-45}$ (VLDFAPPGA) epitope at a DC-to-T-cell ratio of 1:1 for 5 h in the presence of Golgi-stop (1/1500; BD). T2-cells loaded with/without WT1-peptivator (Miltenyi) were used as a respectively positive and negative control. The T-cells were subsequently stained for CD3 (Biolegend), CD8 and extracellular expression of LAMP-1 (BD) and intracellular IFNg (Ebioscience) expression, followed by flow cytometry– based analysis.



## ISOLATION, EXPANSION, AND IDENTIFICATION OF WT1-SPECIFIC T-CELLS

CBDC culture was performed as described above with 1 week of expansion. To isolate antigen-specific T-cells from cord blood the CD34- population was thawed and resuspended in RPMI- 1640 medium (Gibco) supplemented with 5% human serum (Sanquin). A maximum of $1x10^8$ CD34- cells were stimulated by the addition of 1 ug/ml CD28 monoclonal antibody (Miltenyi Biotec) and WT1 peptivator (Miltenyi Biotec). Antigen-reactive CD8+ T-cells were isolated after 24 h using a CD137 microbead kit (Miltenyi Biotec) according to manufacturer's instructions[25]. Antigen-reactive T-cells were co-cultured with autologous matured CBDC in X-Vivo15 medium (Lonza) supplemented with 5% human serum (Sanquin) and 15ng/ml IL-21 (Miltenyi Biotec) at a maximum ratio of 1:100 effector:APC. Plate 1 ml/well in a 24-wells plate. After 24–48 h half of the medium was changed every other day with fresh medium supplemented with 5 ng/ml IL-7, 5 ng/ml IL-15, and 10 ng/ml IL-21 (all Miltenyi Biotec). After 10 days of enrichment and expansion, T-cells were stained to identify tetramer+ T-cells. Tetramer panels were generated according to established protocol[26], consisting of 8 WT1 peptides; WT1(27), WT1(37), WT1(126), WT1(187), WT1(225), WT1(235), WT1(242), WT1(436), CMV-tetramer is used as control. Cells are pre-treated with 50 nM dasatinib (VWR international), to stabilize the TCR on the surface, for 30 min at 37°C prior to staining with the tetramer panel. Cells were washed once with FACS buffer and tetramer panel was prepared by adding approximately 0,1 ug per peptide: MHC complex to brilliant violet staining buffer (BD). Incubated 15 min at 37°C. Without washing a 5x stock of the following antibodies: anti-CD8 (BD), anti-CD4, anti-CD14, anti-CD16, anti-CD19 (all life technologies) and fixable viability dye (Ebioscience) is added to the sample and incubated for 30 min on ice. Cells were

washed twice with FACS buffer, resuspended in FACS buffer and multiparameter analysis was performed on a FACS LSR Fortessa (BD) flow cytometer. Analysis was performed with FACS Diva software (BD).

## KILLING ASSAY

Primary AML cells from the bone marrow were obtained from SKION biobank from the Princess Máxima Center for Pediatric Oncology, obtained after broad informed consent and selected for HLA-A2+ donors. These target AML cells were thawed, washed and suspended in RPMI + 10% human serum. AML cells were confirmed to express HLA-A2 and WT1 levels and after two washes with serum free PBS labeled with 0.4 µM cell trace violet (CTV; Invitrogen), diluted to $1x10^5$/ml and cocultured with WT1-specific T-cells as effector cells in a 10:1 and 40:1 ratio when indicated. HLA-A2+ WT1-expressing cell- line 697 (derived from a patient with acute lymphoblastic leukemia) was used as a positive control and K562 ((A2-WT1+) derived from a patient with chronic myeloid leukemia) and unstimulated CTV-labeled cells serves as a negative control. After 18–24 h, cells were incubated with 7AAD (final dilution 1:200; BD) for 15 min at RT. 10.000 beads (Beckman Coulter) were added just prior to measurement for quantification to measure a fixed amount per sample. All samples were measured on CantoII (BD). Percentage of lysis was calculated by 100% (viable target cell count/viable target cell count of target only condition × 100%).

# RESULTS

## GMP PRODUCTION OPTIMIZATION

A total of five preclinical GMP production runs were performed for the translation of the preclinical culture protocol[16] to generate CBDC into a GMP-production process and to further optimize the procedure. CD34+ cells were enriched from the CB using CliniMACS providing $0.73\times10^6$ ($0.18–2.6\times10^6$) CD34+ cells with a viability of ≥70% and a purity of 72%–92%, and subsequently expanded in culture bags. Run 1 and 2 showed lower expansion using CD34-enriched cells from a frozen CB unit compared to fresh CB. Adding AB serum during the first week of expansion and the addition of a second week of expansion were tested in the next runs to achieve sufficient numbers of cells (Table 1). Next, the expanded cells were differentiated toward DCs and reference vials were obtained. The expansion factor (EF), calculated by the number of live cells ($\times10^6$) divided by the number of cells at the start before the medium change, was high in the expansion phase, increased slightly adding serum, and lower in the differentiation phase in the optimization runs (Figure 1A).



**TABLE 1** | Preclinical good manufacturing practice (GMP) optimization runs.

| | CD34+ (x10^6) | EF expansion week 1 | EF expansion week 2 | EF differentiation week 1 | total EF | total vaccine cells (x10^6) | % CD83 CBDC | Summary protocol |
|---|---|---|---|---|---|---|---|---|
| Run 1 | 0.38 | 16 | x | 2 | 32 | 12 | 19 | 1 week exp |
| Run 2 | 2.6 | 4.6 | x | 5.4 | 25 | 65 | 62 | 1 week exp |
| Run 3 | 0.27 | 12 | 10 | 12 | 1,440 | 368 | 50 | 2 weeks exp |
| Run 3 + 10% AB | 0.27 | 81 | 9 | 3 | 2,187 | 590 | 15 | 2 weeks exp + serum |
| Run 4 | 0.18 | 1.5 | 5.3 | 1.7 | 13.5 | 3 | NA | 2 weeks exp |
| Run 4 + 5% AB | 0.18 | 7.9 | 51.3 | 3.5 | 1418 | 255 | NA | 2 weeks exp + serum |
| Run 4 + 10% AB | 0.18 | 10.8 | 32.1 | 3.1 | 1,075 | 194 | NA | 2 weeks exp + serum |
| Run 5 | 0.24 | 14 | 9.7 | 13.6 | 1,847 | 443 | 39 | 2 weeks exp |
| Run 5 + 5% AB | 0.24 | 51 | 12 | 3.3 | 2,020 | 485 | 33 | 2 weeks exp + serum |

Five test runs from five donors were performed to test for sufficient number of mature CBDCs. Run 1 and 2 were subjected to 1 week expansion, for run 3–5, two weeks of expansion. In run 3–5 either 5% or 10% human serum (AB) was added to the expansion. CD34+ cells after enrichment using CliniMACS. EF dividing the total cell number by the cell number at the beginning of the week. % CD83 CBDC are the mature DCs of total cells at the end of the culture measured using flow cytometry. x= no 2nd week of expansion in the first runs; EF, expansion factor; NA, not assessed; exp, expansion.

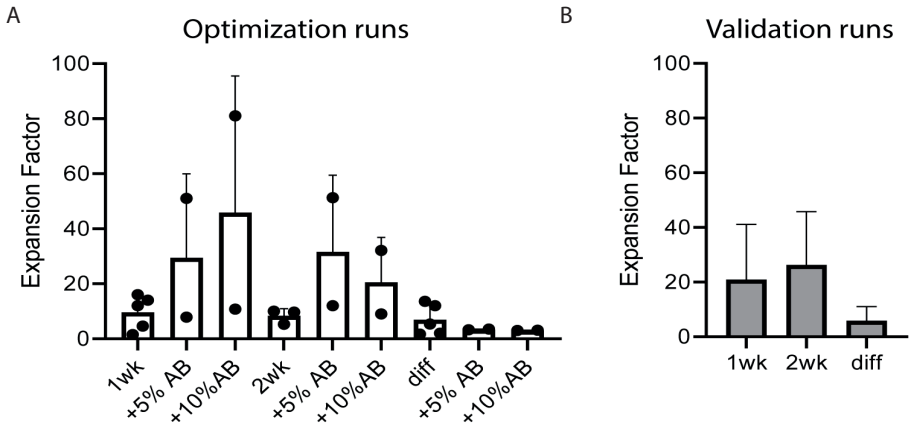





**FIGURE 1 | Cell expansion over time in cord blood (CB)-culture of the five optimization and five validation runs performed under good manufacturing practice (GMP) conditions.** Expansion factor (EF) expansion week 1 means total cells week 1 divided by CD34+ number at start; EF week 2 means total cells week 2 divided by week 1; EF diff is total cells after differentiation divided by expansion week 2. (A) EF of the five optimization runs including five or 10% human serum during expansion and an additional week of expansion (runs 3–5). (B) EF of the five validation runs. Two weeks of expansion (week 1 and week 2) followed by 1 week of differentiation (diff), which contains 5% human serum. Error bar represents SD.

After 1 week expansion followed by differentiation, roughly 12 and $65\times10^6$ DCs (run 1 and 2) were obtained, compared to 3, 388, and $443\times10^6$ DCs (run 3–5) with 2 weeks expansion. Two weeks expansion is necessary to ensure sufficient myeloid precursors to differentiate toward DCs (at least $50\times10^6$ cells) from each batch of CB. This expansion capacity is confirmed in the validation runs (Figure 1B). The addition of serum increased the yield to some extent during expansion, but decreased the total number of mature DCs in some batches (Table 1). It was therefore omitted from the final protocol. From these preclinical test runs we concluded that the final protocol would be a 3-week protocol without addition of human AB serum to the first week.

## GMP PRODUCTION VALIDATION

After completion of the procedure-optimization runs, we performed 5 additional GMP runs to validate the final protocol. $0.75\times10^6$ ($0.34–1.24\times10^6$) CD34+ cells were subjected to expansion and differentiation, generating $235\times10^6$ ($145–300\times10^6$) cells in the final product, i.e. sufficient to obtain the minimum of $50\times10^6$ cells for three rounds of vaccination and the appropriate amount of reference and retention vials. Data from these runs were used to check the feasibility to reach the release criteria for the final product. Next, the phenotype of the CBDC within the live gate was assessed by flow cytometry. The protocol of expansion, differentiation and maturation resulted in 60% (+/-10%) CD11c+ HLA-DR+ cells (Figure 2A). In the validation run CBDC were positive for the maturation marker CD80 (average 58% [43-70%]) and CD83 (average 56% [43-70%]) (Figure 2B). CC-chemokine receptor 7 (CCR7) was highly upregulated on CBDC (average 44% [20-67%]), enabling in vivo migration (Figure 2B). T- (CD3+), B- (CD19+), and NK-cells (CD16+CD56+) were measured in the CB culture after maturation in the 5 validation test runs: all T-, B-and NK-populations were below 0.1% (gating strategy in Supplementary Figure 1). The remaining cells in the culture are primarily myeloid precursors based on cytoplasmic expression of Myeloperoxidase (cMPO) and surface expression of CD33 and CD117. In addition, levels of CD14, CD15 and CD11b are detected in part of the HLA-DR negative fraction (Figure 2C). Altogether, the GMP production protocol resulted in sufficient CBDCs with a highly mature phenotype and purity (release criteria ≥30% CD83/80+ DCs). These CBDCs are subjected to a variety of assays described to determine their potency.

## MIGRATION AND ALLOGENEIC T-CELL ACTIVATION BY CBDCS

CBDCs express high levels of CCR7 on the surface, illustrating the capacity to migrate toward a CCL19 gradient. Indeed, significant migration of 60.000 cells on average was observed to the lower compartment in a transwell system, compared to an average of 560 cells in the absence of CCL19 (Figure 3A). Next, as an outcome measure of DC functionality, allogeneic T-cell proliferation (mixed leukocyte reaction; MLR) was measured using T-cells from a different CB donor. CBDC induced CD4 as well as CD8 proliferation, with a division index of 1.2 and 1 respectively (Figure 3B), showing the capacity to stimulate T-cells in an antigen-independent manner (Figure 3C).

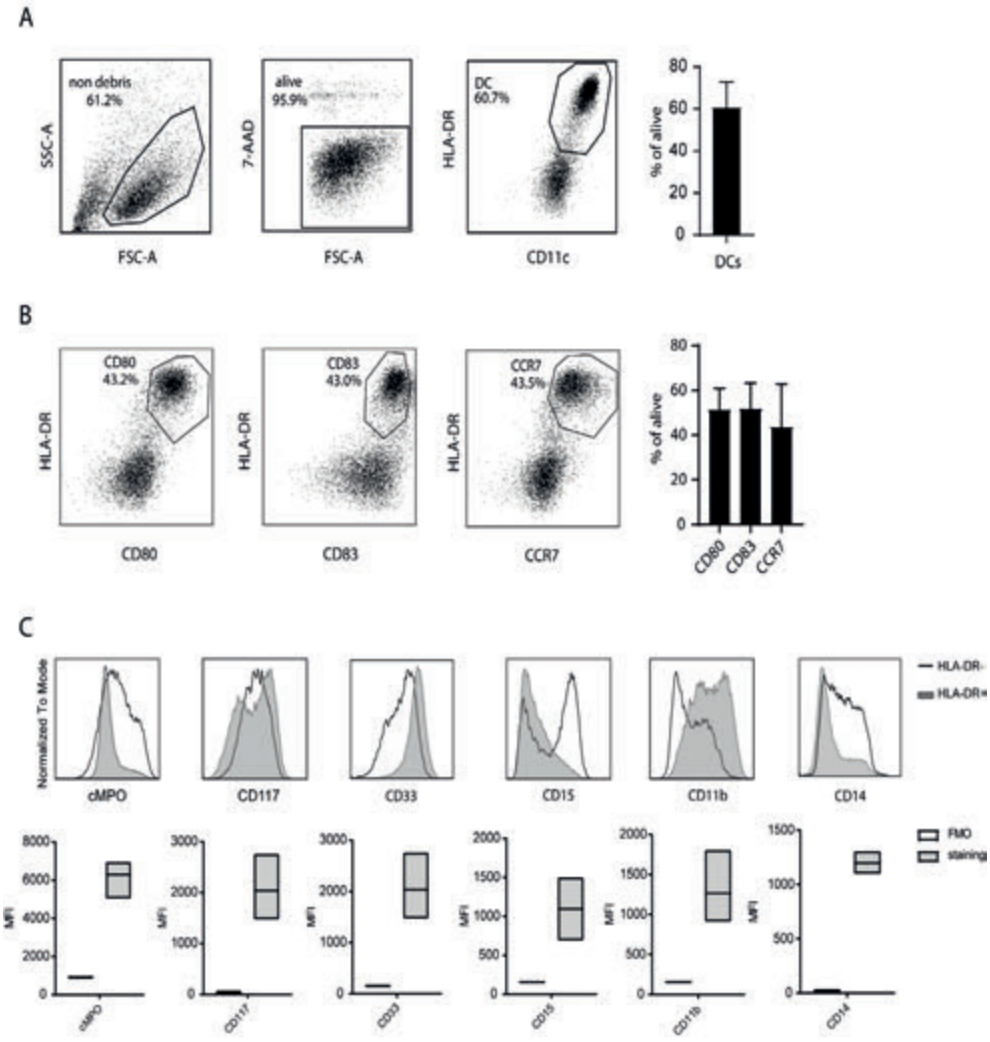



**FIGURE 2 | Phenotype of cord blood (CB)-culture.** (A) Gating strategy for CB-derived dendritic cells (CBDCs) using flow cytometry. (B) Expression of costimulatory levels of CD80, CD83, and CCR7 expression on the 7-AAD- cells in the CB culture. (C) Myeloid expression profile within the HLA-DR negative (black line) and HLADR positive (CBDC; grey) population (histogram) or HLA-DR- compared to fluorescence minus one (FMO) staining (MFI boxplots) in the CB-culture after maturation. (A, B) represent 1 out of 5, the mean of five validation runs is calculated for (C).

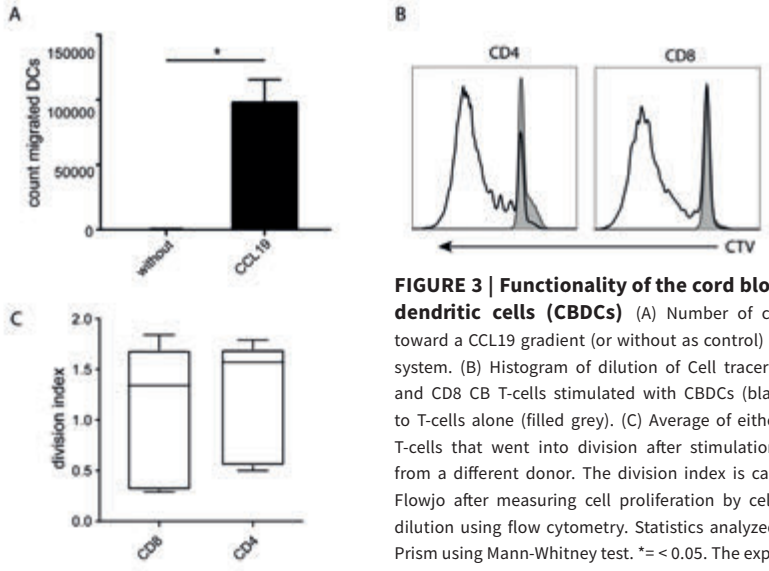



**FIGURE 3 | Functionality of the cord blood -derived dendritic cells (CBDCs)** (A) Number of cells migrating toward a CCL19 gradient (or without as control) in a trans well system. (B) Histogram of dilution of Cell tracer violet of CD4 and CD8 CB T-cells stimulated with CBDCs (black) compared to T-cells alone (filled grey). (C) Average of either CD4 or CD8 T-cells that went into division after stimulation with CBDCs from a different donor. The division index is calculated using Flowjo after measuring cell proliferation by cell tracer violet dilution using flow cytometry. Statistics analyzed in GraphPad Prism using Mann-Whitney test. *= < 0.05. The experiments were performed with five different donors.



## ANTIGEN LOADING AND PRESENTATION

We compared peptivator loading, 15-mers with a 12-mers overlap covering most of the WT1 protein, to electroporation (EP) of mRNA encoding for 2 major isoforms of the full-length WT1 protein. An in vitro T-cell avidity assay with different antigen concentration showed that the dose administered to the CBDC (1ug/ml/peptide) is sufficient for loading CBDC (Supplementary Figure 2). After EP, intracellular WT1 levels were detected on CBDCs using flow cytometry (Figure 4A).

However, EP resulted in extensive loss of cells (Supplementary Figure 3). Next, these DCs were tested for their capacity to activate antigen-specific T-cells using a previously generated CB- derived $WT1_{37\text{-}45}$ T-cell clone. Surface LAMP-1 expression and intracellular IFNg expression in the WT1-specific T-cell clone was measured after a co-culture with either WT1 mRNA electroporated DCs (WT1 EP DCs), WT1-peptivator-pulsed DCs or a combination of both. WT1 EP DCs showed minimal T-cell activation: 19% LAMP-1 and 2,3% IFNg expression, while loading with peptivator increased LAMP-1 and IFNg expression to, 21% and 38,2% respectively. The combination of loading strategies did not show an additional effect compared to peptivator-pulsed DCs alone (13,9 and 35,4%) (Figure 4B). Because of the low WT1 specific T-cell activation, in particular IFNg and high loss of cells in the procedure, EP was ommited from the final GMP production protocol.

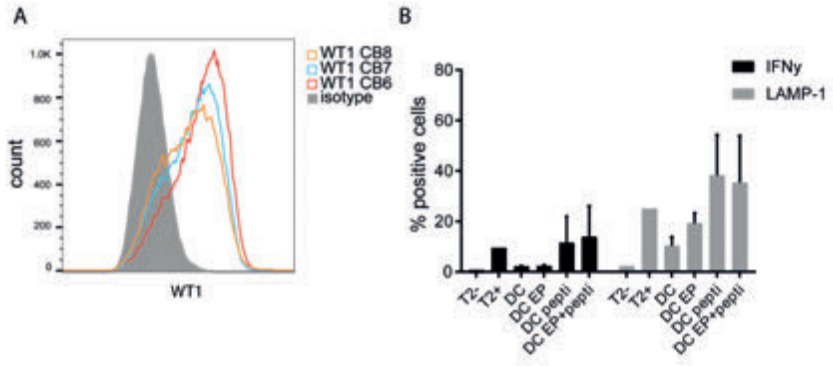



**FIGURE 4 | WT1 expression and presentation.** (A) Intracellular WT1 levels measured by flow cytometry after electroporation of WT1 mRNA compared to isotype control (grey). Validation runs 6–8 are shown. (B) Percentage of intracellular IFNg levels and extracellular LAMP-1 expression by WT1-specific CD8 T-cells activated by cord blood-derived dendritic cells (CBDC) electroporated with WT1 mRNA (DC EP), loaded with peptivator (DC pepti), both (DC EP+pepti) or nothing (DC). T2 cells in the presence or absence of peptide served as controls. 2 CBDC donors were used. Statistical analysis is performed in GraphPad Prism using Kruskal-Wallis test, no significant differences.



## AUTOLOGOUS WT1 T-CELL ASSAY AND AML LYSIS

Next to allogeneic (WT1) stimulation, we tested the stimulatory capacity in an autologous assay. After overnight stimulation of the whole CB unit with WT1 peptivator, WT1-specific T- cells were enriched by CD137+ cell isolation and subsequently re-stimulated with autologous WT1 peptivator-pulsed CBDCs. These T-cells were expanded and characterized by tetramer staining. A few clones were detected of which one dominant HLA-A2; $WT_{37\text{-}45}$ (VLDFAPPGA) of the 8 HLA-A2 tetramers measured (Figure 5A). In Figure 5B the relative contribution of the different T-cells in different donors is shown (Figure 5B). In most cases, the tetramer+ T-cells were FACS-sorted and further expanded. Some WT1+ T-cells were employed for a killing assay using a WT1-expressing cell line (697). Robust target cell lysis was observed in 20%–40% of the cells (Figure 5C). In addition, we added the T-cells to pediatric primary AML cells to observe primary AML lysis. WT1 is not measured routinely and therefore WT1 and HLA-A2 (Figure 5D and Suppl. Figure 4) were stained in parallel. A variety of WT1 levels is observed (mean MFI 316 [25-713]). Lysis was observed in 5 out of 10 samples when compared to background levels of K562 (negative control) (Figures 5E, F). These CB-T-cells stimulated by CBDC suggested antigen specific AML cell lysis.

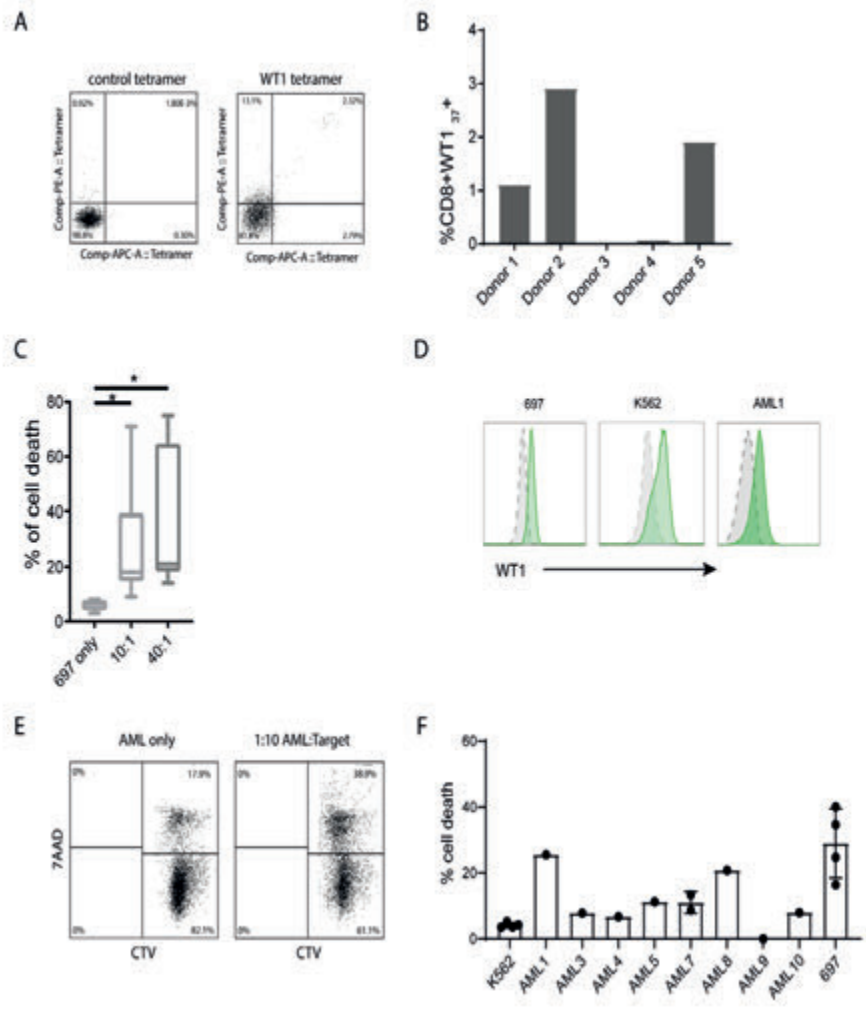



**FIGURE 5 | T-cell activation by cord blood-derived dendritic cell (CBDC) and subsequently acute myeloid leukemia (AML) lysis by CB WT1+ T-cells.** (A) Gating example of WT1-pulsed T-cells, analyzed by either CMV tetramer (negative control) or WT1 tetramer. (B) Frequency of CD8+$WT1_{37}$+ generated from naïve bulk T-cells stimulated by peptivator loaded CBDCs. (C) Percentage of cell death of HLA-A2+ WT1+ 697 cell line in the absence of T-cells or in a 10:1 or 40:1 ratio with CD8+WT1+ T-cells generated from different donors. Target T-cells were labeled with cell tracer dye (CTV) and lysis was assessed detecting % 7-AAD+ CTV+ target T-cells. (D) Example of intracellular WT1 levels (green) of 697, K562 and primary AML1 compared to isotype control (grey). (E) Gating of lysed target cells, pre-gated on CTV+. (F) Percentage of cell death of primary pediatric AML compared to K562 (negative control) or 697 as a positive control. Error bar represents multiple killing assays (2–4) and represents SD. Statistical analysis is performed in GraphPad Prism using Kruskal-Wallis test.

## VIABILITY AND SURVIVAL

Since CBDC will be frozen prior to the use for vaccination, at several time points the effect of cryopreservation is monitored. Less than two months, three months (not shown) and 2 years

after manufacturing CBDC, cells were thawed and subjected to a MLR potency assay. The viability after 2 years was 73%. The CBDC showed expression of CD11c, HLA-DR, CD80 and CD83 (Figure 6A). The division index (the average number of cell divisions) in a MLR was comparable or even increased compared to the CBDC thawed ≤ 2months after freezing (Figure 6B). In conclusion, we are able to generate sufficient mature CBDC, that are not affected by cryopreservation, to use for vaccination after CBT.



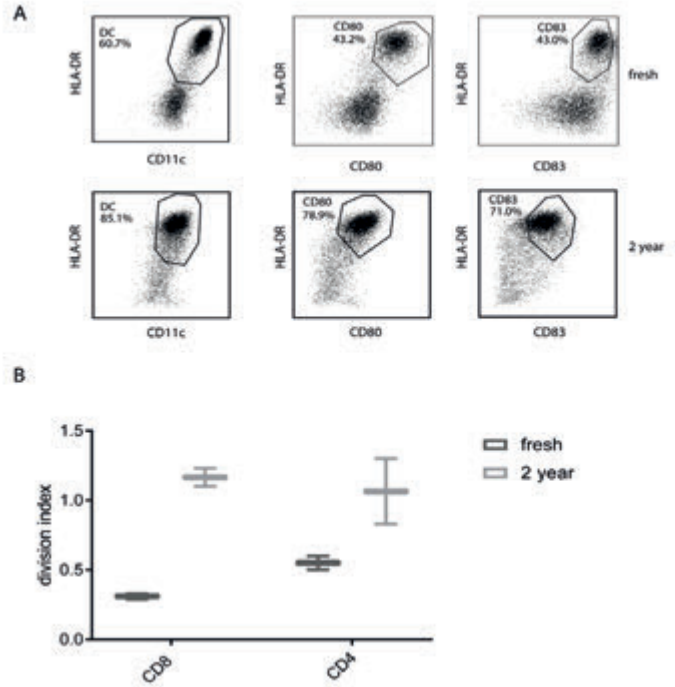



**FIGURE 6 | Phenotype and functionality after thawing.** (A) Phenotype of 7AAD-cord blood-derived dendritic cells (CBDCs) after thawing a reference vial from the test runs either directly after generation or after 2 years. (B) Division index from a mixed leukocyte reaction (MLR) stimulated by directly thawed or CBDC thawed after 2 years. The T-cells used for the MLR are from a different donor, CBDCs from the same validation run.

## RELEASE REQUIREMENTS FOR CBDC VACCINE

The GMP manufacturing process of the CBDC vaccine was optimized in five optimization runs followed by five validation runs. The first five optimization runs were used for translation and optimization of the preclinical culture protocol into a GMP- production process. The validation runs were all performed using the same protocol and provided sufficient information on the efficacy, variation and reproducibility of the manufacturing process, from frozen CB unit to thawed and ready to administer WT1-loaded CBDC vaccine (Table 2). Ultimately, sufficient number of viable cells required for vaccination (at least $5x10^6$ cell/vaccination) will need to be present after thawing and washing of the cryopreserved product. Mature DCs are able to prime naïve T-cells and are potent T-cell activators. As such, the expression of the hallmark surface proteins of mature DC (≥30%), i.e. CD80 and CD83, should be present in the CB culture, while containing a minimum of contaminating cells, like T-, B- and NK-cells (≤5%). Furthermore, microbiological sterility of the thawed vaccine is secured using the reference vial, frozen in addition to the vaccine. All 5 validation runs met these release criteria as indicated in Table 2 and finalized the CBDC protocol (Figure 7).

**TABLE 2** | Quality control testing results of final product n=5 donors (directly thawed reference vials of the validation runs 6–10).

| Test of | Specifications | Runs | | | | |
|---|---|---|---|---|---|---|
| | | 6 | 7 | 8 | 9 | 10 |
| **Cell count** | | | | | | |
| Total viable cells | ≥5x10^6 for max dose | 6.7x10^6 | 7.2x10^6 | 6.0x10^6 | 5.8x10^6 | 6.5x10^6 |
| Viability | ≥ 60% of total cells | 76% | 81% | 79% | 75% | 78% |
| **Phenotype** | | | | | | |
| Mature CBDC (CD83+ HLA-DR+) | ≥ 30% | 70% | 50% | 56% | 50% | 64% |
| Costimulation (CD80+ HLA-DR+) | ≥ 30% | 70% | 47% | 56% | 49% | 60% |
| **Lymphocyte contamination** | | | | | | |
| B cells | ≤ 5% | 0.06% | 0.12% | 0.07% | 0.09% | 0.26% |
| T-cells | ≤ 5% | 0.02% | 0.03% | 0.01% | 0.08% | 0.19% |
| NK cells | ≤ 5% | 0.04% | 0.12% | 0.05% | 0.35% | 0.18% |
| **Microbiological control of cellular products** | Sterile (Ph.Eur.) | Yes | Yes | Yes | Yes | Yes |

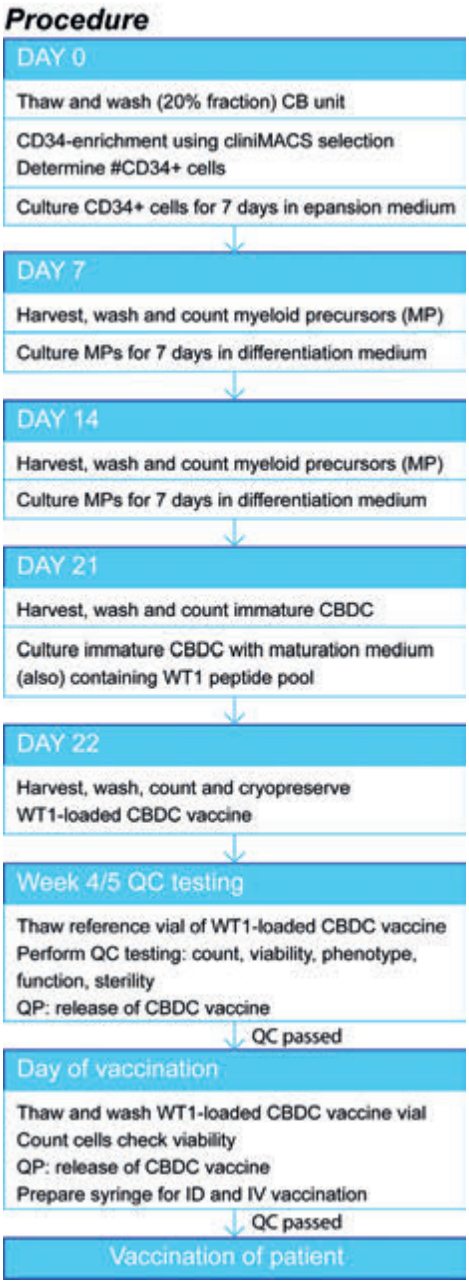



**FIGURE 7 | Flow diagram of production procedure.** QP, quality personal; QC, quality control. ID, intradermal; IV, intravenous.

## DISCUSSION

The DC vaccination field is advancing with implementation of state-of-the-art techniques enabling the selection of different sources of DCs, antigen loading strategies, and genetic modification of the cells[27]. As relapse rates post-HCT are still relatively high in pediatric AML, we hypothesized that selecting and stimulating AML-specific T-cells by DC vaccination post-HCT could protect children from AML relapse. The patients will be vaccinated while in complete remission (CR) and the generation of anti-AML immunity will not be hindered by the suppressive tumor environment as observed in patients with a high tumor burden[28]. Notably, DC vaccination in a minimal residual disease setting or in combination with surgery or chemotherapy, improved its efficacy in other studies[29, 30]. DC vaccinations have been used in a large number of clinical studies, including those performed after allo-HCT, suggesting that DC vaccination post allo-HCT is safe [31–35].

In all these studies the DCs were derived from monocytes. The use of CD34-derived DCs for vaccination has been limited to an autologous DC vaccination setting for the treatment of metastatic melanoma [36, 37]. In addition, no DC vaccinations have

ever been performed after CBT. Since the same graft is used for CBT and generation of DC, optimal DC-T cell communication is warranted. Moreover, CBDCs are loaded with peptides covering the majority of the WT1 protein, therefore no HLA restrictions and concomitantly no HLA-based selection of patients is necessary.

The proposed combination is particularly suitable for children and young adolescents, since adult patients with high risk AML often receive transplants from HLA-matched sibling donors[38]. We previously showed in a preclinical study that CB stem cells can be expanded and differentiated into DCs resembling primary cDC2 cells[16, 39]. In the work presented here, we translated the preclinical protocol into a GMP-grade culture protocol for the generation of an advanced therapy medicinal product. A major difference was the replacement of culture flasks with a closed bag system for cell expansion and differentiation. The DC phenotype and function was similar in the different plastics used for flasks/plates and bags (this manuscript and [16]), but fewer differences were observed between CB donors when using bags. The reason for this has not been studied but could relate to a different organization of the multilayers of cells (microenvironment) in the bags[40, 41]. The cells showed expression of activation markers, migration and MLR capacity, and were able to stimulate WT1-specific cell lines and autologous CB-derived WT1 specific T-cells that subsequently acquired cytolytic capacity to kill primary AML blast.

We showed that sufficient cells can be generated from a low number of isolated CD34+ CB stem cells for at least 3 vaccinations and that the product can be frozen and thawed without losing efficacy. Quality criteria for this product will include the number of viable cells (at least 5x10^6 cells/vaccination) present after thawing and washing of the cryopreserved product, the expression of CD80 and CD83 to show the DC have matured (≥30%), a minimum of contaminating cells, like T-, B- and NK-cells (≤5%), and microbiological sterility of the thawed vaccine using a reference vial. Since the product is made from a separately frozen compartment of the CB graft (20% fraction) and the product will be frozen again before administration to the patient, the generation can be outsourced to a manufacturing facility and shipped to the hospital where the patient received his/her HCT.

So far, we focused on WT1 as a target of pediatric AML. The selection of a tumor associated antigen remains a major challenge for the development of a CBDC vaccine. Neoantigens are of high interest, because of their high tumor specificity [42], but in contrast to, for example pediatric ALL, de novo AML is characterized by a very low burden of genomic alterations: more than one third of pediatric AML cases lacks any identifiable copy number alteration, and more than a quarter of the leukemia's with recurrent translocations lacks any identifiable sequence or

numerical abnormalities[43]. WT1 is overexpressed in the majority (>80-90%) of patients with AML, including cell-cycle quiescent AML stem cells located in the BM[44] and relapses after CBT, which supports the choice of WT1 as a tumor target [45, 46]. Although it has been suggested that consistent high-avidity WT1 T-cell responses could not be demonstrated in vaccination studies[47], others successfully isolated a high-affinity WT1-specific TCR (TCRC4) and reported the first positive results from the clinical trial using WT1 TCR-engineered adoptive T-cell[22]. Moreover, vaccination with mRNA WT1-loaded moDC as a post- remission treatment in high-risk adult AML prevented or delayed relapse in 43% of the 30 patients included in the study [48–52].

The increased survival correlated with the occurrence of WT1-specific T-cell responses, but unfortunately, not all patients responded. Recent results from a phase 2 trial of a multivalent WT1 peptide vaccine showed beneficial clinical outcomes that correlated with an immunological response in a non-transplant setting[53, 54]. We tested different antigen loading techniques: mRNA encoding the full length WT1 protein versus a mix of synthetic overlapping WT1 peptides. The exogenous delivered long peptide antigens will immediately be loaded on MHCII for presentation to CD4 T-cells, but will require uptake and delivery into the MHC class I cross-presentation pathway to stimulate CD8 T-cells. Peptide loading resulted in a better T-cell stimulation efficacy and in a significant reduction in the loss of CBDCs due to the transduction procedure. For these reasons we decided to include the peptide loading as it would simplify the GMP-procedure, increase the number of cells for vaccination while maintaining good efficacy.

In conclusion, with the purpose of developing a clinical grade DC vaccine from CB CD34+, we here established a GMP- protocol to generate sufficient CB-derived DCs in a culture bag for at least three vaccinations post-HCT. The CBDCs are highly mature, able to migrate and activate WT1-specific T-cells, which in turn lyse WT1+ cell lines and primary pediatric AML samples. These CBDCs may augment the reconstituting immune system toward anti-WT1 T-cell activity and thereby prevent relapse in refractory AML patients.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Nolan Cord blood bank. Full informed consent is obtained from expectant mothers in accordance with the Human Tissue Act 2004, the Human Tissue Regulations 2007 and the HTA's Code of Practice in the United Kingdom. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Conceptualization, MP, CH, and SN. MP, VLP, and ED performed the experiments. MP made the figures and wrote the manuscript. AM, CL, and SN critically reviewed the paper. AM provided CB. Supervision by JJB and SN.



## FUNDING

This research was funded by ZonMW/TAS 116003003 and KIKA nr 142.

## ACKNOWLEDGMENTS

The authors appreciate the effort from the nurses to obtain the umbilical cord blood used in this study. They thank Jolanda de Vries and Viggo van Tendeloo for sharing their knowledge and expertise in translating preclinical findings into a GMP-product and the expertise and help of the cell therapy facility to perform the clinical runs.

## BIBLIOGRAPHY

1. de Rooij JD, Zwaan CM, van den Heuvel-Eibrink M. Pediatric AML: From Biology to Clinical Management. J Clin Med (2015) 4(1):127–49. doi: 10.3390/ jcm4010127
2. Bachas C, Schuurhuis GJ, Reinhardt D, Creutzig U, Kwidama ZJ, Zwaan CM, et al. Clinical relevance of molecular aberrations in paediatric acute myeloid leukaemia at first relapse. Br J Haematol (2014) 166(6):902–10. doi: 10.1111/ bjh.12989
3. Creutzig U, van den Heuvel-Eibrink MM, Gibson B, Dworzak MN, Adachi S, de Bont E, et al. Diagnosis and management of acute myeloid leukemia in children and adolescents: recommendations from an international expert panel. Blood (2012) 120(16):3187–205. doi: 10.1182/blood-2012-03-362608
4. Rautenberg C, Germing U, Haas R, Kobbe G, Schroeder T. Relapse of Acute Myeloid Leukemia after Allogeneic Stem Cell Transplantation: Prevention, Detection, and Treatment. Int J Mol Sci (2019)

20(1):228. doi: 10.3390/ ijms20010228

5. Bol KF, Schreibelt G, Gerritsen WR, de Vries IJ, Figdor CG. Dendritic Cell- Based Immunotherapy: State of the Art and Beyond. Clin Cancer Res (2016) 22(8):1897–906. doi: 10.1158/1078-0432.CCR-15-1399
6. Brunstein CG, Gutman JA, Weisdorf DJ, Woolfrey AE, Defor TE, Gooley TA, et al. Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. Blood (2010) 116(22):4693–9. doi: 10.1182/blood-2010-05-285304
7. Eapen M, Rubinstein P, Zhang MJ, Stevens C, Kurtzberg J, Scaradavou A, et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. Lancet (2007) 369(9577):1947–54. doi: 10.1016/S0140-6736(07)60915-5
8. Locatelli F, Masetti R, Rondelli R, Zecca M, Fagioli F, Rovelli A, et al. Outcome of children with high-risk acute myeloid leukemia given autologous or allogeneic hematopoietic cell transplantation in the aieop AML-2002/01 study. Bone Marrow Transplant (2015) 50(2):181–8. doi: 10.1038/ bmt.2014.246
9. Milano F, Gooley T, Wood B, Woolfrey A, Flowers ME, Doney K, et al. Cord- Blood Transplantation in Patients with Minimal Residual Disease. New Engl J Med (2016) 375(10):944–53. doi: 10.1056/NEJMoa1602074
10. Chiesa R, Gilmour K, Qasim W, Adams S, Worth AJ, Zhan H, et al. Omission of in vivo T-cell depletion promotes rapid expansion of naive CD4+ cord blood lymphocytes and restores adaptive immunity within 2 months after unrelated cord blood transplant. Br J Haematol (2012) 156(5):656–66. doi: 10.1111/j.1365-2141.2011.08994.x
11. Cohen G, Carter SL, Weinberg KI, Masinsin B, Guinan E, Kurtzberg J, et al. Antigen-specific T-lymphocyte function after cord blood transplantation. Biol Blood Marrow Transplant J Am Soc Blood Marrow Transplant (2006) 12 (12):1335–42. doi: 10.1016/j.bbmt.2006.08.036
12. Admiraal R, Lindemans CA, van Kesteren C, Bierings MB, Versluijs AB, Nierkens S, et al. Excellent T-cell reconstitution and survival depend on low ATG exposure after pediatric cord blood transplantation. Blood (2016) 128 (23):2734–41. doi: 10.1182/blood-2016-06-721936
13. Flinsenberg TW, Spel L, Jansen M, Koning D, de Haar C, Plantinga M, et al. Cognate CD4 T-cell licensing of dendritic cells heralds anti-CMV CD8 T-cell immunity after human allogeneic umbilical cord blood transplantation. J Virol (2014) 89(2):1058–69. doi: 10.1128/JVI.01850-14
14. Hiwarkar P, Gaspar HB, Gilmour K, Jagani M, Chiesa R, Bennett-Rees N, et al. Impact of viral reactivations in the era of pre-emptive antiviral drug therapy following allogeneic haematopoietic SCT in paediatric recipients. Bone Marrow Transplant (2013) 48(6):803–8. doi: 10.1038/bmt.2012.221
15. Admiraal R, de Koning CCH, Lindemans CA, Bierings MB, Wensing AMJ, Versluys AB, et al. Viral reactivations and associated outcomes in the context of immune reconstitution after pediatric hematopoietic cell transplantation. J Allergy Clin Immunol (2017) 140(6):1643–1650 e1649. doi: 10.1016/j.jaci.2016.12.992
16. de Haar C, Plantinga M, Blokland NJ, van Til NP, Flinsenberg TW, Van Tendeloo VF, et al. Generation of a cord blood-derived Wilms Tumor 1 dendritic cell vaccine for AML patients treated with allogeneic cord blood transplantation. Oncoimmunology (2015) 4(11):e1023973. doi: 10.1080/ 2162402X.2015.1023973
17. Schreibelt G, Bol KF, Westdorp H, Wimmers F, Aarntzen EH, Duiveman-de Boer T, et al. Effective Clinical Responses in Metastatic Melanoma Patients after Vaccination with Primary Myeloid Dendritic Cells. Clin Cancer Res (2016) 22(9):2155–66. doi: 10.1158/1078-0432.CCR-15-2205
18. Van Craenenbroeck AH, Smits EL, Anguille S, Van de Velde A, Stein B, Braeckman T, et al. Induction of cytomegalovirus-specific T cell responses in healthy volunteers and allogeneic stem cell recipients using

vaccination with messenger RNA-transfected dendritic cells. Transplantation (2015) 99 (1):120–7. doi: 10.1097/TP.0000000000000272

19. Van Driessche A, Van de Velde AL, Nijs G, Braeckman T, Stein B, De Vries JM, et al. Clinical-grade manufacturing of autologous mature mRNA- electroporated dendritic cells and safety testing in acute myeloid leukemia patients in a phase I dose-escalation clinical trial. Cytotherapy (2009) 11 (5):653–68. doi: 10.1080/14653240902960411

20. Saito Y, Kitamura H, Hijikata A, Tomizawa-Murasawa M, Tanaka S, Takagi S, et al. Identification of therapeutic targets for quiescent, chemotherapy- resistant human leukemia stem cells. Sci Trans Med (2010) 2(17):17ra19. doi: 10.1126/scitranslmed.3000349

21. Bolouri H, Farrar JE, Triche TJr, Ries RE, Lim EL, Alonzo TA, et al. The molecular landscape of pediatric acute myeloid leukemia reveals recurrent structural alterations and age-specific mutational interactions. Nat Med (2018) 24(1):103–12. doi: 10.1038/nm.4439

22. Chapuis AG, Egan DN, Bar M, Schmitt TM, McAfee MS, Paulson KG, et al. T cell receptor gene therapy targeting WT1 prevents acute myeloid leukemia relapse post-transplant. Nat Med (2019) 25(7):1064–72. doi: 10.1038/s41591- 019-0472-9

23. Poulin LF, Salio M, Griessinger E, Anjos-Afonso F, Craciun L, Chen JL, et al. Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. J Exp Med (2010) 207 (6):1261–71. doi: 10.1084/jem.20092618

24. Van Tendeloo VF, Ponsaerts P, Lardon F, Nijs G, Lenjou M, Van Broeckhoven C, et al. Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. Blood (2001) 98(1):49–56. doi: 10.1182/ blood.V98.1.49

25. Schmied S, Gostick E, Price DA, Abken H, Assenmacher M, Richter A. Analysis of the functional WT1-specific T-cell repertoire in healthy donors reveals a discrepancy between CD4(+) and CD8(+) memory formation. Immunology (2015) 145(4):558–69. doi: 10.1111/imm.12472

26. Rodenko B, Toebes M, Hadrup SR, van Esch WJ, Molenaar AM, Schumacher TN, et al. Generation of peptide-MHC class I complexes through UV- mediated ligand exchange. Nat Protoc (2006) 1(3):1120–32. doi: 10.1038/ nprot.2006.121

27. Garg AD, Coulie PG, Van den Eynde BJ, Agostinis P. Integrating Next- Generation Dendritic Cell Vaccines into the Current Cancer Immunotherapy Landscape. Trends Immunol (2017) 38(8):577–93. doi: 10.1016/j.it.2017. 05.006

28. Lindau D, Gielen P, Kroesen M, Wesseling P, Adema GJ. The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. Immunology (2013) 138 (2):105–15. doi: 10.1111/imm.12036

29. Bol KF, Aarntzen EH, Hout FE, Schreibelt G, Creemers JH, Lesterhuis WJ, et al. Favorable overall survival in stage III melanoma patients after adjuvant dendritic cell vaccination. Oncoimmunology (2016) 5(1):e1057673. doi: 10.1080/2162402X.2015.1057673

30. Wheeler CJ, Das A, Liu G, Yu JS, Black KL. Clinical responsiveness of glioblastoma multiforme to chemotherapy after vaccination. Clin Cancer Res (2004) 10(16):5316–26. doi: 10.1158/1078-0432.CCR-04-0497

31. Feuchtinger T, Opherk K, Bicanic O, Schumm M, Grigoleit GU, Hamprecht K, et al. Dendritic cell

vaccination in an allogeneic stem cell recipient receiving a transplant from a human cytomegalovirus (HCMV)-seronegative donor: induction of a HCMV-specific T(helper) cell response. Cytotherapy (2010) 12 (7):945–50. doi: 10.3109/14653241003587645

32. Fujii S, Shimizu K, Fujimoto K, Kiyokawa T, Tsukamoto A, Sanada I, et al. Treatment of post-transplanted, relapsed patients with hematological malignancies by infusion of HLA-matched, allogeneic-dendritic cells (DCs) pulsed with irradiated tumor cells and primed T cells. Leukemia Lymphoma (2001) 42(3):357–69. doi: 10.3109/10428190109064592

33. Grigoleit GU, Kapp M, Hebart H, Fick K, Beck R, Jahn G, et al. Dendritic cell vaccination in allogeneic stem cell recipients: induction of human cytomegalovirus (HCMV)-specific cytotoxic T lymphocyte responses even in patients receiving a transplant from an HCMV-seronegative donor. J Infect Dis (2007) 196(5):699–704. doi: 10.1086/520538

34. Kitawaki T, Kadowaki N, Kondo T, Ishikawa T, Ichinohe T, Teramukai S, et al. Potential of dendritic-cell immunotherapy for relapse after allogeneic hematopoietic stem cell transplantation, shown by WT1 peptide- and keyhole-limpet-hemocyanin-pulsed, donor-derived dendritic-cell vaccine for acute myeloid leukemia. Am J Hematol (2008) 83(4):315–7. doi: 10.1002/ ajh.21127

35. Saito S, Yanagisawa R, Yoshikawa K, Higuchi Y, Koya T, Yoshizawa K, et al. Safety and tolerability of allogeneic dendritic cell vaccination with induction of Wilms tumor 1-specific T cells in a pediatric donor and pediatric patient with relapsed leukemia: a case report and review of the literature. Cytotherapy (2015) 17(3):330–5. doi: 10.1016/j.jcyt.2014.10.003

36. Banchereau J, Palucka AK, Dhodapkar M, Burkeholder S, Taquet N, Rolland A, et al. Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res (2001) 61 (17):6451–8.

37. 37. Di Nicola M, Carlo-Stella C, Mortarini R, Baldassari P, Guidetti A, Gallino GF, et al. Boosting T cell-mediated immunity to tyrosinase by vaccinia virus- transduced, CD34(+)-derived dendritic cell vaccination: a phase I trial in metastatic melanoma. Clin Cancer Res (2004) 10(16):5381–90. doi: 10.1158/1078-0432.CCR-04-0602

38. Majhail NS, Brunstein CG, Shanley R, Sandhu K, McClune B, Oran B, et al. Reduced-intensity hematopoietic cell transplantation in older patients with AML/MDS: umbilical cord blood is a feasible option for patients without HLA-matched sibling donors. Bone Marrow Transplant (2012) 47(4):494–8. doi: 10.1038/bmt.2011.114

39. Plantinga M, de Haar CG, Dunnebach E, van den Beemt D, Bloemenkamp KWM, Mokry M, et al. Cord-Blood-Stem-Cell-Derived Conventional Dendritic Cells Specifically Originate from CD115-Expressing Precursors. Cancers (Basel) (2019) 11(2):181–95. doi: 10.3390/cancers11020181

40. Buchler T, Kovarova L, Musilova R, Bourkova L, Ocadlikova D, Bulikova A, et al. Generation of dendritic cells using cell culture bags–description of a method and review of literature. Hematology (2004) 9(3):199–205. doi: 10.1080/10245330410001701486

41. Fekete N, Beland AV, Campbell K, Clark SL, Hoesli CA. Bags versus flasks: a comparison of cell culture systems for the production of dendritic cell-based immunotherapies. Transfusion (2018) 58(7):1800–13. doi: 10.1111/trf.14621

42. Ott PA, Hu Z, Keskin DB, Shukla SA, Sun J, Bozym DJ, et al. An immunogenic personal neoantigen vaccine for patients with melanoma. Nature (2017) 547 (7662):217–21. doi: 10.1038/nature22991

43. Radtke I, Mullighan CG, Ishii M, Su X, Cheng J, Ma J, et al. Genomic analysis reveals few genetic alterations in pediatric acute myeloid leukemia. Proc Natl Acad Sci U S A (2009) 106(31):12944–9. doi: 10.1073/pnas.0903142106

44. Saito Y, Uchida N, Tanaka S, Suzuki N, Tomizawa-Murasawa M, Sone A, et al. Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. Nat Biotechnol (2010) 28(3):275–80. doi: 10.1038/nbt.1607

45. Zhang W, Lu X, Cui P, Piao C, Xiao M, Liu X, et al. Phase I/II clinical trial of a Wilms' tumor 1-targeted dendritic cell vaccination-based immunotherapy in patients with advanced cancer. Cancer Immunol Immunother CII (2019) 68 (1):121–30. doi: 10.1007/s00262-018-2257-2

46. Nakata J, Oji Y, Oka Y, Sugiyama H. What should we tackle next in acute myeloid leukemia? Wilms tumor gene 1 vaccine therapy would be a promising and versatile strategy for acute myeloid leukemia. Expert Rev Hematol (2019) 1– 3. doi: 10.1080/17474086.2019.1593824

47. Falkenburg WJ, Melenhorst JJ, van de Meent M, Kester MG, Hombrink P, Heemskerk MH, et al. Allogeneic HLA-A*02-restricted WT1-specific T cells from mismatched donors are highly reactive but show off-target promiscuity. J Immunol (2011) 187(5):2824–33. doi: 10.4049/jimmunol.1100852

48. Anguille S, Van de Velde AL, Smits EL, Van Tendeloo VF, Juliusson G, Cools N, et al. Dendritic cell vaccination as postremission treatment to prevent or delay relapse in acute myeloid leukemia. Blood (2017) 130(15):1713–21. doi: 10.1182/ blood-2017-04-780155

49. Coosemans A, Vanderstraeten A, Tuyaerts S, Verschuere T, Moerman P, Berneman Z, et al. Immunological response after WT1 mRNA-loaded dendritic cell immunotherapy in ovarian carcinoma and carcinosarcoma. Anticancer Res (2013) 33(9):3855–9.

50. Shimodaira S, Sano K, Hirabayashi K, Koya T, Higuchi Y, Mizuno Y, et al. Dendritic Cell-Based Adjuvant Vaccination Targeting Wilms' Tumor 1 in Patients with Advanced Colorectal Cancer. Vaccin (Basel) (2015) 3(4):1004– 18. doi: 10.3390/vaccines3041004

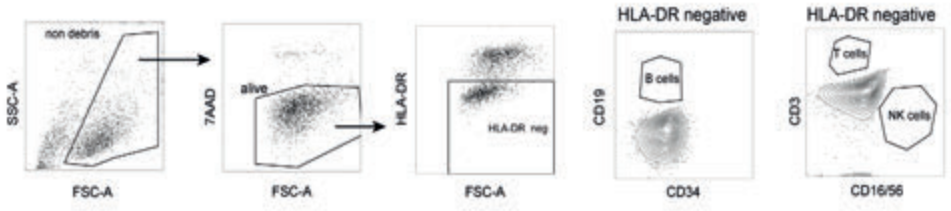
51. Takahashi H, Okamoto M, Shimodaira S, Tsujitani S, Nagaya M, Ishidao T, et al. Impact of dendritic cell vaccines pulsed with Wilms' tumour-1 peptide antigen on the survival of patients with advanced non-small cell lung cancers. Eur J Cancer (2013) 49(4):852–9. doi: 10.1016/j.ejca.2012.11.005

52. Van Tendeloo VF, Van de Velde A, Van Driessche A, Cools N, Anguille S, Ladell K, et al. Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. Proc Natl Acad Sci U S A (2010) 107(31):13824–9. doi: 10.1073/pnas.1008051107

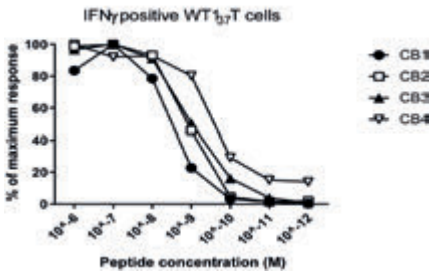
53. Maslak PG, Dao T, Bernal Y, Chanel SM, Zhang R, Frattini M, et al. Phase 2 trial of a multivalent WT1 peptide vaccine (galinpepimut-S) in acute myeloid leukemia. Blood Adv (2018) 2(3):224–34. doi: 10.1182/bloodadvances.2017014175

54. Chaise C, Buchan SL, Rice J, Marquet J, Rouard H, Kuentz M, et al. DNA vaccination induces WT1-specific T-cell responses with potential clinical relevance. Blood (2008) 112(7):2956–64. doi: 10.1182/blood-2008-02-137695

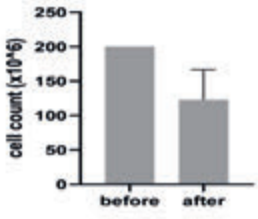
# SUPPLEMENTARY MATERIAL




**SUPPLEMENTARY FIGURE 1 |** Gating strategy of purity after the whole procedure. Reference vial from the 5 validation runs were used to perform purity staining using flow cytometry. 1 representative shown.

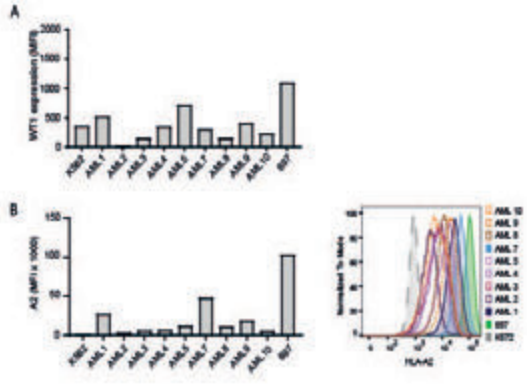



**SUPPLEMENTARY FIGURE 2 |** T-cell avidity assay. Normalized IFNg production of WT1-specific T-cells stimulated by four different CBDCs, pulsed with different WT1-antigen concentrations (M).




**SUPPLEMENTARY FIGURE 3 |**

Loss of cell number after EP. Cell count before and after EP. 200x10^6 cells were electroporated for each sample.




**SUPPLEMENTARY FIGURE 4 |** WT1 and HLA-A2 expression by primary AML samples. All obtained AML samples were measured using flow cytometry. (A) Mean fluorescent intensity (MFI) of intracellular WT1 levels of the different primary pediatric AML samples. WT1 positive AML samples were selected for the killing assay (figure 5) (B) MFI of HLA-A2 levels expressed in bars (left) or histogram (right) to show variable expression although selected from the database on genetically positive levels of A2.

# COMBINED CRISPR-CAS9 AND LENTIVIRAL VECTOR FOR TCR EXCHANGE GENERATES A SAFE AND EFFICIENT CORD BLOOD DERIVED T CELL THERAPY PRODUCT FOR RELAPSED AML PEDIATRIC PATIENTS

5

Vania Lo Presti[1,2], Angelo Meringa[2], Ester Dunnebach[1,2], Aida Valera Moreira[1], Maud Plantinga[2], Annelisa Cornel[1,2], Zsolt Sebestyen[2], Jurgen Kuball[2], Niek van Til[2,3,4], Stefan Nierkens[1,2]

[1] Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands

[2] Center for Translational Immunology, UMC Utrecht, The Netherlands

[3] AVROBIO, Inc., Cambridge, MA, USA.

[4] Child Neurology, Emma Children's Hospital, Amsterdam University Medical Centers, Vrije Universiteit and Amsterdam Neuroscience, Amsterdam, the Netherlands.

*Manuscript in preparation*

## ABSTRACT

Hematopoietic cell transplantation (HCT) is an effective treatment for pediatric patients with high risk, refractory, or relapsed acute myeloid leukemia (AML). However, a large proportion of transplanted patients eventually die due to relapse. To improve overall survival, we propose a combined strategy based on cord blood (CB)-HCT combined with the application of cellular therapy derived from the same CB graft. The proposed cell therapy product account for CB-CD8+ T cells expressing a recombinant T cell receptor (rTCR) against Wilms tumor 1 (WT1) while lacking endogenous TCR (eTCR) expression to avoid mispairing and TCR competition. Highly efficient CRISPR-Cas9 multiplexing approach, targeting the constant region of the endogenous TCRα (TRAC) and TCRβ (TRBC) chains, resulted up to 95% gene-editing efficiency. Next, using an efficient method for lentiviral transduction, more than 70% of the cells were genetically modified to express rWT1-TCR. WT1-TCR engineered T cells lacking the expression of their eTCR (eTCRαβ$^{-/-}$+WT1-TCR) showed increased surface expression of rTCR and cytotoxic cytokines production, such as Granzyme A and B, perforin, IFNɣ and TNFα upon antigen recognition, when compared to WT1-TCR engineered T cells expressing their eTCR. CRISPR-Cas9 editing did not affect immunophenotypic characteristics, T cell activation and did not induce inhibitory molecules. Moreover, eTCRαβ$^{-/-}$+WT1-TCR show effective killing capacity when in co-culture with neoplastic cell lines and primary AML blasts, but did not show toxicity towards healthy cells.

In summary, we show the feasibility to develop a potent CB-derived CD8+ T cell product targeting WT1, providing an alternative option for post-transplant allogeneic immune cell therapy to prevent relapse or as an off-the-shelf product to increase overall survival chances in pediatric AML patients.

## INTRODUCTION

Relapse after hematopoietic cell transplantation (HCT) in AML pediatric patients is still a leading cause of mortality, accounting for approximately 30% of deaths in the transplanted AML patients[1]. Additional therapeutic approaches are therefore essential to increase the overall survival. Considering the great results achieved in the treatment of pediatric acute lymphoblastic leukemia, T cell therapy approaches have also been explored for the treatment of acute myeloid leukemia (AML). Adoptive T cell therapy for AML has focused on chimeric antigen receptor (CAR)-T cells targeting CD33 and CD123; or on TCR-T cells targeting tumor associated antigens, such as Wilm's Tumor 1 (WT1), preferentially expressed antigen in melanoma (PRAME), and minor histocompatibility antigens (MiHA) [2–5].

WT1 over-expression is reported in 75 to 90% of AML adult and pediatric patients[6] and it has been proposed as a potential marker for minimal residual disease[7]. The use of TCR-T cells targeting WT1, post-HCT, recently showed promising results in term of safety and prevention of relapse in adults[8], prompting the question whether WT1-TCR mediated therapy could also be implemented in pediatric AML patients. Generating tailor-made TCR-T cell products for pediatric leukemia patients are complex and often not applicable due to lymphocytopenia, inherent to the disease condition and/or cancer treatment (e.g. chemotherapy)[9]. In addition, the time between cell collection and availability of the product remains a limiting factor, especially in patients at later stage of the disease whom experience a rapid deterioration of their health condition[10], even though increasing effort has been invested to optimize the TCR-T cell production protocol and to decrease many challenges remain unresolved. The use of cord blood (CB) derived allogeneic T cells for adoptive T cell therapy products is a valid option, in combination with CB-HCT or as an off-the-shelf treatment option [11].

CB-HCT has shown to be an effective treatment for relapsed or refractory AML, especially in pediatric patients, who lack a matched bone marrow donor[12]. Around 20% of the available CB unit is stored as a frozen product separately from the original unit and serves mostly for quality control or research purposes, but can also be used for generating complementary cellular immunotherapy products. CB-T cells, have an intrinsic capacity to stimulate Graft versus Leukemia (GvL) responses without increasing the probability of developing Graft versus Host Disease (GvHD)[13]. Therefore, the transfer of a recombinant TCR (rTCR) tumor specific can further increase the efficacy of the anti-leukemia response of CB derived T cells.

However, the simulteneous presence of the rTCR and the endogenous TCR can induce mispairing of TCRs. TCRs mispairing is extremely unpredictable in the sense that it can re-target to self-antigen. Hence, it resulted in devastating autoimmune-like consequences in a mouse model treated with TCR-T cell products[14]. Gene editing techniques eliminating endogenous TCR expression can further improve their efficacy and safety. Here we show a strategy of gene editing in CB-T cells using CRISPR-Cas9, that eliminates the expression of both the alpha and beta chains of the endogenous TCR (eTCR), coupled with the use of lentiviral vectors coding for a WT1 specific TCR (WT1-TCR).

As previously described, eliminating both chains of the eTCR abolishes the possibility to generate TCR mispairing and increases the potential of the final retrovirally engineered rTCR T cell product [15,16]. Moreover, the cell surface expression of the rTCR competes with the eTCR for co-receptor binding, such as CD3 and CD4/CD8, limiting efficient TCR signaling [17].



In this paper we show an efficient and relatively short procedure to generate WT1-TCR T cells from CB-CD8+ T cells whom have been gene edited using CRISPR/Cas9 to eliminate the expression of the eTCR, named eTCRαβ$^{-/-}$ WT1-TCR CB-T cells. eTCRαβ$^{-/-}$ WT1-TCR CB-T cells are characterized by high proliferation rate and viability. In addition, we also investigated the differences between eTCRαβ$^{-/-}$ -WT1-TCR CB CD8+ T cells and CD8+ T cells transduced only with WT1-TCR, but without TCR gene editing (eTCRαβ$^{+/+}$ -WT1-TCR) CB CD8+ T cells in terms of immunophenotype, cytokine production and killing capacity of cells naturally expressing WT1, both immortalized and primary AML cells.

## MATERIALS AND METHODS

### CORD BLOOD DERIVED CD8+ T CELL ISOLATION AND EXPANSION

Fresh umbilical cord blood (CB) was collected after informed consent was obtained according to the Declaration of Helsinki. The ethics committee of the University Medical Center Utrecht approved these collection protocols. CB was processed to isolate CD8+ T cells using Ficoll (GE Healthcare Bio-Sciences AB) separation and CD8 positive magnetic bead separation according to the manufacturer protocol (Miltenyi Biotech). CD8+ T cells were subsequently cultured in RPMI (Fisher Scientific) supplemented with 10% human serum, 1% penicillin/streptomycin (P/S) cytotoxic T lymphocytes media (CTL media), 50U of IL2/ml, 5ng/ml IL7 and 5ng/ml IL15 (all from Miltenyi Biotech), activated for 3 days with anti CD3/CD28 Dynabeads (GIBCO, Thermo Fisher Scientific) in a 1:3 ratio (beads : T cells).



### CRISPR-CAS9 MEDIATED GENE EDITING OF CB CD8+ T CELLS

Ribonucleoprotein (RNP) complexes were generated using an equal concentration (45µM) of Alt-R® S.p. Cas9 Nuclease V3 (IDT, 1081059) and Alt-R® CRISPR-Cas9 crRNA:Alt-R® CRISPR-Cas9 tracrRNA in a 1:1 volume ratio and incubated for 15 min at room temperature. CB CD8+ T cells were selected after 3 days of culture and magnetic separation was applied to remove beads. 5x105 cells per condition were resuspended in T Buffer (Neon™ Transfection System 10µL Kit, Thermo Fisher). Electroporation was performed following the producer suggested settings for Jurkat cells (1600 V/10 ms/3 pulses). After electroporation cells were transferred in a 96 well plate with CTL media supplemented with 200U of IL2/ml, 20ng/ml IL7 and 20ng/ml IL15 (Supplemented CTL media) and incubated at 37°C and 5% CO2.

### LENTIVIRAL VECTOR PRODUCTION AND TITRATION

HIV-derived self-inactivating third generation lentiviral vectors were constructed using the CMV promoter driving the expression of the viral transcript (pCCL plasmid backbone)[18,19]. Additionally to the packaging signal (ψ), the Rev response element (RRE) and the central polypurine tract (cPPT), the transfer vector plasmid contains a modified Woodchuck posttranslational regulatory element (bPRE4)[20]. A Wilms' Tumor 1 (WT1) specific codon-optimized TCR sequence[21] was cloned into the lentiviral transfer vector plasmid under the control of the MND promoter [22].Lentiviral vectors were produced by transient co-transfection of HEK293T cells with the lentiviral

transfer vector plasmid (LV.WT1-TCR) and the respective packaging plasmids (pRSV-Rev, pMDLg/pRRE and pMD2-VSV-G) using CalPhos™ Mammalian Transfection Kit (Clontech), as previously described. Viral supernatants were filtered through 0.45 µm low-protein binding filters, concentrated by ultracentrifugation at 20,000g for 2 hours, resuspended in StemMACS HSC expansion media (Miltenyi Biotech) and stored at -80°C. Vesicular stomatitis virus (VSV)-g protein pseudotyped lentiviral particles were titrated on Jurkat cells. Concentrated viral vector supernatants were serially diluted on Jurkat T cells. At 72h post transduction, cells were harvested and analyzed by flow cytometry for the expression of TCRVβ21.3, expressed by the WT1-TCR, to determine viral titer.

## CB CD8+ T CELL TRANSDUCTION

Expanded and stimulated CD8+ T cells were transduced overnight in StemMACS HSC Expansion Media XF (Miltenyi Biotech) supplemented with 1%P/S at MOI 10 using LV.WT1-TCR. After 14-16 hrs transduction media was replaced with complete CTL-media. T cells were mixed and incubated with the lentiviral vector for 14-16 hrs after which transduction media was replaced with complete supplemented CTL-media. Transduction was performed in the presence of the transduction adjuvant LentiBOOST (1:100 of the total volume; SIRION Biotech).

## FLOW CYTOMETRY

Cells were stained with fluorescently labeled antibodies against CD3-Pacific Blue (clone UCHT1, ref. 558117, BD); CD8-PE (clone RPA-T8, ref. 301008, Biolegend); Fixable Viability Dye eFluor™ 780 (ref. 65-0865-14, ThermoFisher); CD45-PeCy7 (clone HI30, ref. 557748, BD); TCR-panαβ (clone IP6, ref. 17-9986-42, eBioscience) to evaluate knock-out (KO) efficiency and TCRVβ21.3 (clone IG125, ref. PN IM1483, Beckman Coulter) to evaluate transduction efficiency. Antigen recognition was confirmed using tetramer staining (PE-WT1126 / APC-WT1-126), generated as previously described 11,23. To determine the immunophenotype, transduced CD8+ T cells were stained with fluorescently labeled antibodies against Dye eFluor™ 780 (ref. 65-0865-14, ThermoFisher), CD62L-BV650 (clone DREG-56, ref. 2124160, Sony Biotechnology), CD25-PerCP Cy5.5 (clone BC96, ref. 2113130, Sony Biotechnology), TIM3-APC (clone 34482, ref. FAB2365A, R&D), CD3-AF700 (clone UCHT1, ref. 300424, Sony Biotechnology), CD45RA-BV421 (clone HI100, ref. 304118, Biolegend), PD1-BV711 (clone EH12.2H7, ref. 329928, Biolegend), LAG3-PE (ref. FAB2319P, R&D), and CD8-PeCy7 (clone SK1, ref. 335822, BD). HLA-A2 and WT1 positivity were determined

in target cells K562, K562 HLA-A2+, AML485, AML703) by flow cytometry (HLA-A2: clone BB7.2, ref. 558570, BD Pharmingen; clone WT1: F6-H2, Dako). Flow cytometry analysis was performed on a BD Fortessa using FACSDiva, with acquisition of a fixed number.

## IMAGESTREAM

Amnis ImageStream® X Mark II multispectral imaging system was used. 1x106 unmanipulated CB CD8+ cells and eTCR-/- were washed and stained with antibodies against CD3 (clone OKT3, ref.317306, Biolegend), CD8-PE (clone RPA-T8, ref. 301008, Biolegend), TCR-panαβ (clone IP6, ref. 17-9986-42, eBioscience) to detect the membrane expression. Subsequently cells where fixed, permeabilized and stained with anti-CD3 (clone UCHT1, ref. 300420, Biolegend) to determine the cytoplasmatic expression of the co-receptor.

## FUNCTIONAL ASSAYS



T2, K562, K562 HLA-A2+, AML485, AML703 cells were kept in culture in complete RPMI (Glutamax, 1%P/S, 10%FCS) at a concentration of $0.3x10^6$/ml.

**For the activation assay**: eTCRαβ$^{+/+}$-WT1-TCR and eTCRαβ$^{-/-}$-WT1-TCR CB-CD8+ T cells were incubated overnight in four conditions: (1) cells only, (2) in the presence of PMA-Ionomycin, (3) with T2 cells and (4) with T2 cells loaded with the WT1p126. Effector cells and target cells were maintained in a ratio of 1:1. After 14-16hrs supernatant was collected to measure the concentration of secreted cytokines. Cells were washed and stained with CD3, CD8, CD137 and TCRVb21.3. Percentage of activation of T cells in co-culture with T2s was calculated proportionally to the activation after PMA-Ionomycin treatment. The LEGEND-Plex CD8/NK cells (Biolegend) kit was used to detect the amount of cytokines released in the supernatant. The experiment was performed following manufacturer's protocol and analyzed using BD-Fortessa. Data analysis was performed using the Qognit software supplied by Biolegend.

**For the killing assay:** CTRL-untransduced, eTCRαβ$^{+/+}$-WT1-TCR and eTCRαβ$^{-/-}$-WT1-TCR CB-CD8+ T cells were incubated overnight with tumor cell lines HLA-A2+ naturally expressing WT1. K562 (HLA-A2$^{-}$) cells were used as a negative CTRL to determine bystander killing. Effector cells and target cells were maintained in a ratio of 5:1(K562 and K562 HLA-A2+) or 10:1 (AML485-AML703). Target cells were labeled with CellTraceViolet before co-culture. After 12-14hrs cells where washed, stained with Fixable Viability Dye eFluor™ 780 (ref. 65-0865-14, ThermoFisher) and analyzed for cell death at the BD-Fortessa.

## 3D MODEL

The 3D bone marrow model experiments were performed according to the previously described protocol[24]. In short, primary pediatric AML blasts (SKION biobank) were selected for relapse diagnosis, HLA-A2 and WT1 positivity. The WT1+ tumor cells were dyed with Vybrant DiO (Thermo Fisher, United States) and seeded in Matrigel (Corning, United States) combined with endothelial progenitor cells (EPCs) and multipotent, mesenchymal stromal cells (MSCs). The EPCs and MSCs were dyed with Vybrant DiD (Thermo Fisher, United States). After a four day incubation, untransduced T lymphocytes or WT1 TCR+ T lymphocytes were dyed with Vybrant DiI (Thermo Fisher, United States) and administered to the 3D models. Two days post T-cell administration, Matrigel was dissolved using Cell Recovery solution (Corning, United States) to retrieve a single cell suspension. Subsequently, tumor cells, T lymphocytes and stromal cell numbers were quantified by flow cytometry using Flow count Fluorospheres (Beckman Coulter, United States).

## STATISTICAL ANALYSIS

Error bars represent standard deviation across at least three donors. Significance was calculated using Student's T test or two way ANOVA tests in GraphPad Prism Prism 8.3. Statistical significance is represented as *p< 0.05; **p<0.01; ***p <0.001; ****p<0.000

## EXPERIMENTAL SET-UP

The experimental set up is visualized in figure 1, including a timeline of the experiments. In short, cord blood CD8+ T cells were isolated from fresh CB units and expanded with a cytokine mix (IL2, IL7 and IL15) and αCD3/CD28 beads. Expanded cells were electroporated with the RNP complex (gRNAs:Cas9) targeting the endogenous TCR alpha and beta chains (eTCRαβ) and cultured for 72hrs. eTCRαβ$^{-/-}$ T cells and unedited T cells were transduced with lentiviral vectors, coding for the WT1-TCR. eTCRab$^{+/+}$ - WT1-TCR T cells and eTCRαβ$^{-/-}$-WT1-TCR T cells were tested and compared for activation and cytokines production upon antigen recognition at day 12 of the procedure. Cytotoxic functionality was evaluated upon co-culture with cell lines and primary cells naturally expressing WT1.



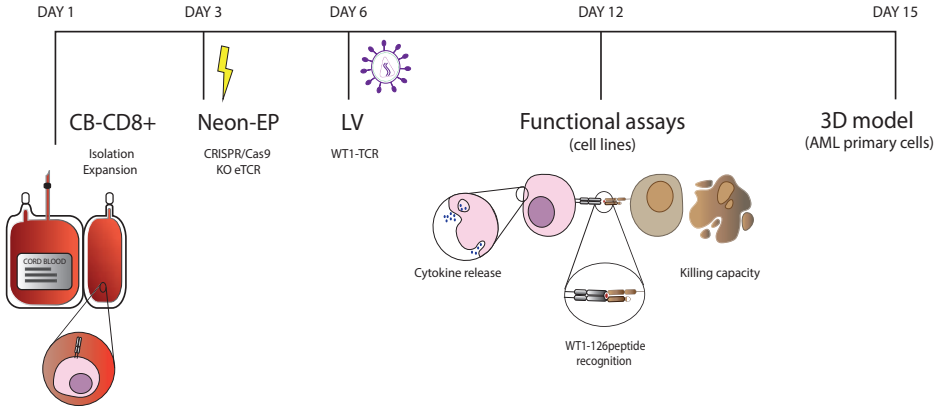



**Figure 1.** Visual representation of the procedure and experimental set-up for generation and functionality assessment of eTCRαβ-/-+WT1-TCR CB CD8+ T cells.

# RESULTS

## HIGH EFFICIENT KO OF TCRαβ USING CRISPR-CAS9 MULTIPLEXING APPROACH TO TARGET TRAC AND TRBC

eTCRαβ $^{-/-}$ in CB-CD8+ T cells was performed using RNP complex electroporation (gRNAs:Cas9) with gRNAs targeting both the constant region of the α chain (TRAC) and the constant region of the β chain (TRBC). The use of single gRNAs showed a KO efficiency, measured as lack of TCR expression, of 82.4% ±10.6 for α-chain and 96.8 ±1.5 for the β-chain. Concomitant use of both gRNAs showed a 96.5±1.5. As expected the eTCRαβ $^{-/-}$ lacked membrane expression of both eTCRαβ and the co-receptor CD3 (Figure 2A-B). Single cell images produced using ImageStream analyses confirmed complete absence of the eTCRαβ and CD3, but edited CB-CD8+ T cells retained the expression of CD8 and the intracellular presence of CD3 (Figure 2C).



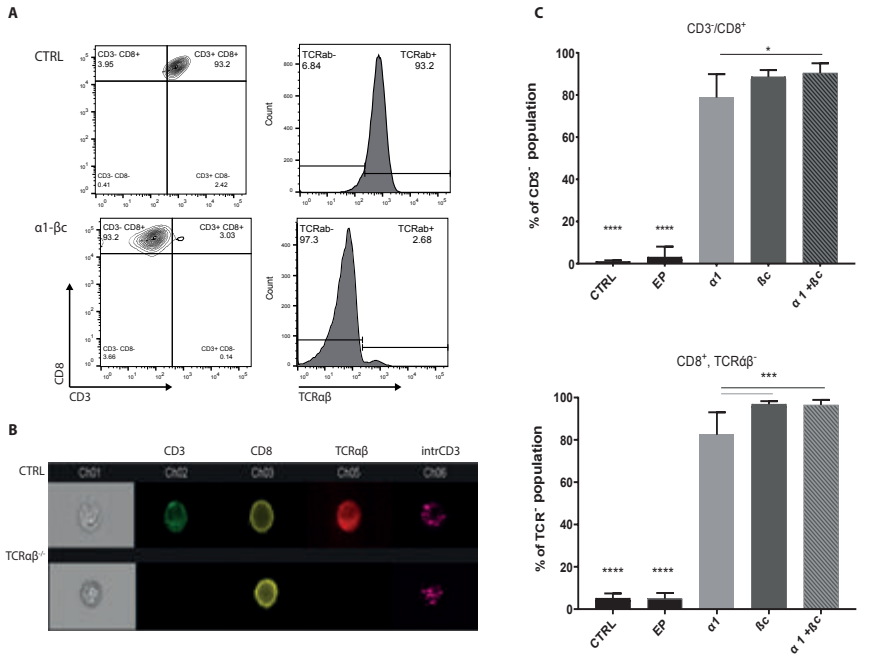



**Figure 2. CRISPR-Cas9 mediated TCRαβ gene editing targeting alpha and beta chain in CB CD8+ T cells.**

A) Representative FACS plot of TCRαβ expression in expanded and unedited CB CD8+ T cells (CTRL) and CB CD8+ transfected with RNP complex targeting simultaneously the TRAC and TRBC locus. Flow cytometry analysis was performed 4 days after electroporation.. B) Representative images of single cells using Image Stream. On the top CTRL, on the bottom gene edited with TRAC+TRBC. C) Percentage of CD3- population (on top) and TCRαβ- population comparing single targeting of TRAC (n=5), TRBC (n=5), simultaneous TRAC+TRBC (n=6) with CTRL cells The data are shown as average ± standard deviation; *p< 0.05; ***p<0.001; ****p<0.0001

## eTCRαβ$^{-/-}$ CB-CD8+ T CELLS SHOW HIGHER TRANSDUCTION EFFICIENCY COMPARED TO CB CD8+ T CELLS

eTCRαβ$^{-/-}$ CB-CD8+ T cells and paired unedited eTCRαβ$^{+/+}$ CB-CD8+ T cells were transduced with a lentiviral vector coding for WT1-TCR, with an MOI of 10. Transduction efficiency was determined after 6 days of culture via detection of expression of the specific Vbeta chain characterizing the WT1-TCR (Vb21.3) and by tetramer reactivity. Transduction efficiency was high in both eTCR$^{\alpha\beta}$ gene-edited and unedited cells. However, eTCRαβ $^{-/-}$ CB-CD8+ T cells, showed an increase in the percentage of cells expressing the WT1-TCR, with an average of 79.8% ± 9.1 compared to an average of 73.8 ± 17.2 for eTCRαβ$^{+/+}$ CB-CD8+ T cells . In addition, eTCRαβ$^{-/-}$ -WT1-TCR compared to eTCRαβ$^{+/+}$ -WT1-TCR CB-CD8+ T cells showed an higher expression of WT1-TCR calculated as mean fluorescent intensity (MFI), respectively 2151± 700 and 1775 ± 731 (Figure 3A-C). In both products, reactivity to the target antigen was confirmed by staining with fluorescent tetramer molecules, confirming that the WT1-TCR expressing cells were fully recognizing the cognate peptide (Figure 3D-E).



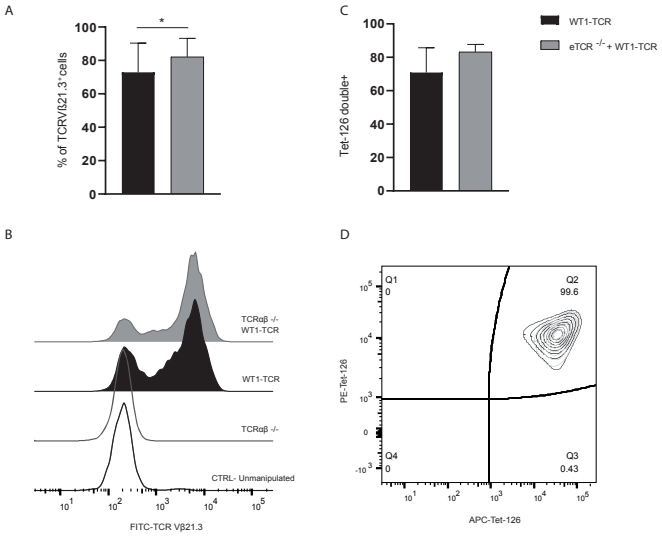



**Figure 3. eTCR$^{-/-}$ CB CD8+ T cells show an increased transduction efficiency and expression of rTCR after LV transduction.**

A) Percentage and median fluorescence intensity of TCRVb21.3+ cells after 6 days from LV transduction of eTCRαβ$^{+/+}$ (black) and of eTCRαβ$^{-/-}$ (grey) CB CD8+ T cells. Data were acquired with flow cytometry. B) Representative comparative FACS plot of TCRVb21.3 (WT1-TCR) expression in transduced eTCRαβ$^{+/+}$ (black; n=7) and of eTCRαβ$^{-/-}$ (grey; n=7) CB CD8+ T cells compared to untransduced eTCRαβ$^{+/+}$ and of eTCRαβ$^{-/-}$ CB CD8+ T cells (White lines). C) Tetramer reactivity of eTCRαβ$^{+/+}$ -WT1-TCR (black; n=3) and of eTCRαβ$^{-/-}$ -WT1-TCR (grey; n=3) CB CD8+ T cells. D) Representative FACS plot of tetramer binding in eTCRαβ$^{+/+}$ -WT1-TCR (black; n=3) and of eTCRαβ$^{-/-}$ (grey; n=3) CB CD8+ T cells. Data are shown as average ± standard deviation.

## eTCRαβ$^{-/-}$ DOES NOT INFLUENCE CB-CD8+ T CELLS PHENOTYPE, BUT INDUCE ENHANCED ACTIVATION AND CYTOTOXIC CYTOKINES PRODUCTION COMPARED eTCRαβ$^{+-+}$-WT1-TCR CB-CD8+ T CELLS

Extensive immunophenotyping of the transduced cells showed that the CRISPR-Cas9 editing did not affect the phenotype of the cells. Both eTCRαβ $^{-/-}$-WT1-TCR and eTCRαβ$^{+/+}$ - WT1-TCR T cells expressed similar levels of CD45RA and CD62L and were characterized by three populations: T1 (CD45RA+, CD62L-); T2 (CD45RA+CD62L+) and T4 (CD45RA-CD62L-). Moreover, the expression of activation and inhibitory markers remained unaltered between the two products, with the only exception of an increased expression of CD127 (IL7Ra) in eTCRαβ$^{-/-}$+WT1-TCR (Figure 4A-C). To determine if there was any difference in activation upon antigen recognition in eTCRαβ$^{-/-}$-WT1-TCR and eTCRαβ$^{+/+}$ -WT1-TCR T cells, the cells were co-cultured for 24hrs with T2 loaded with the cognate WT1 peptide (T2+). After co-culture with T2+, eTCRαβ$^{-/-}$ -WT1-TCR T cells showed a higher level of activation defined by CD137 expression (Figure 4D). Moreover, the supernatant was collected and the production of thirteen cytokines was analyzed using the platform LegendPlex (Biolegend). A trend of higher cytotoxicity levels by eTCRαβ$^{-/-}$-WT1-TCR cells was observed, especially for TNFα, IFNɣ and Granzyme A and B (Figure 4E), but this was not significant.

## eTCRαβ$^{-/-}$-WT1-TCR CB-CD8+ T CELLS SHOW AN INCREASED KILLING CAPACITY TOWARDS CELL LINES NATURALLY EXPRESSING WT1

K562 have been reported to lack expression of HLA class I and II molecules [25], but WT1 positive and were considered as a negative control for HLA-independent reactivity of the T cells. In addition, K562 were modified to express HLA-A2 (K562 HLA-A2+) to monitor reactivity toward WT-1 peptide presented in A2. To assess the direct cytotoxic capacity of the aforementioned cell products, a preliminary screening on AML patient-derived cell lines was performed to define the expression of HLA-A2 and WT1. Two lines were selected for further experiments: further referred to as AML485 and AML703 (Figure 5A). Overnight co-culture experiments with different ratios of effector and target cells were performed to define the best ratio (5:1 for K562s and 10:1 for AML485/AML703) (data not shown). In line with the increased CD137 and cytokine expression, in all cases, the cytotoxicity of eTCRαβ$^{-/-}$-WT1-TCR was more pronounced than of eTCRαβ$^{+/+}$ -WT1-TCR T cells (Figure 5B-C).

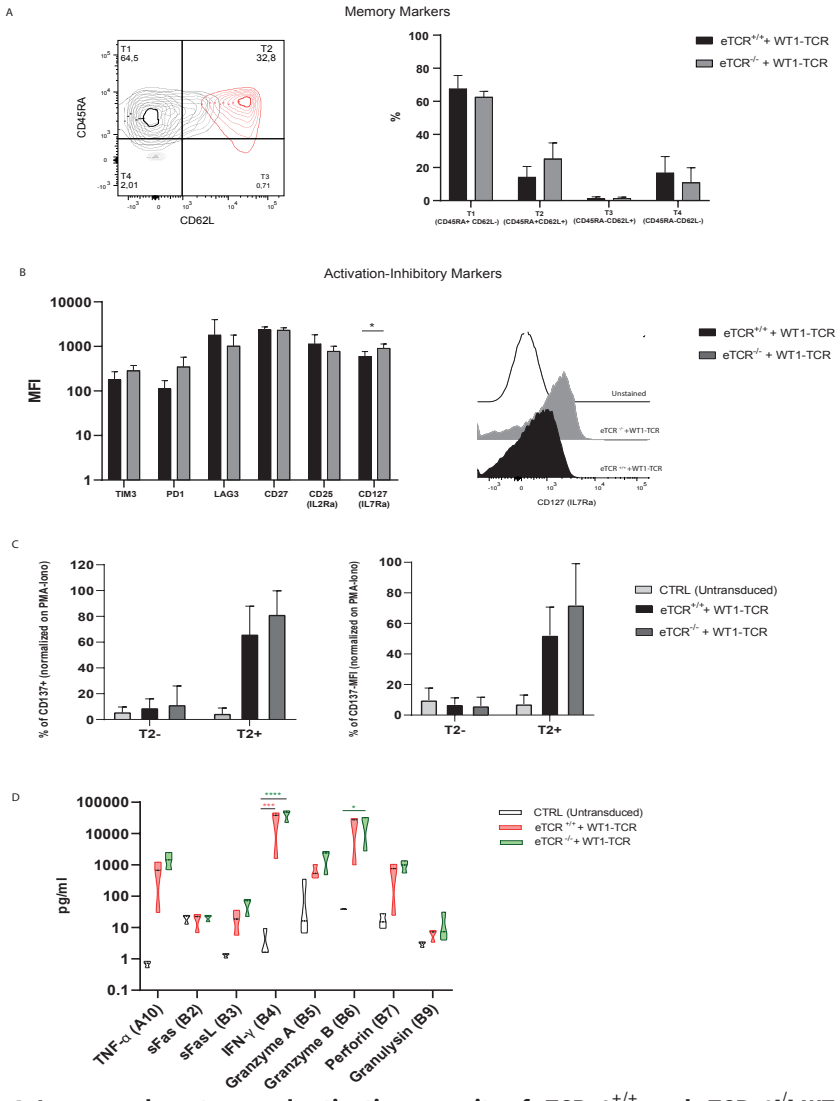



**Figure 4- Immune phenotype and activation capacity of eTCRαβ$^{+/+}$- and eTCRαβ$^{-/-}$-WT1-TCR**

A) On the left a representative FACS plot of CD45RA and CD62L expression in eTCRαβ$^{+/+}$-WT1-TCR (black) and eTCRαβ$^{-/-}$-WT1-TCR (grey) compared to freshly isolated CB CD8+ T cells (red); unstained cells are depicted in light grey. On the right, percentage of cells characterized by several maturation markers, eTCRαβ$^{+/+}$-WT1-TCR (black; n=5) and of eTCRαβ$^{-/-}$-WT1-TCR (grey; n=5) CB CD8+ T cells. In order subpopulations were defined as T1 (CD45RA+, CD62L-), T2 (CD45+, CD62L+), , T3 (CD45RA-, CD62L+), T2 (CD45+, CD62L+) and T4 (CD45RA-, CD62L-). B) On the left, expression of multiple receptors on the surface of eTCRαβ$^{+/+}$+WT1-TCR (black) and eTCRαβ$^{-/-}$-WT1-TCR (grey) calculated as MFI. Data are shown as average ± standard deviation from ≥ 5 independent experiments. C) Percentage (on the left) and MFI (on the right) of CD137+ T cells in CTRL (white), eTCRαβ$^{+/+}$-WT1-TCR (black) and eTCRαβ$^{-/-}$-WT1-TCR (grey) after 24hrs of co-culture with T2 cells (T2-) or T2 cells loaded with the specific WT1 peptide (T2+). Cytokines production after 24hrs of co-culture with T2+ cells of CTRL (white), eTCRαβ$^{+/+}$-WT1-TCR (red) and eTCRαβ$^{-/-}$+WT1-TCR (green) CB CD8 T cells. Data are shown as average ± standard deviation from n=4 independent experiments. Statistical significance is pictured as *p< 0.05; **p <0.01; ***p <0.001; ****p<0.0001

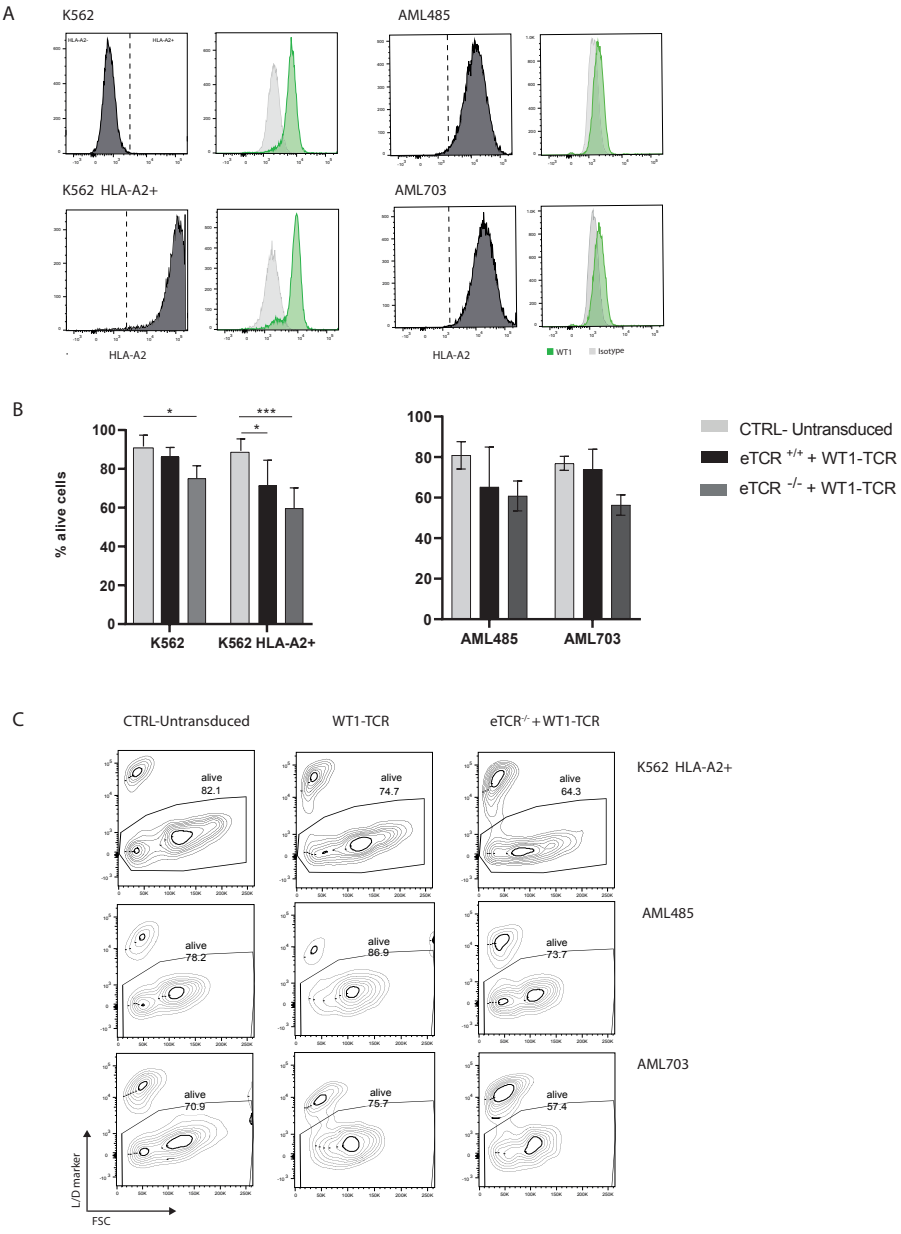



**Figure 5. Cytotoxic capacity of eTCRαβ$^{+/+}$-WT1-TCR and eTCRαβ$^{-/-}$-WT1-TCR**

A) HLA-A2 expression (grey) and WT1 intracellular expression (green) in target tumor cell lines (in order K562, K562- HLA A2+, AML485, AML703). B) Percentage of viable target cells after 16hrs of co-culture with CTRL (white), eTCRαβ$^{+/+}$-WT1-TCR (black) and eTCRαβ$^{-/-}$-WT1-TCR (dark grey). Percentage of viable target cells in co-culture with T cells was standardized on the percentage of viable target cells in standard culture. C) Representative FACS plot depicted target cell viability after 16hrs of co-culture with CB CD8+ T cells. Data are shown as average ± standard deviation from n=5 independent experiments. Statistical significance is pictured as *p< 0.05; ***p <0.001.

## eTCRαβ$^{-/-}$-WT1-TCR CB-CD8+ T CELLS SHOW CYTOTOXIC CAPACITY IN A 3D MODEL RESEMBLING THE PATIENTS' BONE MARROW NICHE

A 3D bone marrow niche model was used for determining the cytotoxic capacity towards primary AML blasts from pediatric patients. Supportive multipotent mesenchymal stromal cells were co-cultured with endothelial progenitor cells, sufficient requirements to facilitating the survival and proliferation of primary CD34+ AML blasts derived from pediatric patients at relapse. The three AML samples were tested for HLA-typing and WT1 expression prior to the experiment. Figure 6A visually summarizes the components of the 3D model and the timeline of the experiment. eTCRαβ$^{-/-}$ -WT1-TCR CB CD8+ T cells were tested for killing capacity alongside untransduced and unedited (CTRL) cells. As shown in figure 6B eTCRαβ$^{-/-}$-WT1-TCR CB CD8+ T cells have a killing capacity that range from 20 to 70% of the primary AML cells, normalized on the killing capacity of CTRL cells. Moreover, eTCRαβ$^{-/-}$ -WT1-TCR CB CD8+ T cells showed a statistically significant increase of T cells migrating towards the target cells and the stroma (Figure 6C). Additionally, to determine the safety of the T cell products and the non-specific killing, the number of stroma cells left in the 3D system was calculated. No differences were detectable between CTRL T cells and eTCRαβ $^{-/-}$ -WT1-TCR CB CD8+ T cells (Figure 6D).

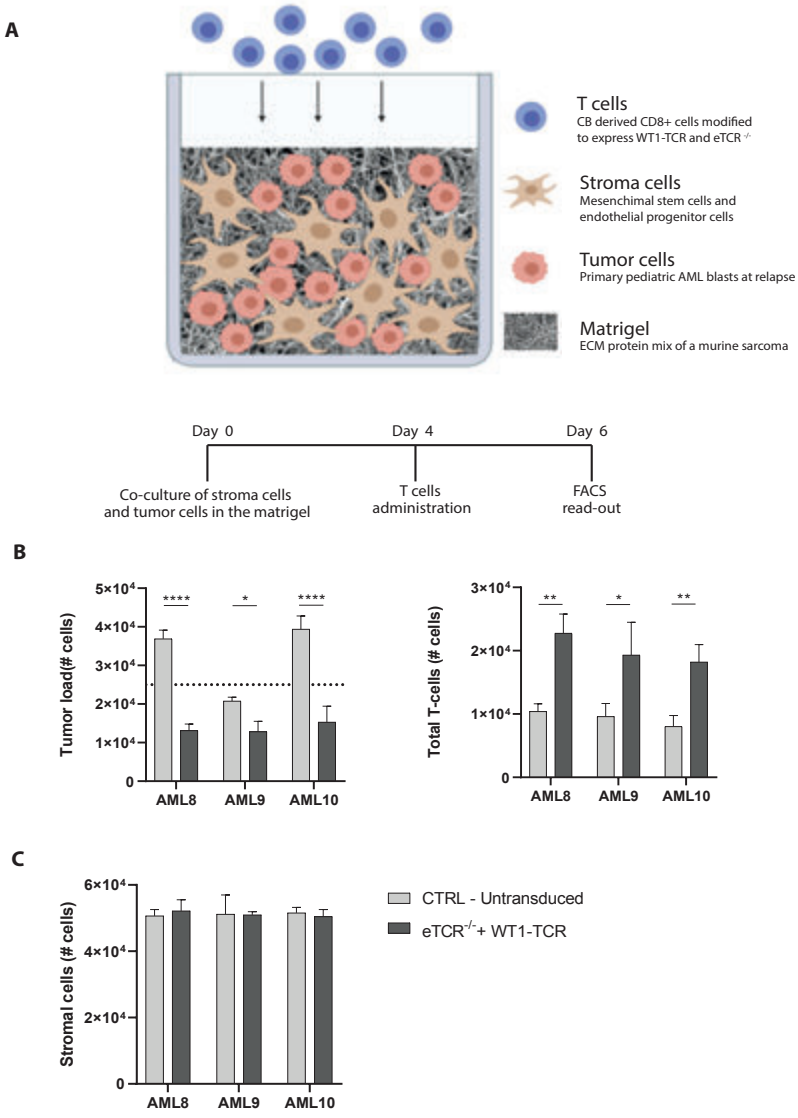



**Figure 6. Cytotoxic capacity of eTCRαβ$^{-/-}$-WT1-TCR in a 3D model of pediatric AML**

A) Representative illustration of the main components of the 3D model and timeline of cell administration. B) Cytotoxic capacity of eTCRαβ$^{-/-}$ + WT1-TCR (grey) and CTRL-Untransduced (white) CB CD8+ T cells after 4 days of co-culture in the 3D model, based on the number of AML primary cells left in the model. Cell number is measured via flow cytometry and compared to the initial number of tumor cells used on Day 0 (dotted line). C) Proliferation capacity upon antigen recognition of eTCRαβ$^{-/-}$ -WT1-TCR (grey) and CTRL-Untransduced (white) CB CD8+ T cells after 4 days of co-culture in the 3D model. D) Viability of stromal cells after 4 days of co-culture in the 3D model, respectively in the presence of eTCRαβ$^{-/-}$ -WT1-TCR (grey) and CTRL-Untransduced (white) CB CD8+ T cells. Cell number is measured via flow cytometry. Data are shown as average ± standard deviation from n=3 biological replicates for eTCRαβ$^{-/-}$ -WT1-TCR and n=1 for CTRL-untransduced. Statistical significance is pictured as *p= 0.05; **p =0.01; ***p =0.001.

## DISCUSSION

CB-HCT is a commonly used treatment option in pediatric hematological malignancies, such as AML, when a matching bone marrow donor is not available. Medical practice has generally improved in the last 20 years, decreasing the chance of graft failure and fatal GvHD reaction, but the relapse rate in pediatric AML patients is still around 30%[1]. Most of the AML patients, both adult and pediatric, are characterized by the over-expression of WT1, a zinc-finger motif nuclear protein. WT1 has been considered as a powerful target for adoptive cell application and for TCR-T cells[26]. Previous in vitro and in vivo studies have successfully demonstrated the effectiveness of WT1 specific T cells in eliminating cell lines naturally expressing WT1[27–30]. A recent clinical trial corroborated the results achieved in pre-clinical studies, showing the success in preventing relapse when treating AML adult patients with WT1-TCR expressing T cells[31]. However, efficacy and safety of the TCR-T cell therapy approach can be hampered by the presence of the endogenous TCR, due to the possibility of mispairing with the recombinant TCR and the competition for co-receptors[17]. Gene-editing techniques, such as Zinc Finger Nucleaseas (ZFNs) and Transcription Activator-like Effector Nucleases (TALENs), have shown successful results to eliminate the expression of the endogenous TCR[32,33], and in a case obtained approval for clinical testing[34]. However, designing ZFNs, and to a lesser extend TALENs is not user-friendly and complex and slowed down their broad applicability. The recent advent of an easy to access and clinically translatable, gene editing technique, known as CRISPR technology, is promoting the fast application of this tools to improve cell therapy products[35].

In this manuscript we generated an efficient and safe T cell product originating from CB, characterized by the knock-out of both alpha and beta chains of the eTCR and gene modified to express a recombinant WT1-TCR; to be used as an additional therapy after CB-HCT in pediatric AML patients. It has been shown that the elimination of either the alpha and beta chains of the endogenous TCR can increase the efficacy of the final product, measured as percentage of transduced cells, lytic capacity and IFNɣ production when in culture with target cells[36,37]. However, more recently, it has been described that, even if eliminating only one of the two chains (alpha or beta) can improve the functionality of the edited T cells, the procedure can increase the probability of mispairing between the un-edited chain and the recombinant TCR[16]. A more extensive comparison between single chain edited T cells versus eTCRαβ$^{-/-}$ demonstrated that cells edited for both alpha and beta chains were significantly more prone to express the rTCRs transferred via retroviral vectors, produced more

IFNɣ and had an higher lytic efficacy[15] . In this manuscript, we confirmed the high efficiency of eliminating both alpha and beta chains of the eTCRαβ , also when target different regions of the TRAC and TRBC genes, using the electroporation of the CRISPR-Cas9 RNP complex without affecting viability and proliferation capacity of CB-CD8+ T cells (Figure 2).

Higher transduction efficiency, using lentiviral vectors was also detected in this study in eTCRαβ$^{-/-}$ cells compared to unedited cells (Figure 3). To further investigate the differences between the two T cell products and expand what have already been presented in literature, an extensive immunophenotype analysis was performed. Overall WT1-TCR transduced cells and eTCRαβ$^{-/-}$ -WT1-TCR cells do not differ. Based on the expression of CD45RA and CD62L three populations were detectable that we defined as T1(CD62L-CD45RA+); T2 (CD62L+CD45RA+) and T4(CD62L-CD45RA-). In both products the expression activation and exhaustion markers remained comparable throughout the culture; with the exception of CD127(IL7Ra) that appeared to be expressed more in eTCR$^{-/-}$-WT1-TCR T cells. Interestingly, both products maintained an elevated expression of the co-stimulatory receptor CD27. These phenotypical features in CB derived T cell products, suggest the potential presence of a subpopulation with similar feature to the previously described T stem cells memory (scm)[38].

Cellular products derived from T(n) fraction of PB CD8+ T cells expanded with IL-7 and IL-15, have shown an increased capacity of persist and proliferate in in vivo studies and have been categorized as T(scm)[39]. Additional phenotypic markers, such as the presence of CD95 and CCR7, and more in depth genome expression analysis, using RNAseq, will be necessary to better characterize the phenotype of the CB derived final product; however its homogenous initial naïve composition is a good indicator of a favorable phenotype. Moreover, maintenance of CD27 andCCR7 expression, together with the presence of longer telomerase, positively correlated with the likelihood to achieve an objective clinical response after adoptive cell therapy (ACT)[40,41] . New approaches are being investigated to further select and instruct the generation of Tscm cell product, such as the use of AKT inhibitors[42,43], opening perspective for the future.

WT1-TCR T cells with eTCRαβ$^{-/-}$ -WT1-TCR T cells were further compared based on grade of activation and cytokines production upon antigen recognition. eTCRαβ$^{-/-}$ +WT1-TCR T cells showed an increased population expressing CD137, both in term of number of cells expressing the marker as well as mean fluorescent intensity (MFI); suggesting a better/faster response upon cognate peptide recognition. This result

was also corroborated by the production, by eTCRαβ$^{-/-}$ +WT1-TCR cells, of an overall greater amount of cytotoxic cytokines (Figure 4), broadening the findings already presented before on the capacity to produce higher levels of IFNg by eTCRαβ$^{-/-}$ [15,16]. In our study, edited and only transduced cells were also compared in more relevant assays with cell lines naturally expressing WT1 and with primary cells derived from relapsed pediatric patients, grown in a 3D model. As shown in figure 5, eTCRαβ$^{-/-}$ -WT1-TCR T cells performed better than unedited transduced T cells. In both cases, it is worth mentioning that the effector to target ratio was optimized to achieve an efficient result without the use of extremely high cell numbers.

Due to the high death rate of primary AML cells in in vitro and the difficulty to generate in vivo models using primary AML cells, we decided to test the efficacy of our proposed eTCRαβ$^{-/-}$ +WT1-TCR product in a 3D model of the BM niche[24]. The presence of a supportive microenvironment allows to keep high viability of the primary AML cells, specifically derived from three pediatric patients at time of relapse. Using this method, we were able to define both the migration capacity of the and their specific killing capacity. On both aspects, eTCRαβ$^{-/-}$ +WT1-TCR T cells showed increased anti-leukemic capacity, achieving up to 65% of active killing of primary tumor cells (Figure 6); similarly or better than the results achieved by previous studies with AML primary cells [8,33].



In this manuscript we proposed one specific application involving gene-editing techniques to improve cell therapeutic products; especially in the field of TCR-T therapy. The same application might not be valid for other type of T cell products, as it was recently shown that the elimination of the TCR impairs the functionality of CAR-T cells in vivo[44]. However, the use of gene-editing techniques, especially CRISPR/Cas9, for its the feasibility and translational capacity, can be implemented to target different molecules involved in the efficacy of final products, such us checkpoint inhibitors [45,46]; or even to target several molecules at the same time [47,48].

Multiplex approaches, such as specifically targeting the three genes eTCR, CD52, and HLA class I (*via* B2M targeting), can move faster the use of CB-T cells as a source for the development of "off-the-shelf" cell therapy products, and it was already tested in CAR-T cells[49–51]. However, with multiplexing approaches it is important to take in consideration the probability of off-target effects. The off-target effect is categorized as non-specific genome modification due to the use of CRISPR platform and is a reason of concern in the field of gene-editing applied in a clinical context. Many strategies are being investigated to minimize the probability of the off-target effect to occur[52] and, at the moment, no critical related side-effects for the patients have been reported in the clinical trials using gene-edited T cells[48,53].

In conclusion, we demonstrated the use of CRISPR/Cas9 to eliminate the expression of the eTCR is a feasible approach for CB-CD8+ T cells, to improve the efficacy and safety . eTCRαβ$^{-/-}$ -WT1-TCR CB-CD8+ T cells showed an overall superior efficacy when compared to eTCRαβ$^{+/+}$ - WT1-TCR CB-CD8+ T cells. Conclusively eTCRαβ$^{-/-}$ -WT1-TCR CB-CD8+ T cells showed great killing capacity when co-cultured with AML blasts derived from pediatric patients at the moment of relapse, the most relevant target cells for proving the potential of the product to prevent relapse after CB-HCT. Moreover, eTCRαβ$^{-/-}$ -WT1-TCR CB-CD8+ T cells showed a safe profile and did not show any effect on the stroma cells. Overall, a feasible and short protocol was developed for production of gene-edited and tumor-antigen redirected cord blood CD8+ T cells, which showed an improved in vitro functionality supporting further pre-clinical development and safety assessment.

## BIBLIOGRAPHY


1. Rasche, M., Zimmermann, M., Borschel, L., Bourquin, J.P., Dworzak, M., Klingebiel, T., Lehrnbecher, T., Creutzig, U., Klusmann, J.H., and Reinhardt, D. (2018). Successes and challenges in the treatment of pediatric acute myeloid leukemia: a retrospective analysis of the AML-BFM trials from 1987 to 2012. Leukemia, 1–11.
2. Bolouri, H., Farrar, J.E., Triche, T., Ries, R.E., Lim, E.L., Alonzo, T.A., Ma, Y., Moore, R., Mungall, A.J., Marra, M.A., et al. (2018). The molecular landscape of pediatric acute myeloid leukemia reveals recurrent structural alterations and age-specific mutational interactions. Nat. Med. 24, 103–112.
3. Xue, S.A., Gao, L., Hart, D., Gillmore, R., Qasim, W., Thrasher, A., Apperley, J., Engels, B., Uckert, W., Morris, E., et al. (2005). Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. Blood 106, 3062–3067.
4. Amir, A.L., Van Der Steen, D.M., Van Loenen, M.M., Hagedoorn, R.S., De Boer, R., Kester, M.D.G., De Ru, A.H., Lugthart, G.J., Van Kooten, C., Hiemstra, P.S., et al. (2011). PRAME-specific allo-HLA-restricted T cells with potent antitumor reactivity useful for therapeutic T-cell receptor gene transfer. Clin. Cancer Res. 17, 5615–5625.
5. van Balen, P., Jedema, I., van Loenen, M.M., de Boer, R., van Egmond, H.M., Hagedoorn, R.S., Hoogstaten, C., Veld, S.A.J., Hageman, L., van Liempt, P.A.G., et al. (2020). HA-1H T-Cell Receptor Gene Transfer to Redirect Virus-Specific T Cells for Treatment of Hematological Malignancies After Allogeneic Stem Cell Transplantation: A Phase 1 Clinical Study. Front. Immunol. 11, 1–11.
6. Rosenfeld, C., Cheever, M.A., and Gaiger, A. (2003). WT1 in acute leukemia, chronic myelogenous leukemia and myelodysplastic syndrome: Therapeutic potential of WT1 targeted therapies. Leukemia 17, 1301–1312.
7. Bergmann, L., Miething, C., Maurer, U., Brieger, J., Karakas, T., Weidmann, E., and Hoelzer, D. (1997). High levels of Wilms' tumor gene (wt1) mRNA in acute myeloid leukemias are associated with a worse long-term outcome. Blood 90, 1217–25.
8. Chapuis, A.G., Egan, D.N., Bar, M., Schmitt, T.M., McAfee, M.S., Paulson, K.G., Voillet, V., Gottardo, R., Ragnarsson, G.B., Bleakley, M., et al. (2019). T cell receptor gene therapy targeting WT1 prevents acute myeloid leukemia relapse post-transplant. Nat. Med. 25, 1064–1072.
9. Graham, C., Jozwik, A., Pepper, A., and Benjamin, R. (2018). Allogeneic CAR-T Cells : More than Ease of Access ? Cells, 1–11.
10. Roddie, C., O'Reilly, M., Dias Alves Pinto, J., Vispute, K., and Lowdell, M. (2019). Manufacturing chimeric antigen receptor T cells: issues and challenges. Cytotherapy 21, 327–340.
11. Lo Presti, V., Cornel, A.M., Plantinga, M., Dünnebach, E., Kuball, J., Boelens, J.J., Nierkens, S., and van Til, N.P. (2021). Efficient lentiviral transduction method to gene modify cord blood CD8+ T cells for cancer therapy applications. Mol. Ther. - Methods Clin. Dev. 21, 357–368.
12. Rocha, V., Labopin, M., Sanz, G., Arcese, W., Schwerdtfeger, R., Bosi, A., Jacobsen, N., Ruutu, T., Lima, M. De, Finke, J., et al. (2004). Transplants of Umbilical-Cord Blood or Bone Marrow from Unrelated Donors in Adults with Acute Leukemia.
13. Hiwarkar, P., Qasim, W., Ricciardelli, I., Gilmour, K., Quezada, S., Saudemont, A., Amrolia, P.,

and Veys, P. (2017). Cord blood T cells mediate enhanced antitumor effects compared with adult peripheral blood T cells. Blood 126, 2882–2892.

14. Bendle, G.M., Linnemann, C., Hooijkaas, A.I., Bies, L., de Witte, M.A., Jorritsma, A., Kaiser, A.D.M., Pouw, N., Debets, R., Kieback, E., et al. (2010). Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. Nat. Med. 16, 565–570.
15. Morton, L.T., Reijmers, R.M., Wouters, A.K., Kweekel, C., Remst, D.F.G., Pothast, C.R., Falkenburg, J.H.F., and Heemskerk, M.H.M. (2020). Simultaneous Deletion of Endogenous TCRαβ for TCR Gene Therapy Creates an Improved and Safe Cellular Therapeutic. Mol. Ther. 28, 64–74.
16. Schober, K., Müller, T.R., Gökmen, F., Grassmann, S., Effenberger, M., Poltorak, M., Stemberger, C., Schumann, K., Roth, T.L., Marson, A., et al. (2019). Orthotopic replacement of T-cell receptor α- and β-chains with preservation of near-physiological T-cell function. Nat. Biomed. Eng.
17. Ahmadi, M., King, J.W., Xue, S.A., Voisine, C., Holler, A., Wright, G.P., Waxman, J., Morris, E., and Stauss, H.J. (2011). CD3 limits the efficacy of TCR gene therapy in vivo. Blood 118, 3528–3537.
18. Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. (1998). A Third-Generation Lentivirus Vector with a Conditional Packaging System. J. Virol. 72, 8463–8471.
19. Zufferey, R., Dull, T., Mandel, R.J., Bukovsky, a, Quiroz, D., Naldini, L., and Trono, D. (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J. Virol. 72, 9873–80.
20. Van Til, N.P., Sarwari, R., Visser, T.P., Hauer, J., Lagresle-Peyrou, C., Van Der Velden, G., Malshetty, V., Cortes, P., Jollet, A., Danos, O., et al. (2014). Recombination-activating gene 1 (Rag1)-deficient mice with severe combined immunodeficiency treated with lentiviral gene therapy demonstrate autoimmune Omenn-like syndrome. J. Allergy Clin. Immunol. 133, 1116–1123.
21. Kuball, J., Dossett, M.L., Wolfl, M., Ho, W.Y., Voss, R.H., Fowler, C., and Greenberg, P.D. (2007). Facilitating matched pairing and expression of TCR chains introduced into human T cells. Blood 109, 2331–2338.
22. Li, M., Husic, N., Lin, Y., Christensen, H., Malik, I., McIver, S., Daniels, C.M.L.P., Harris, D.A., Kotzbauer, P.T., Goldberg, M.P., et al. (2010). Optimal promoter usage for lentiviral vector-mediated transduction of cultured central nervous system cells. J. Neurosci. Methods 189, 56–64.
23. Rodenko, B., Toebes, M., Hadrup, S.R., van Esch, W.J.E., Molenaar, A.M., Schumacher, T.N.M., and Ovaa, H. (2006). Generation of peptide–MHC class I complexes through UV-mediated ligand exchange. Nat. Protoc. 1, 1120–1132.
24. Braham, M.V.J., Minnema, M.C., Aarts, T., Sebestyen, Z., Straetemans, T., Vyborova, A., Kuball, J., Öner, F.C., Robin, C., and Alblas, J. (2018). Cellular immunotherapy on primary multiple myeloma expanded in a 3D bone marrow niche model. Oncoimmunology 7.
25. Drew, S.I., Terasaki, P.I., Billing, R.J., Bergh, O.J., Minowada, J., and Klein, E. (1977). Group specific human granulocyte antigens on a chronic myelogenous leukemia cell line with a Philadelphia chromosome marker. Blood 49, 715–718.
26. Cebinelli, G.C.M., DE Sousa Pereira, N., Sena, M.M., DE Oliveira, C.E.C., Fujita, T.C., DA Rocha,

S.P.D., DE Abreu Oliveira, F.J., Marinello, P.C., and Watanabe, M.A.E. (2016). Immunotherapy in Acute Leukemias: Implications and Perspectives Using Wt1 Antigen. Anticancer Res. 36, 3795–802.

27. Gao, L., Bellantuono, I., Elsässer, A., Marley, S.B., Gordon, M.Y., Goldman, J.M., and Stauss, H.J. (2000). Selective elimination of leukemic CD34+ progenitor cells by cytotoxic T lymphocytes specific for WT1. Blood 95, 2198–2203.
28. Xue, S.A., Gao, L., Hart, D., Gillmore, R., Qasim, W., Thrasher, A., Apperley, J., Engels, B., Uckert, W., Morris, E., et al. (2005). Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. Blood.
29. Stauss, H.J., Thomas, S., Cesco-Gaspere, M., Hart, D.P., Xue, S.A., Holler, A., King, J., Wright, G., Perro, M., Pospori, C., et al. (2008). WT1-specific T cell receptor gene therapy: Improving TCR function in transduced T cells. Blood Cells, Mol. Dis.
30. Xue, S.A., Gao, L., Thomas, S., Hart, D.P., Xue, J.Z., Gillmore, R., Voss, R.H., Morris, E., and Stauss, H.J. (2010). Development of a Wilms' tumor antigen-specific T-cell receptor for clinical trials: Engineered patient's T cells can eliminate autologous leukemia blasts in NOD/SCID mice. Haematologica 95, 126–134.
31. Chapuis, A.G., Egan, D.N., Bar, M., Schmitt, T.M., McAfee, M.S., Paulson, K.G., Voillet, V., Gottardo, R., Ragnarsson, G.B., Bleakley, M., et al. (2019). T cell receptor gene therapy targeting WT1 prevents acute myeloid leukemia relapse post-transplant. Nat. Med.
32. Poirot, L., Philip, B., Schiffer-Mannioui, C., Le Clerre, D., Chion-Sotinel, I., Derniame, S., Potrel, P., Bas, C., Lemaire, L., Galetto, R., et al. (2015). Multiplex genome-edited T-cell manufacturing platform for "off-the-shelf" adoptive T-cell immunotherapies. Cancer Res. 75, 3853–3864.
33. Provasi, E., Genovese, P., Lombardo, A., Magnani, Z., Liu, P.-Q., Reik, A., Chu, V., Paschon, D.E., Zhang, L., Kuball, J., et al. (2012). Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. Nat. Med. 18, 807–815.
34. Qasim, W., Zhan, H., Samarasinghe, S., Adams, S., Amrolia, P., Stafford, S., Butler, K., Rivat, C., Wright, G., Somana, K., et al. (2017). Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. Sci. Transl. Med. 9, eaaj2013.
35. Ghaffari, S., Khalili, N., and Rezaei, N. (2021). CRISPR/Cas9 revitalizes adoptive T-cell therapy for cancer immunotherapy. J. Exp. Clin. Cancer Res. 40, 1–18.
36. Mastaglio, S., Genovese, P., Magnani, Z., Ruggiero, E., Landoni, E., Camisa, B., Schiroli, G., Provasi, E., Lombardo, A., Reik, A., et al. (2019). NY-ESO-1 TCR single edited stem and central memory T cells to treat multiple myeloma without graft-versus-host disease SE. 130, 606–619.
37. Legut, M., Dolton, G., Mian, A.A., Ottmann, O.G., and Sewell, A.K. (2018). CRISPR-mediated TCR replacement generates superior anticancer transgenic t cells. Blood 131, 311–322.
38. 38. Gattinoni, L., Klebanoff, C.A., and Restifo, N.P. (2012). Paths to stemness: Building the ultimate antitumour T cell. Nat. Rev. Cancer 12, 671–684.
39. Cieri, N., Camisa, B., Cocchiarella, F., FOrcato, M., Oliviera, G., Provasi, E., Bondanza, A., Bordignon, C., Peccatori, J., Ciceri, F., et al. (2013). IL-7 and IL-15 instruct the generation of human memory stemTcells from naive precursors. Blood 121, 573–585.
40. Fraietta, J.A., Lacey, S.F., Orlando, E.J., Pruteanu-Malinici, I., Gohil, M., Lundh, S., Boesteanu,

A.C., Wang, Y., O'connor, R.S., Hwang, W.T., et al. (2018). Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. Nat. Med. 24, 563–571.

41. Xu, Y., Zhang, M., Ramos, C.A., Durett, A., Liu, E., Dakhova, O., Liu, H., Creighton, C.J., Gee, A.P., Heslop, H.E., et al. (2014). Closely related T-memory stem cells correlate with in vivo expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15. Blood 123, 3750–3759.
42. Mousset, C.M., Hobo, W., Ji, Y., Fredrix, H., De Giorgi, V., Allison, R.D., Kester, M.G.D., Falkenburg, J.H.F., Schaap, N.P.M., Jansen, J.H., et al. (2018). Ex vivo AKT-inhibition facilitates generation of polyfunctional stem cell memory-like CD8+ T cells for adoptive immunotherapy. Oncoimmunology 7, 1–14.
43. Klebanoff, C.A., Crompton, J.G., Leonardi, A.J., Yamamoto, T.N., Chandran, S.S., Eil, R.L., Sukumar, M., Vodnala, S.K., Hu, J., Ji, Y., et al. (2017). Inhibition of AKT signaling uncouples T cell differentiation from expansion for receptor-engineered adoptive immunotherapy. JCI Insight 2.
44. Stenger, D., Stief, T.A., Kaeuferle, T., Willier, S., Rataj, F., Schober, K., Vick, B., Lotfi, R., Wagner, B., Grünewald, T.G.P., et al. (2020). Endogenous TCR promotes in vivo persistence of CD19-CAR-T cells compared to a CRISPR/Cas9-mediated TCR knockout CAR. Blood 136, 1407–1418.
45. Hu, W., Zi, Z., Jin, Y., Li, G., Shao, K., Cai, Q., Ma, X., and Wei, F. (2019). CRISPR/Cas9-mediated PD-1 disruption enhances human mesothelin-targeted CAR T cell effector functions. Cancer Immunol. Immunother. 68, 365–377.
46. Choi, B.D., Yu, X., Castano, A.P., Darr, H., Henderson, D.B., Bouffard, A.A., Larson, R.C., Scarfò, I., Bailey, S.R., Gerhard, G.M., et al. (2019). CRISPR-Cas9 disruption of PD-1 enhances activity of universal EGFRvIII CAR T cells in a preclinical model of human glioblastoma. J. Immunother. Cancer 7, 1–8.
47. Ren, J., Liu, X., Fang, C., Jiang, S., and June, C.H. (2017). Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition. Clin. Cancer Res., 2255–2267.
48. Stadtmauer, E.A., Fraietta, J.A., Davis, M.M., Cohen, A.D., Weber, K.L., Lancaster, E., Mangan, P.A., Kulikovskaya, I., Gupta, M., Chen, F., et al. (2020). CRISPR-engineered T cells in patients with refractory cancer. Science (80-. ). 367, 1–64.
49. Philip, L.P.B., Schiffer-Mannioui, C., Le Clerre, D., Chion-Sotinel, I., Derniame, S., Potrel, P., Bas, C., Lemaire, L., Galetto, R., Lebuhotel, C., et al. (2015). Multiplex genome-edited T-cell manufacturing platform for "off-the-shelf" adoptive T-cell immunotherapies. Cancer Res. 75, 3853–3864.
50. Ren, J., Zhang, X., Liu, X., Fang, C., and Jiang, S. (2017). A versatile system for rapid multiplex genome-edited CAR T cell generation. Oncotarget 8, 17002–17011.
51. Liu, X., Zhang, Y., Cheng, C., Cheng, A.W., Zhang, X., Li, N., Xia, C., Wei, X., Liu, X., and Wang, H. (2017). CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells. Cell Res. 27, 154–157.
52. Naeem, M., Majeed, S., Hoque, M.Z., and Ahmad, I. (2020). Latest Developed Strategies to Minimize the Off-Target Effects in CRISPR-Cas-Mediated Genome Editing. Cells 9, 1–23.
53. Lu, Y., Xue, J., Deng, T., Zhou, X., Yu, K., Deng, L., Huang, M., Yi, X., Liang, M., Wang, Y., et al. (2020). Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. Nat. Med. 26, 732–740.

# INTEGRATION DEFICIENT LENTIVIRAL VECTORS CAN BE EXPLOITED AS DONOR TEMPLATE DELIVERY SYSTEMS FOR CRISPR-CAS9 MEDIATED HOMOLOGY DIRECTED REPAIR IN T CELL LINES AND PRIMARY T CELLS

6

Vania Lo Presti[1], Doris Logtenberg[1], Stefan Nierkens[1] and Niek van Til[1]

[1] Center for Translational Immunology, University Medical Center Utrecht, Utrecht, Netherlands

*Manuscript in preparation*

## ABSTRACT

Genetically modified autologous or allogeneic T cells expressing chimeric antigen receptor (CAR) and recombinant T cell receptors (rTCR) transgenes have been investigated in clinical trials to treat cancer patients. The use of integrating lentiviral vectors are currently the most commonly applied vectors to stably transfer these transgenes to the target T cells. However, integration of these vectors into the host genomes are semi-random and transgene expression is directed by vector variegation. Additionally, although no records are available at present in T cells, uncontrolled vector integration into the genome could lead to insertional oncogenesis. The fast-growing use of the CRISPR-Cas9 technology to efficiently induce double strand breaks (DSB) has opened up the possibility to selectively insert donor gene of interest in a precise genomic location via the homologous directed repair (HDR). In this study, we tested the use of CRISPR-Cas9 technology with an integration deficient lentiviral vector as a delivery system of our gene of interest. Optimization of the delivery method, timing and T cell stimulation parameters showed feasibility of integration deficient lentiviral vectors as a donor delivery system in CB derived CD8+ T cells with precise HDR efficiency up to 4%. These promising results could provide an initial platform to generate defined cellular gene therapy products for treatment of cancer therapy.

## INTRODUCTION

Adoptive T cell therapies using chimeric antigen receptor (CAR) and recombinant TCRs have shown effectiveness in the treatment of several types of cancer. Those cell therapy products are characterized by the expression of recombinant proteins, either a CAR or a TCR, that are able to recognize a specific tumor associated antigen (TAA)[1]. Gammaretroviral and lentiviral vectors are most often used to transduce and express those recombinant proteins, because they efficiently integrate in the host genome providing long-term expression[2]. Particularly, third generation self-inactivating lentivirus vectors are currently used for the production of T-cell therapy product Kymriah approved by Food and Drug Administration (FDA) and European Medical Agency (EMA) for the treatment of pediatric acute lymphoblastic leukemia (ALL)[3]. The transduction efficiency of lentiviral vectors is efficient in T-lymphocytes [4], but the integration sites occur in a semi-random pattern with a bias for transcriptional units in actively transcribed genes, and cannot be selected, hence there is a risk of insertional mutagenesis and/or disruption of expression of pivotal genes[5,6]. Moreover, in the context of TCR engineered T cell therapy, the introduced gene is expressed complementary to the endogenous TCR expression, increasing the possibility to generate mispairing of TCRs and to compete with co-receptors engagement [7,8]. The combination of the lentiviral vector expression of recombinant TCRs and simultaneous CRISPR-Cas9 mediated elimination of endogenous TCR expression is a promising strategy to increase safety and efficacy [9–11]. However, this approach would not address the LV semi-random integration into the host genome.

More recently, it has been shown that homologous mediated repair (HDR) after a double strand break (DSB) using CRISPR-Cas9 technology could create safer and more efficient cell therapy products, with low expression of inhibitory molecules. A donor DNA template with overlapping flanking sequences is required to enable HDR [12]. For this purpose a modified lentiviral vector, deficient to integrate into the genome can be used. Integration deficient lentiviral vectors (IDLV) can be generated by interfering with different steps of the integration process[13]. The most common approach is to use a mutated HIV integrase with a single amino acid substitution (D64V) [14]. In this manuscript, an IDLV containing a reversed GFP expression cassette flanked with homologous arms was generated allowing the cassette to integrate into the T cell receptor constant region (TRAC), simultaneously eliminating endogenous TCR expression. We report the optimization of these IDLVs for HDR in T cell lines, and primary cord blood CD8+ T lymphocytes so that these could be exploited for further development to deliver T cell receptor transgenes in the TRAC locus.

## MATERIALS AND METHODS

For the collection of umbilical cord blood (CB) informed consent according to the Declaration of Helsinki was obtained. The collection protocol for CB is approved by the ethical committee of the University Medical Center Utrecht (UMCU), the Netherlands.

### CELL CULTURE

The human Jurkat T cell line was cultured in complete RPMI-1640 with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (PS). HEK 293T cells were cultured in complete DMEM (10% FCS and 1% PS). CB mononuclear cells (CBMC) were separated from fresh CB over a Ficoll-Paque PLUS solution (Ficoll-Paque, Amersham). Primary CD8+ T cells were isolated from CBMC using Magnetic-Activated Cell Sorting (MACS, Stem Cell Technologies) with CD8+ magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). 5x $10^6$ CD8+ T cells/mL were cultured for 3 days at 37°C/5% CO2 in complete CTL medium (RPMI-1640, 10% human serum, 1% PS) supplemented with IL-2 (50U) IL-15 (5 ng/mL), (IL-7 (5 ng/mL) and activated with CD3/CD28 Dynabeads (in a ratio 1:3 beads:cells, Thermo Fisher Scientific). All cytokines were obtained from Miltenyi Biotech.



### PLASMID CONSTRUCTS

HIV-1, third-generation, self-inactivating (SIN) lentiviral plasmids were used for virus production [15]. The envelope glycoprotein plasmid pMD2-VSVg, the packaging plasmid pMDLg/pRRE, for integration proficient LV (IPLV) production, or pMDLg/pD64V[16], for IDLV production, and the regulatory plasmid pRSV-REV were previously designed and produced at Plasmid Factory. Transfer vectors for HDR purposes were based on a pCCL backbone[17]. The pCCL.cPPT.1A.LHA.PGK.GFP.pA.RHA.RV.bPRE4.SIN (referred to as LHA_GFP_RHA) transfer vector contains a gene cassette encoding GFP sequence driven by an internal PGK promoter, which was placed in a reverse orientation to the lentiviral vector transcript. A bovine growth hormone polyA was placed downstream of the GFP sequence. The transgene cassette is flanked by sequences homologous to exon 1 of the TRAC locus. The size of the homologous arms for LHA_GFP_RHA was 350 – 450 bp (Supplementary Figure 1). The plasmids were designed and synthesis was performed at Genscript. Plasmids were amplified in STBL3 bacterial cells.

## VIRUS PRODUCTION

Transient transfection of the packaging plasmids and the transfer vector was performed on HEK 293T cells by overnight calcium phosphate co-precipitation. After 17 hours of incubation, the medium was refreshed. Viral vector supernatant was collected 48 hours after transfection, filtered through 0.45 µm filters (Fisher scientific) and concentrated by ultracentrifugation (20,000 rpm x 2 hours at 4˚C). Four different vector lots were produced: (1) GFP IDLV, (2) GFP IPLV, (3) LHA_GFP_ RHA_ IDLV and (4) LHA_GFP_RHA_ IPLV; for the latter an extra Nodamuravirus B2 protein (Nov-B2) plasmid was added to the plasmid mix to increase the viral titer (Supplementary Figure 2). VSV-G pseudotyped lentiviral particles were titrated by serial dilution on Jurkat cells (clone E6-1; ATCC TIB-152). At 72 h post-transduction, cells were harvested and analyzed by flow cytometry for GFP expression to determine viral vector titer.

## TRANSDUCTION OF JURKAT T CELLS

Per well 5 x $10^5$ or 10 x $10^5$ Jurkat cells/well were seeded in 1 mL of the RPMI-1640 complete medium. Jurkat T cells were transduced with GFP IDLV with a multiplicity of Infection (MOI) of 10. After 15 – 18 hours, medium was refreshed and Jurkat cells were kept in culture up to 13 days.



## TRANSDUCTION CB CD8+ T CELLS

CB CD8+ T cells were isolated from fresh CB and expanded as described previously. CB CD8+ T cells were either transduced after the activation phase upon removal of aCD3/CD28 Dynabeads, or alternatively, the CB CD8+ T cells were transduced during the activation phase 12 hours after the addition of aCD3/CD28 Dynabeads and LentiBOOST. CB CD8+ T cells were transduced with a range of MOI 10-50 in StemMACS medium (1% PS). After 15 – 18 h, cells were washed and resuspended in complete CTL medium supplemented with IL-2 (50 U/ml).

## TCR-KO GENERATION VIA ELECTROPORATION OF RNP COMPLEX.

Two gRNAs were designed to target the TRAC locus in exon 1, sequences are available in Supplementary Figure 1D. First, Alt-RR CRISPR-Cas9 tracrRNA (200 µM) was annealed to Alt-RR CRISPR-Cas9 crRNA (200 µM) in Nuclease-Free IDTE Buffer and heated at 95°C, to form gRNA by diluting Alt-R Cas9 enzyme to a final concentration of 36 µM by mixing 0.6 µl AltR-Cas9 enzyme (61 µM stock) with 0.4 µL

resuspension buffer R (Neon System Kit), per condition undergoing electroporation. The ribonucleoprotein (RNP) complex was formed by combining 0.5 µL of both gRNAs with the diluted Cas9. The Neon Transfection System was set at 1600 V, 10 ms pulse width and 3 pulses. Before the electroporation procedure 1134 x $10^6$ IDLV transduced and untransduced cells were washed twice with phosphate buffered saline (PBS) and centrifuged at 600 rpm room temperature (RT). Control samples were directly transferred to the plate containing complete CTL medium supplemented with IL-2 (200U) IL-15 (20 ng/mL) and IL-7 (10 ng/mL). Cells were cultured at 37°C/5% CO2 up to 30 days post electroporation; from 6 days after electroporation cell were maintaned in CTL medium supplemented with IL-2 (100U) IL-15 (10 ng/mL) and IL-7 (10 ng/mL)

## FLOW CYTOMETRY

Samples were analysed on a FACS LSR Fortessa (BD Biosciences) flow cytometer. Cells were stained with anti Pan-αβTCR monoclonal (clone IP26, APC, eBioscienceTM) and anti-human CD3 mouse IgG1 monoclonal (clone UCHT1, Pacific BlueTM, BD PharmingenTM) and incubated at 4°C for 15 min. Cells were selected based on the forward and side scatter and the expected cell size. Dead cells were excluded by staining with 7-Amino Actinomycin D (7-AAD) (BD Pharmingen). To determine transduction efficiency, cells were selected for GFP expression compared to an untransduced control sample. The endogenous TCR knock out efficiency was determined by flow cytometry based on the percentage of αβTCR and CD3+ positive cells compared to untreated. FlowJo software (Tree Star, Inc., Ashland, OR, USA) was used to analyse data. All stainings and measurements were performed in FACS buffer (1x PBS, 1% FCS, 0.1% sodium azide).

## VECTOR COPY NUMBER ANALYSIS

CB CD8+ T cells were analysed with quantitative polymerase chain reaction (qPCR) 48 hours after transduction to confirm vector copy number (VCN). 5 – 10 x $10^5$ cells per condition were collected for DNA isolation following the protocol of NucleoSpin Tissue Column (Macherey-Nagel). Mastermixes for human β-actin (hBAC) and HIV primers specific for vector cDNA were prepared as follows, per sample: 2x Syber green (10 µL), forward primer (20 pmol), reversed primer (20 pmol), DNA sample (100 ng), Milli-Q H2O (4 µL). Samples were compared to a reference DNA sample from transduced cells carrying an average VCN ~1.

## HDR INTEGRATION CONFIRMATION

Genomic DNA was extracted from (1) untransduced, (2) IDLV transduced and (3) IDLV transduced + RNP complex electroporated CB CD8+ T cells, . Polymerase chain reaction was performed using a set of primer targeting the extremity of the LHA (FW: ATCCTCCGGCAAACCTCTG) and the GFP sequence (RV: GGCGGACTTGAAGAAGTCGTG).

## STATISTICAL ANALYSIS

For statistical analysis T-tests were performed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, La Jolla California USA). Bars indicate mean ± standard deviation (SD) of at least two independent experiments. Significance is indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $P < 0.0001$ (****).

## RESULTS

### ANALYSIS OF IDLV TRANSDUCTION EFFICIENCY AND KINETICS IN JURKAT T CELLS

In order to maximize the efficiency of homologous recombination, the transduction efficiency and kinetics of GFP protein content after IDLV transduction was assessed. Jurkat T cells were transduced with an MOI of 8.5 and analysed on flow cytometry over time. GFP expression was observed at 8 hours post-transduction and signal was lost ~9 days after transduction (Figure 1A). This confirms transient gene expression and lack of integration into the host genome with the IDLV, because the integrative proficient lentiviral vector provided long-term GFP expression. GFP mean fluorescence intensity (MFI) increased nearly 7-fold between 24 hours and 48 hours post-transduction, reaching the peak 48hrs post-transduction (Figure 1B). VCN analysis, with primers amplifying a fragment of the proviral vector DNA, confirmed the presence of viral genomes in the transduced cells (Figure 1C). Next, Jurkat T cells were transduced with IDLV encoding GFP flanked by homologous arms (MOI 10). The presence of the flanking regions did not affect the transduction capacity of the LV and no difference in term of percentage was observed when analysed by flow cytometry 48 hours post-transduction compared to GFP IDLV; with an average ~95% GFP positive cells (Figure 1D).



### COMBINATION OF CRISPR-CAS9- AND IDLV LEADS TO HDR MEDIATED INTEGRATION IN JURKAT T CELLS

The observation of the marked increase in GFP MFI 48 hours post-transduction and the VCN measured, which was higher than 4 between 12 and 36 hours, suggested vector DNA present in the nucleus. Forty-eight hours post-transduction was chosen as the timepoint for subsequent CRISPR-Cas9 transfection. To determine whether CRISPR-Cas9-mediated integration of the LHA_GFP_RHA_short transgene cassette could be achieved, LHA_GFP_RHA_short IDLV transduced Jurkat T cells were electroporated with CRISPR-Cas9 RNP-complex combining two gRNAs targeting the TRAC locus and Cas9 enzyme. CRISPR-Cas9 transfection determined a 97% decrease in αβTCR expression (Figure 2A). Jurkat T cells that were transduced with IDLV and transfected with CRISPR-Cas9 RNP-complex showed a reduction in viability compared to IDLV transduced cells only (Figure 2B). Cells were measured for GFP protein by flow cytometry 48 hours post-transduction, prior to RNP-Cas9 protein electroporation (Figure 2C (Blue histogram)).

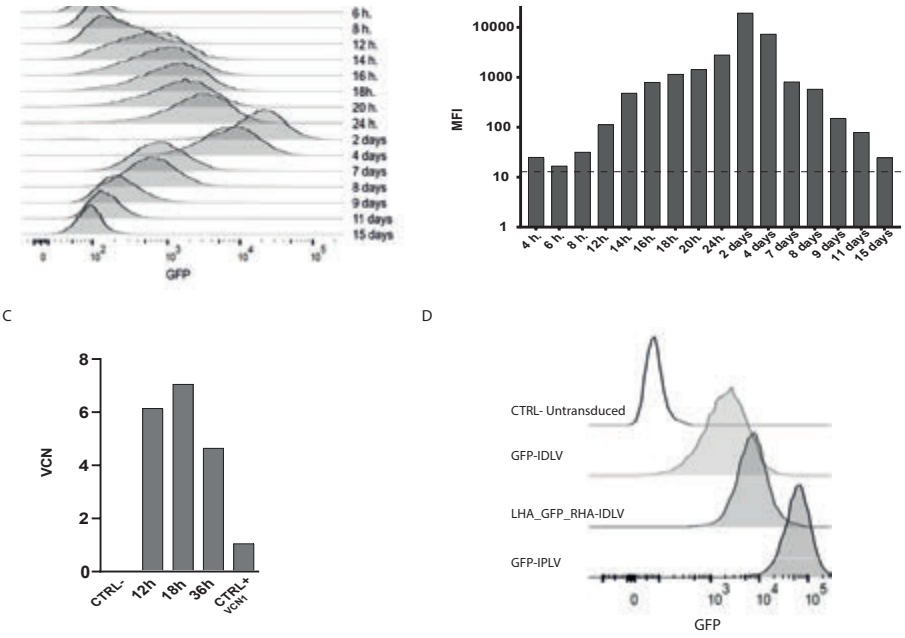



**Figure 1 - IDLV kinetics after transduction of Jurkat T cells.**

A) GFP expression over time after transduction with GFP-IDLV. B) Mean Fluorescent Intensity (MFI) of GFP. Both A and B show results from 4hrs to 15 days after transduction. Dotted line represent MFI of untransduced Jurkat cells. C) VCN analysis based on qPCR detection of proviral DNA extracted from transduced cells at different timepoints after transduction. D) Comparison of GFP intensity between GFP-IDLV and GFP-IPLV.



At 9 – 13 days after transduction and subsequent RNP electroporation, αβTCR and GFP protein expression were analyzed by flow cytometry. GFP protein fluorescence analyzed over time by flow cytometry confirmed a gradual loss of GFP signal, as was seen before for GFP-IDLV (Figure 2C). An isolated GFP-positive population of ~4.0% (n=3) was observed when analyzed 9 – 13 days after transduction and CRISPR-Cas9 RNP-complex transfection, compared to transduced cells without CRISPR-Cas9 treatment (Figure 2D).

Based on the observed kinetics of GFP protein in GFP IDLV transduced Jurkat T cells, LHA_GFP_RHA that has not been integrated by means of HDR, should have disappeared after ~9 days. Therefore, these results suggest that the isolated GFP-expressing population indicates successful integration of the LHA_GFP_RHA_short cassette into the genome of Jurkat T cells.

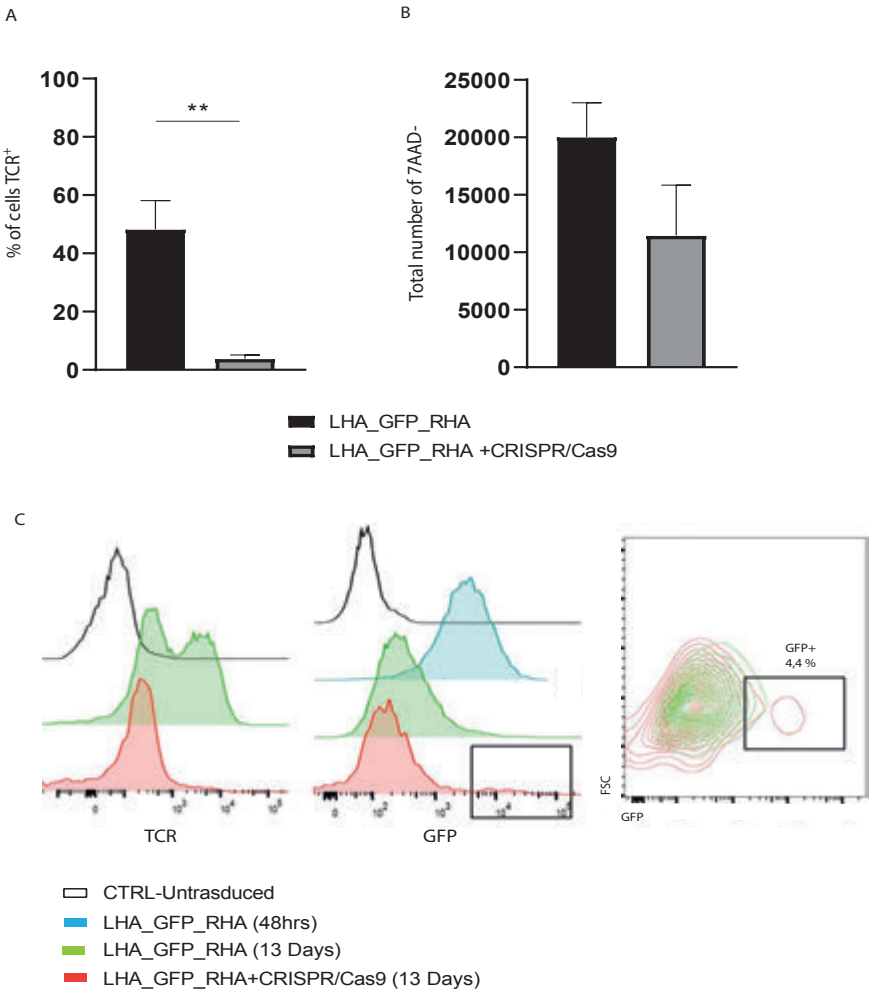



**Figure 2 – CRISPR-Cas9 and IDLV mediated HDR efficiency**

A) KO efficiency expressed as percentage of Jurkat T cells expressing the endogenous TCR after electroporation with RNP complex. B) Viability comparison between LHA_GFP_RHA transduced and LHA_GFP_RHA transduced and electroporated with CRISPR-Cas9 RNP complex C) On the left, TCR expression of unedited Jurkat T cells (green) and KO cells (red). On the right GFP expression over time: 48hrs after transduction (blue), 13 days after transduction with or without CRISPR-Cas9 editing (respectively red and green curves). D) Representative FACS plot of potentially integrated GFP+ population 13 days after transduction. Error bars represent standard deviation (SD) across 3 donors. Statistical testing was performed using Student's t-test and p-values are depicted as * =<0.05; **=<0.01.

## IDLV TRANSDUCTION OF CB- CD8+ T CELLS IS CHARACTERIZED BY LACK OF TRANSGENE PROTEIN EXPRESSION BUT PRESENCE OF VIRAL GENOME

Using IDLV showed efficient transduction (~95%) and gene expression in Jurkat T cells. When CB CD8+ T cells were transduced with LHA_GFP_RHA IDLV, following activation and expansion with CD3/CD28 Dynabeads, low percentages between 3 – 5% GFP positive cells were observed (Figure 3A) at 5 hours, 7 hours, 24 hours and 48 hours after transduction. To increase the population of GFP-expressing cells, MOI 1, MOI 10 and MOI 50 were used, showing only a slight increase in GFP+ cells after 48 hours when transduced with an MOI 50 (Figure 3B). The transgene vector architecture did not influence the transduction efficiency, since LHA_GFP_RHA IPLV vectors were able to transduce primary CB CD8+ T cells and express detectable levels of GFP protein (up to 73% of transduced cells); while LHA_GFP_RHA IDLV vectors were not showing GFP signal (Figure 3C). The results obtained from the transduction of CB CD8+ T cells show a distinct difference between primary CB CD8+ T cells and Jurkat T cells. Surprisingly, in both LHA_GFP_RHA IPLV and LHA_GFP_RHA IDLV, the amount of vector copies present inside the cells was comparable (Figure 3D-E), even though the expression of GFP was not detectable in the LHA_GFP_RHA IDLV conditions, confirming the efficiency of the transduction, but the lack of expression.



## ADDITION OF aCD3/CD28 BEADS AND TRANSDUCTION ENHANCER TO THE CULTURE MEDIA IMPROVES IDLV TRANSDUCTION AND HDR IN CB CD8+ T CELLS

The presence of viral genome in both IDLV and IPLV transduced cells, suggested the possible presence of an impaired transport to the nucleus, due to the D64V mutation in the integrase, in primary CB CD8+ T cells. Therefore, cells were transduced, with LHA_GFP_RHA IDLV at an MOI of 50, in the presence of aCD3/CD28 beads and LentiBOOST to facilitate the formation of pores in both the cellular and nuclear membrane. Transduced cells were then electroporated 12hrs post transduction with CRISPR-Cas9 RNP-complex combining two gRNAs targeting the TRAC locus and Cas9 enzyme. At day 2, LHA_GFP_RHA IDLV transduced cells showed an increased population of GFP+ cells compared to previous experiment, with an average of 6,1% of GFP+ cells. Fig. 4A shows successful loss of TCR expression and the presence of a distinct TCR-GFPhigh population at day 4, interpreted as efficient HDR (Figure 4A) and a stable GFPhigh population overtime at day 12 and 21 (Figure 4B).

Additionally, a genomic analysis based on the amplification of the LHA and GFP demonstrated that only IDLV+CRISPR/Cas9 treated cells showed a PCR product at the predicted size; confirming integration in the exact genomic location (Figure 4C).

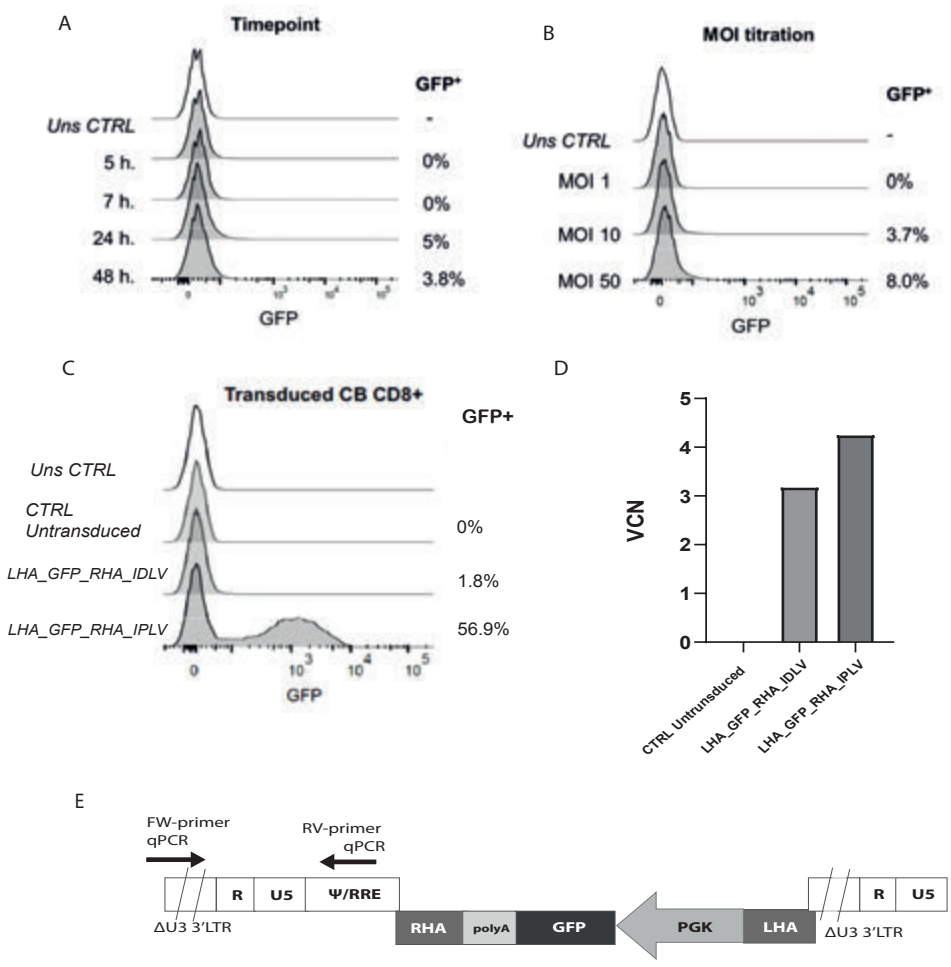



**Figure 3 – IDLV transduction efficiency and kinetics in CB CD8+ T cells**

A) GFP expression from 5hrs to 48hrs after transduction with LHA_GFP_RHA B) GFP expression after transduction with LHA_GFP_RHA at different MOIs C) Comparison of transduction efficiency of IDLV and IPLV when using the same transgene plasmid (LHA_GFP_RHA). The graph is representative of 4 independent experiments across different donors. D) VCN analysis based on qPCR detection of proviral DNA extracted from transduced cells with IDLV and IPLV. E) Graphic representation of primers binding site for VCN analysis.

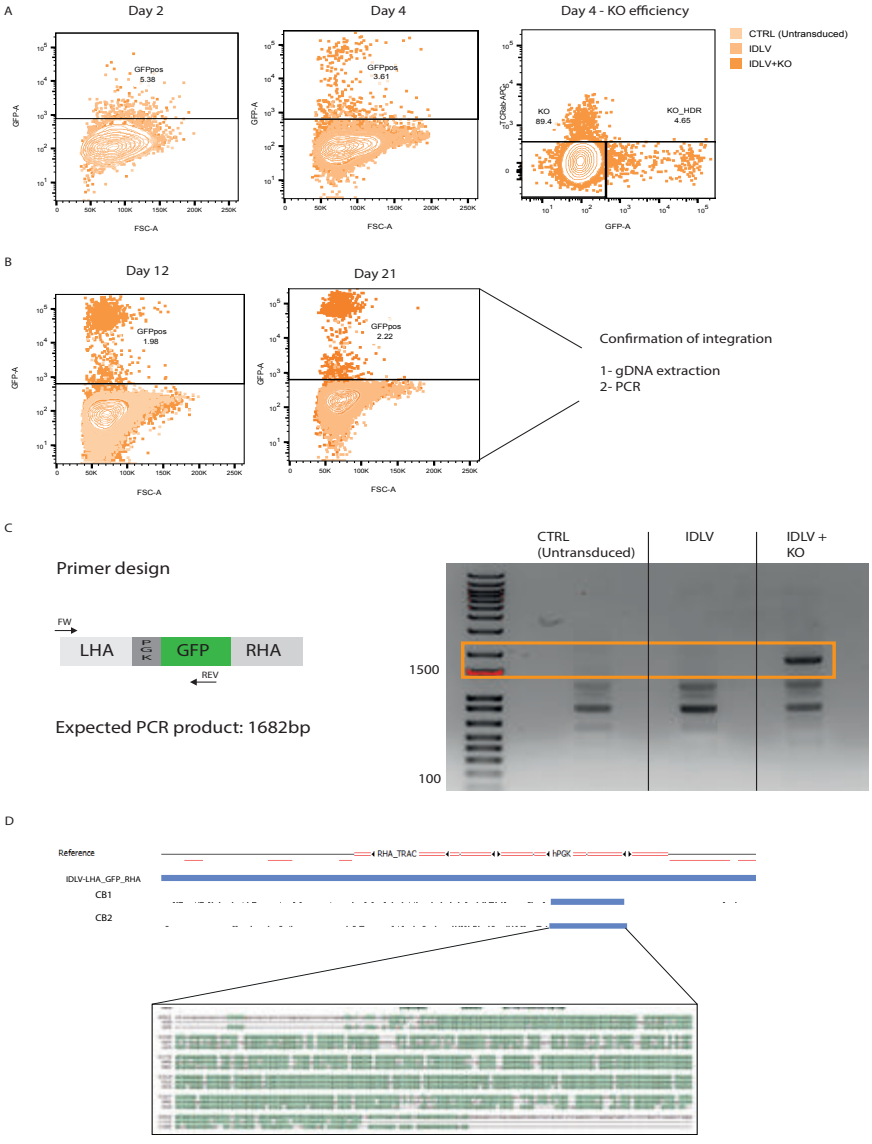



**Figure 4 – IDLV transduction efficiency and CRISPR-Cas9 mediated HDR in CB CD8+ T cells.**

A) On the left, GFP expression 2 and 4 days after transduction with LHA_GFP_RHA and electroporation with CRISPR/Cas9 RNP complex. On the right, KO and HDR efficiency defined as lack of TCR expression and presence of a GFP+high population B) GFP expression 12 and 21 days after transduction with LHA_GFP_RHA and electroporation with CRISPR/Cas9 RNP complex. C) On the left, PCR primers design for confirming the integration of the GFP construct in the TRAC locus. On the right, PCR amplification results on gDNA derived from untransduced cells, IDLV transduced and IDLV transduced and CRISPR/Cas9 RNP complex electroporated cells. D) Sanger sequencing of PCR product from IDLV transduced and CRISPR/Cas9 RNP complex electroporated cells compared to original plasmid. A-B FACS plot are representative of 3 independent experiments with different CB donors. C-D are representative of 2 independent experiments with different CB donors

## DISCUSSION

The user-friendly application of CRISPR-Cas9 technology for gene-editing has created possibilities of applying gene-editing to improve the safety and efficacy of adoptive T cell therapy[18,19]. After creating a DSB break through CRISPR/Cas9 gene-editing, HDR may be used to allow integration of a gene-of-interest in a specific genomic locus. Limitations of this approach are the low HDR efficiency, particularly in primary cells, compared to non –homologous end joining (NHEJ), and the inefficiency to deliver of exogenous DNA to allow integration of the donor cassette to occur [20]. In this manuscript we describe how to successfully use IDLV to deliver the exogenous DNA template into Jurkat T cells and more importantly in CB primary CD8+ T cells. To the best of our knowledge there are no previous studies that showed delivery of a donor template using IDLV to facilitate HDR dependent CRISPR-Cas9 in primary cord blood T cells. As a proof of concept, we assessed different parameters to optimize the delivery of genetic material by IDLVs for HDR, using Jurkat T cell lines. IDLV transduction of Jurkat T cells was followed by a gradual increase in GFP fluorescence with a peak at 48hrs (Figure 1). Comparing the mean fluorescent intensity of GFP IDLV transduced cells with integration proficient lentiviral vector (IPLV) confirmed that IDLVs produce lower levels of transgene product, because of lack of integration, as reported before[21].



However, although GFP fluorescence levels were lower, VCN numbers early after transduction were in similar ranges for both the IPLV and IDLV, and HDR when the cells were electroporated with the CRISPR-Cas9 ribonucleoprotein (RNP) complex was still feasible in Jurkat T cells (Figure 2). Subsequently, feasibility of this approach was assessed in CB-CD8+ T cells. Unexpectedly, the GFP fluorescence after IDLV transduction in primary CB-T cells was completely abrogated compared to previous results on Jurkat cells, indicating lower transduction efficiency. The discrepancy between the Jurkat cells and primary T cells has been previously described by Joglekar *et al.*, noticing that a reduction in transduction capacity, VCN and protein produced was clearly visible between highly dividing tumor cell lines and primary T cells[22]. However, in contrast to the lack detectable transgene product GFP, proviral vector copies up to three VCN were detected in the target cells, confirming the intact binding to the cell membrane and entry capacity of the lentiviral vector(Figure 3). The transport to the nucleus is actively mediated by the formation of the pre-integration complex (PIC), consisting of viral cDNA, cellular proteins, the viral proteins integrase (IN) and reverse transcriptase (RT). The D64V substitution of the integrase, which was used for the production of IDLV, has not

been reported to functionally affect the PIC formation and nuclear DNA import, however, detailed studies are lacking[23]. With the aim to induce cell cycling and opening up of the nuclear membrane, T cells were transduced in the presence of aCD3/CD28 beads and a transduction enhancer (LentiBOOST), which resulted in a detectable GFP fluorescence after 48hrs. Moreover, the electroporation of the RNP complex induced an efficient KO of the targeted TCR alpha chain and a concomitant well-defined KO population expressing high levels of GFP fluorescence; suggesting a successful HDR. Further confirmation of specific integration in the TRAC locus was obtained with PCR analysis and sequencing (Figure 4).

Overall this study shows the possibility to use IDLV as a delivery system for CRISPR-Cas9 mediated HDR, however the efficiency remains low and further optimization steps are needed to achieve significant percentages for future translational applications, as well as testing of therapeutic TAA specific rTCR for cellular immunotherapies. Incremental modifications such as optimization of the timing of delivery of the RNP complexes after transduction, increasing the vector dose, improved purification of the lentiviral vectors lots, optimal stimulation of T cells and addition of compounds that might promote HDR may lead to significant efficiencies. If the D64V substitution affects the nuclear transport, it is possible to abrogate the integration capacity of the LV using different strategies, such us the use of clinically approved integration inhibitors (such as Raltegravir) or via mutations in the long terminal repeats[24].



However, it is important to mention that other viral-based template delivery techniques have become available, such as the use of adeno-associated vectors (AAV), which are able to obtain high HDR efficiency. Specifically, using AAV serotype 6 vectors, efficient transient transduction of T cells, as well as donor template delivery and insertion in the TRAC locus have been reported [25–27]. The use of AAV6 has shown promising results when introducing a chimeric antigen receptor (CAR) molecule into the TRAC locus, with HDR efficiency ranging from 30-70% [28–30]. More recently, to completely eliminate the use of a viral vector delivery system, the use of dsDNA and ssDNA molecules during the electroporation of the RNP complex has been also investigated. This method has shown the possibility to efficiently target the TRAC locus with a rTCR sequence, achieving a range of 3 to 12% of HDR efficiency [9,31]. Several adjustment are being tested to further increase the HDR efficiency with this approach, such as modification of the donor sequence, use of different Cas proteins, and treatment with small molecule interfering with NHEJ [32–36].

To summarize, we have shown proof-of-concept of HDR using IDLV as donor template in cord-blood derived CD8+ T cells to deliver an expression cassette in the TRAC locus, but optimization steps are required to bring this technology to medical applications.



## ACKNOWLEDGEMENTS

The pMDLg/pRRE-D64V was kindly provided by Dr. Rafael Yáñez-Muñoz (Centre of Gene and Cell Therapy, Centre for Biomedical Sciences, Royal Holloway University of London, Egham, United Kingdom.)

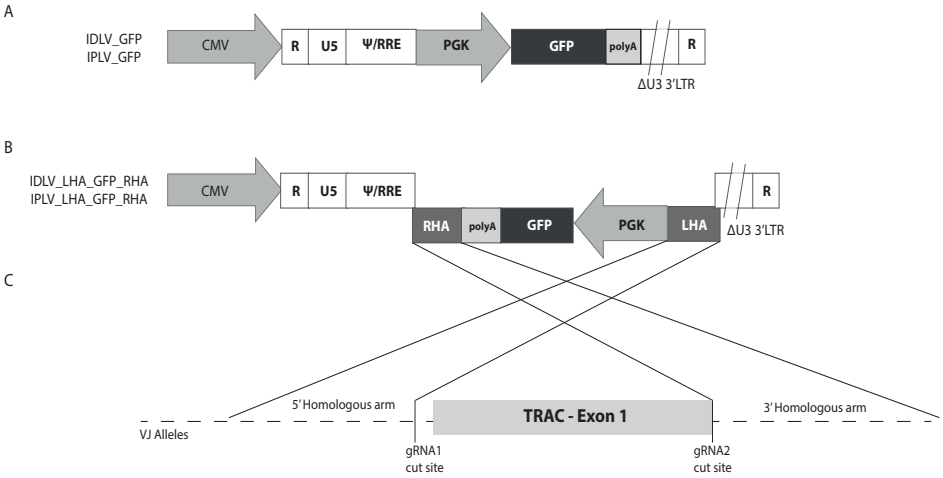
## BIBLIOGRAPHY


1. Restifo, N.P., Dudley, M.E., and Rosenberg, S.A. (2012). Adoptive immunotherapy for cancer: Harnessing the T cell response. Nat. Rev. Immunol. 12, 269–281.
2. Morgan, R.A., and Boyerinas, B. (2016). Genetic modification of T cells. Biomedicines 4.
3. O'Leary, M.C., Lu, X., Huang, Y., Lin, X., Mahmood, I., Przepiorka, D., Gavin, D., Lee, S., Liu, K., George, B., et al. (2019). FDA Approval summary: Tisagenlecleucel for treatment of patients with relapsed or refractory b-cell precursor acute lymphoblastic leukemia. Clin. Cancer Res. 25, 1142–1146.
4. Frecha, C., Lévy, C., Cosset, F.-L., and Verhoeyen, E. (2010). Advances in the field of lentivector-based transduction of T and B lymphocytes for gene therapy. Mol. Ther. 18, 1748–57.
5. Mitchell, R.S., Beitzel, B.F., Schroder, A.R.W., Shinn, P., Chen, H., Berry, C.C., Ecker, J.R., and Bushman, F.D. (2004). Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. PLoS Biol. 2.
6. Biffi, A., Bartholomae, C.C., Cesana, D., Cartier, N., Aubourg, P., Ranzani, M., Cesani, M., Benedicenti, F., Plati, T., Rubagotti, E., et al. (2011). Lentiviral vector common integration sites in preclinical models and a clinical trial reflect a benign integration bias and not oncogenic selection. Blood 117, 5332–5339.
7. Shao, H., Zhang, W., Hu, Q., Wu, F., Shen, H., and Huang, S. (2010). TCR mispairing in genetically modified T cells was detected by fluorescence resonance energy transfer. Mol. Biol. Rep. 37, 3951–3956.
8. Heemskerk, M.H.M., Hagedoorn, R.S., Van Der Hoorn, M.A.W.G., Van Der Veken, L.T., Hoogeboom, M., Kester, M.G.D., Willemze, R., and Falkenburg, J.H.F. (2007). Efficiency of T-cell receptor expression in dual-specific T cells is controlled by the intrinsic qualities of the TCR chains within the TCR-CD3 complex. Blood 109, 235–243.
9. Schober, K., Müller, T.R., Gökmen, F., Grassmann, S., Effenberger, M., Poltorak, M., Stemberger, C., Schumann, K., Roth, T.L., Marson, A., et al. (2019). Orthotopic replacement of T-cell receptor α- and β-chains with preservation of near-physiological T-cell function. Nat. Biomed. Eng.
10. Morton, L.T., Reijmers, R.M., Wouters, A.K., Kweekel, C., Remst, D.F.G., Pothast, C.R., Falkenburg, J.H.F., and Heemskerk, M.H.M. (2020). Simultaneous Deletion of Endogenous TCRαβ for TCR Gene Therapy Creates an Improved and Safe Cellular Therapeutic. Mol. Ther. 28, 64–74.
11. Legut, M., Dolton, G., Mian, A.A., Ottmann, O.G., and Sewell, A.K. (2018). CRISPR-mediated TCR replacement generates superior anticancer transgenic t cells. Blood 131, 311–322.
12. Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D. a., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281–2308.
13. Shaw, A., and Cornetta, K. (2014). Design and Potential of Non-Integrating Lentiviral Vectors. 14–35.
14. Leavitt, A.D., Robles, G., Alesandro, N., and Varmus, H.E. (1996). Human immunodeficiency virus type 1 integrase mutants retain in vitro integrase activity yet fail to integrate viral DNA efficiently during infection. J. Virol. 70, 721–728.

15. Zufferey, R., Dull, T., Mandel, R.J., Bukovsky, a, Quiroz, D., Naldini, L., and Trono, D. (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J. Virol. 72, 9873–80.
16. Yáñez-Muñoz, R.J., Balaggan, K.S., MacNeil, A., Howe, S.J., Schmidt, M., Smith, A.J., Buch, P., MacLaren, R.E., Anderson, P.N., Barker, S.E., et al. (2006). Effective gene therapy with nonintegrating lentiviral vectors. Nat. Med. 12, 348–53.
17. Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. (1998). A Third-Generation Lentivirus Vector with a Conditional Packaging System. J. Virol. 72, 8463–8471.
18. Zhang, X., Cheng, C., Sun, W., and Wang, H. (2020). Engineering T Cells Using CRISPR/Cas9 for Cancer Therapy. In RNA Interference and CRISPR Technologies, pp. 503–509.
19. Bailey, S.R., and Maus, M. V. (2019). Gene editing for immune cell therapies. Nat. Biotechnol.
20. Yeh, C.D., Richardson, C.D., and Corn, J.E. (2019). Advances in genome editing through control of DNA repair pathways. Nat. Cell Biol. 21, 1468–1478.
21. Kantor, B., Ma, H., Webster-Cyriaque, J., Monahan, P.E., and Kafria, T. (2009). Epigenetic activation of unintegrated HIV-1 genomes by gut-associated short chain fatty acids and its implications for HIV infection. Proc. Natl. Acad. Sci. U. S. A. 106, 18786–18791.
22. Joglekar, A. V., Hollis, R.P., Kuftinec, G., Senadheera, S., Chan, R., and Kohn, D.B. (2013). Integrase-defective lentiviral vectors as a delivery platform for targeted modification of adenosine deaminase locus. Mol. Ther. 21, 1705–1717.
23. Saeed, M.Q., Dufour, N., Bartholmae, C., Sieranska, U., Knopf, M., Thierry, E., Thierry, S., Delelis, O., Grandchamp, N., Pilet, H., et al. (2014). Comparison Between Several Integrase-defective Lentiviral Vectors Reveals Increased Integration of an HIV Vector Bearing a D167H Mutant.
24. Banasik, M.B., and McCray, P.B. (2010). Integrase-defective lentiviral vectors: Progress and applications. Gene Ther. 17, 150–157.
25. Santiago-Ortiz, J.L., and Schaffer, D. V. (2016). Adeno-associated virus (AAV) vectors in cancer gene therapy. J. Control. Release 240, 287–301.
26. Xu, C.L., Ruan, M.Z.C., Mahajan, V.B., and Tsang, S.H. (2019). Viral delivery systems for crispr. Viruses 11, 1–12.
27. David, R.M., and Doherty, A.T. (2017). Viral Vectors: The road to reducing genotoxicity. Toxicol. Sci. 155, 315–325.
28. Eyquem, J., Mansilla-soto, J., Giavridis, T., Stegen, S.J.C. Van Der, Hamieh, M., Cunanan, K.M., Odak, A., Gönen, M., and Sadelain, M. (2017). Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nat. Publ. Gr. 543, 113–117.
29. Hale, M., Lee, B., Honaker, Y., Leung, W.H., Grier, A.E., Jacobs, H.M., Sommer, K., Sahni, J., Jackson, S.W., Scharenberg, A.M., et al. (2017). Homology-Directed Recombination for Enhanced Engineering of Chimeric Antigen Receptor T Cells. Mol. Ther. - Methods Clin. Dev. 4, 192–203.

30. Wiebking, V., Lee, C.M., Mostrel, N., Lahiri, P., Bak, R., Bao, G., Roncarolo, M.G., Bertaina, A., and Porteus, M.H. (2020). Genome editing of donor-derived T-cells to generate allogenic chimeric antigen receptor-modified T cells: Optimizing αβ T cell-depleted haploidentical hematopoietic stem cell transplantation. Haematologica, haematol.2019.233882.
31. Roth, T.L., Puig-saus, C., Yu, R., Shifrut, E., Carnevale, J., Li, J., Hiatt, J., Saco, J., Krystofinski, P., Li, H., et al. (2018). Repromming human T cell function and specificity with non-viral genome targeting. Nature.
32. Song, F., and Stieger, K. (2017). Optimizing the DNA Donor Template for Homology-Directed Repair of Double-Strand Breaks. Mol. Ther. - Nucleic Acids 7, 53–60.
33. Richardson, C.D., Ray, G.J., DeWitt, M.A., Curie, G.L., and Corn, J.E. (2016). Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. Nat. Biotechnol. 34, 339–344.
34. Zhang, J.P., Li, X.L., Li, G.H., Chen, W., Arakaki, C., Botimer, G.D., Baylink, D., Zhang, L., Wen, W., Fu, Y.W., et al. (2017). Efficient precise knockin with a double cut HDR donor after CRISPR/Cas9-mediated double-stranded DNA cleavage. Genome Biol. 18, 1–18.
35. Chang, H.H.Y., Pannunzio, N.R., Adachi, N., and Lieber, M.R. (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. Nat. Rev. Mol. Cell Biol. 18, 495–506.
36. Schubert, M.S., Thommandru, B., Woodley, J., Turk, R., Yan, S., Kurgan, G., McNeill, M.S., and Rettig, G.R. (2021). Optimized design parameters for CRISPR Cas9 and Cas12a homology-directed repair. Sci. Rep. 11, 1–15.

# SUPPLEMENTARY INFORMATION

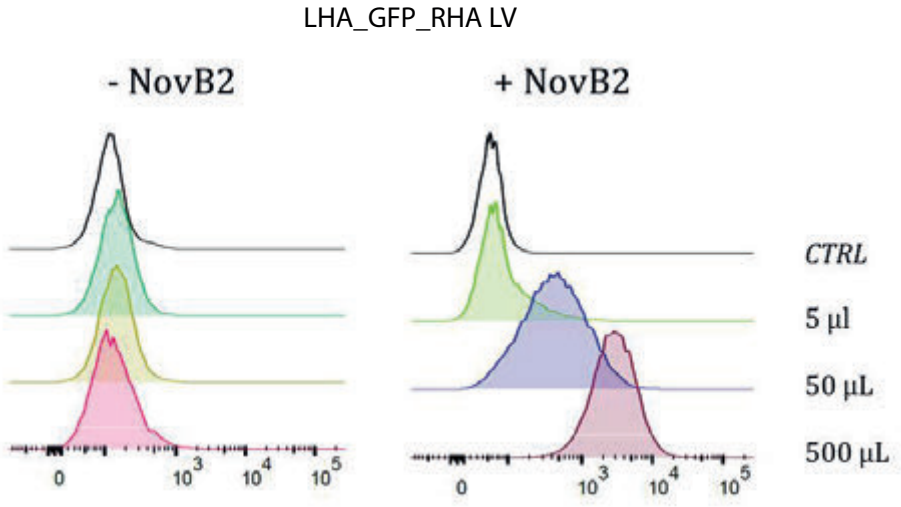



| ID | Sequence |
|---|---|
| gRNA 1 | GCAGGGTTCTGGATATCTGT |
| gRNA 2 | GAGAATCAAAATCGGTGAAT |



**Supplementary Figure 1 - Graphic representation of the transfer plasmids used for the generation of IDLV and IPLV vectors.**

A) Transfer plasmid, in a pCCL backbone, expressing the GFP under the control of a PGK promoter. B) Transfer plasmid, in a pCCL backbone, expressing the GFP under the control of a PGK promoter flanked by homology arms targeting the TRAC locus of the endogenous TCR. C) Graphic visualization of HDR mediated predicted integration into the TRAC locus, including gRNAs target sites. D) gRNAs sequences used for the CRISPR-Cas9 editing.






**Supplementary Figure 2- Nov-B2 plasmid drastically increase viral titer.**

Viral titers are calculated considering the percentage of GFP-expressing cells during titration experiments LHA_GFP_RHA transfer vector contains a viral promoter in sense and an internal promoter in antisense, HEK293T transcribe two transcripts that are (partly) complementary. Nov-B2 binds to dsRNA and prevents degradation by anti-viral mechanisms. Addition of Nov-B2 (5 µg) to the plasmids mixture led to a substantial increase in GFP expression and thus higher titers in LHA_GFP_RHA produced IDLV and IPLV.

# 7 GENE EDITING OF CHECKPOINT MOLECULES IN CORD BLOOD-DERIVED DENDRITIC CELLS AND CD8+ T CELLS USING CRISPR-CAS9

Vania Lo Presti[1,2], Alessandro Cutilli[2*], Yvonne Dogariu[2*], Konradin F. Müskens[2*], Ester Dünnebach[1,2], Denise A.M.H. van den Beemt[2], Annelisa M. Cornel[1,2], Maud Plantinga[2*] and Stefan Nierkens[1,2*]

[1] Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands
[2] Center for Translational Immunology, UMC Utrecht, The Netherlands

*Those authors contributed equally

*Major revisions - The CRISPR Journal, 2021*

## ABSTRACT

Immunotherapies targeting checkpoint inhibition and cell therapies are considered breakthroughs for cancer therapy. However, only a part of patients benefit from these treatments and resistance has been observed. Combining both approaches can potentially further enhance their efficacy. With the advent of gene editing techniques, such as CRISPR-Cas9, the elimination of checkpoint molecules became available as an option in good manufacturing practice (GMP) conditions to improve persistency and efficacy. However, no data of CRISPR-Cas9 application has been reported in cord blood (CB)-derived immune cells, potentially usable for allogeneic cell therapy purposes. In this manuscript, we describe the optimization of a protocol to deplete checkpoint molecules, at the genomic level using CRISPR/Cas9 technology, from CB-Dendritic Cells (DCs) and CB-CD8+ T cells. The protocol is based on the electroporation of a ribonucleoprotein (RNP) complex, easily translatable to clinical settings. In both cell types the knock-out (KO) was successful and did not affect cell viability. CB-DCs showed a decrease in expression of the targeted protein ranging from 50 to 95%; while CB-CD8+ T cells showed reduction in a range of 25-45%. The procedure did not affect the stimulatory function of the CB-DC or the response of CD-CD8+ T cells (proliferation or TNF-α production). In conclusion, we optimized a protocol to eliminate checkpoint molecules from CB-derived DCs and CD8+ T cells, with the aim to further implement allogeneic cell therapies for cancer.

## INTRODUCTION

Immunotherapy employs the body's own immune system to defend itself against a variety of diseases, e.g. autoimmunity and cancer [1,2]. Most cancer patients can be treated to minimal residual disease (MRD) levels with standard treatment regimens, but part of the patients is still at risk for relapse. Cell therapy approaches, such as gene engineered T cells and dendritic cell (DC) vaccination have evolved as a potential cure in different type of tumors[3,4]. Even though they have already entered clinical practice, several aspects require improvement, such as persistency and expansion, limiting cellular dysfunction and reducing toxicity. Zooming in on cellular dysfunction, it is widely recognized that cancer cells upregulate immune checkpoint inhibition (ICI) receptors to prevent T cell-mediated killing[5]. Similarly, in DC-T cell interactions, ligation of inhibitory receptors causes immune regulation, rather than stimulation. Therefore eliminating ICI receptor expression in cell therapy products might improve the efficacy.

Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-Cas9) technology significantly expanded the potential of gene editing and is highly efficient and specific [6–8]. The use of CRISPR-Cas9 has been shown a feasible and efficient approach to eliminate the expression of checkpoint inhibitors on gene engineered peripheral blood T cells [9–13]. Recently, this technique was successfully used to enhance cell therapy approaches for a clinical trial using NY-ESO specific TCR engineered T cells[14]. However, cellular dysfunction is often exacerbated in patient-derived T cells, due to the underlying disease and/or previous treatment regimens. Therefore, the use of an allogeneic source of cells, such as cord blood (CB), might pose an advantageous alternative. Up to date however, no studies have investigated the expression of checkpoint receptors on allogeneic CB-derived T cells or DCs, and no protocol to gene edit these CB-derived cells has been proposed.

Previously we developed a GMP-grade protocol to generate CD34+-derived CB-DCs [15,16] and optimized a method to isolate and gene modify CB-CD8+ T cells for cancer cell therapy applications[17]. We here describe an efficient protocol to genetically edit CB-DCs and CB-CD8+ T cells using CRISPR/Cas9 electroporation; as a proof of principle we focused on PD-L1/PD-1 and analysed the short term functional consequences of the procedure on proliferation and TNF-α production.

## MATERIALS AND METHODS

### CB COLLECTION AND CD34, CD8 ISOLATION

Umbilical cord blood was collected after informed consent was obtained according to the Declaration of Helsinki. The ethics committee of the University Medical Center Utrecht approved this collection protocol (TC-bio 15-345). CB mononuclear cells were isolated from human umbilical CB by density centrifugation over Ficoll-Paque solution (GE Healthcare Bio-Sciences AB, Chicago, Illinois, USA). CD34+ and CD8+ cells were isolated from fresh CB using magnetic bead separation (Miltenyi Biotec, Bergisch Gladbach, Germany) resulting in an 80–95% pure CD34+ or CD8+ population as determined with flow cytometry.

### CB-DC CULTURE

To generate CB-DCs, we used a protocol as previously described[15]. In short, $5 \times 10^4$ CD34+ cells/mL were expanded in the presence of Flt3L (50 ng/mL), SCF (50 ng/mL), IL-3 (20 ng/mL), and IL-6 (20 ng/mL) for 7 days. After washing, $2 \times 10^5$ cells/mL were differentiated in medium containing 5% human serum, Flt3L (100 ng/mL), SCF (20 ng/mL), GM-CSF (20 ng/mL), and IL-4 (20 ng/mL) for another 7 days. Recombinant cytokines were all obtained from Miltenyi Biotec. To induce maturation, cytokine mix; a combination of IL-1β, IL-6, and TNF-α (all used at 10 ng/mL) and PGE2 (1 µg/mL) from Pfizer was added to the DCs in differentiation medium for 24 h. For flow cytometric analysis, anti-HLA-DR (L243), anti-ILT4 (42D1) were purchased from Biolegend. Anti-CD11c (B-ly6), anti-CD83 (HB15e) were purchased from BD Bioscience. Anti-CD276 (7-517), anti-CD40 (5C3), anti-PD-L1 (MIH1) were purchased from Invitrogen. Anti-CCR7/CD197 (FR11-11E8) was purchased from R&D systems. After 30 minutes incubation, cells were washed and measured on an LSR Fortessa (BD) flow cytometer. Dead cells and doublets were excluded by scatter/width gating. Analysis was performed using FlowJo software 10.7 (Tree Star, Inc.).

### CD8+ T CELL CULTURE

CD8+ T cells (fresh or thawed) were cultured in complete Human RPMI (HuRPMI - RPMI 1640 medium + 10% human serum + 1% Penicillin/Streptomycin + 50uM Beta-mercaptoethanol) at a concentration of $0.5 \times 10^6$ cells/mL in a T25 flask. The HuRRPMI medium was supplemented with 50 U/mL IL-2, 5 ng/mL IL-7, and 5 ng/mL IL-15 (Miltenyi Biotech) unless otherwise specified. For initial T cell expansion, CD3/CD28 dynabeads (ThermoFisher Scientific, ref. 11161D) were used in a ratio of 1:2.5

aCD3/CD28 dynabeads to cells (or 10uL dynabeads for 1 x $10^6$ cells).

## GRNA-MEDIATED GENE KNOCK-OUT USING NEON TRANSFECTION SYSTEM

Cells at indicated time points were counted, 0.5 × $10^6$ cells were prepared for electroporation (EP) with Neon Transfection system and were used according to manufacturer instructions (ThermoFisher Scientific) regarding CRISPR-Cas9-mediated knockout using Ribonucleoprotein Complexes. Briefly, 1.8 µM crRNA:tracrRNA, 1.5 µM Cas9 nuclease and 1.8 µM Neon Electroporation Enhancer were combined with the 0.5 × $10^6$ cells. Cells were pulsed three times with a voltage of 1,600 and 10ms. gRNAs sequences can be found in table T1.

| *GENE* | *SEQUENCE (5' – 3')* |
|---|---|
| PD-1 | CGTCTGGGCGGTGCTACAAC |
| PD-L1 | ACCGTTCAGCAAATGCCAGT |
| CD276 | TGGCACAGCTCAACCTCATC |
| ILT4 | GTATTGATCTTAGACGGAGT |

To enrich for KO DCs, cells were sorted after maturation based on PD-L1 expression. After sorting (>95% purity), cells were subjected to T cell assays. For the genomic analysis, purified PCR products were sequenced using the EZ-Seq platform offered by Macrogen Europe (EZ-Seq (macrogen-europe.com)). Lastly, the sequenced PCR products were analyzed for knockout efficiency and predicted insertions and deletions (indels) using the TIDE software (Tide (nki.nl), 18).



## MIXED LEUKOCYTE REACTION

For mixed leukocyte reaction (MLR), allogeneic lymphocytes (1 × $10^6$/mL) were labeled with cell trace violet (5 µM; Invitrogen) and cocultured with sorted CB-DCs (2 × $10^5$/mL) in a 96-well round-bottom plate (Corning) at a stimulator:responder ratio of 1:5. Unstimulated cell-trace-violet-labeled cells served as negative control. After 4 days, cells were stained with anti-CD3 (UCHT1; Biolegend), anti-CD8α (RPA-T8), and incubated at 4 °C. After 30 minutes incubation, cells were washed and multiparameter analysis was performed on an LSR Fortessa (BD) flow cytometer. Dead cells were excluded by a fixable live/dead marker (Life sciences). T cell proliferation analysis was performed using the proliferation tool in the FlowJo software (Tree Star, Inc.), providing the division index.

## ELISA

Cell culture supernatants were collected following functional assays and frozen at -20˚C until later use. The TNF-α Human Uncoated ELISA kits (ref. 88-7346-88 respectively; Invitrogen) were used according to the manufacturer's instructions, acquired with the iMark™ Microplate Absorbance Reader (Bio-Rad, 168-1130), and analyzed using the Microplate Manager® Software.

## STATISTICS

Statistical analysis was performed using GraphPad Prism 8.3. Student's T-test analysis was performed to determine statistical significance. Statistical significance is reached when p-value is <0.05 and depicted as * =<0.05; **=<0.01; ***=<0.001 ****=<0.0001



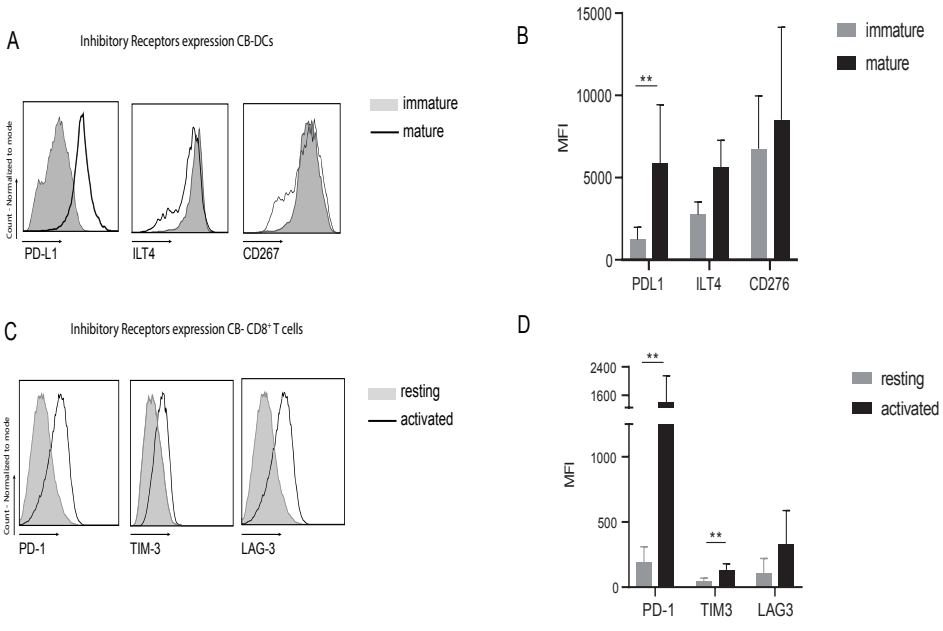
**Figure 1. CB-DCs and CB-CD8+ T cells expression of inhibitory molecules in different time-points of culture**

A) A representative histogram of the expression profile for inhibitory molecules of one CB-DC donor is shown. Mature DCs (black) are compared to immature DCs (grey). B) The MFI of co-stimulatory molecule expression by immature (grey) and mature (black) DCs. C) A representative histogram of the expression profile for inhibitory molecules of one CB-CD8+ T cells donor is shown. Activated CB-CD8+ T cells (black) are compared to resting CB-CD8+ T cells (grey). D) The MFI of inhibitory molecule expression by resting (grey) and activated (black) CB-CD8+ T cells. The average of at least 3 experiments is shown. T- tests were used for significance, p-values are depicted as * =<0.05; **=<0.01; ***=<0.001 ****=<0.0001

# RESULTS

## INHIBITORY RECEPTORS EXPRESSION PATTERN DURING MATURATION IN CB-DCS AND AFTER ACTIVATION IN CB-CD8+ T CELLS

Cord blood-derived CD34+ stem cells were expanded to obtain sufficient numbers for DC generation, followed by differentiation, resulting in 50% [20-80%] CB-DCs. Subsequently, the CB-DCs were matured with cytokine mix. This inflammatory cytokine cocktail significantly upregulated the levels of HLA-DR, CD40 and CCR7, and CD83; confirming the mature status of CB-DCs. (Supplementary Figure 1A-B). Both immature and mature, CB-DCs expressed CD276 (H7-B3), ILT4 and PD-L1, which slightly further increased in mature DCs after overnight stimulation (Figure 1A-B). CB-CD8+ T cells were isolated from fresh CB unit and kept in culture with a mix of cytokines (gating strategy explained in Supplementary Figure 1C). The expression of PD-1, TIM-3 and LAG-3 was evaluated in resting cells and cells activated with aCD3/CD28 beads. Resting CB-CD8+ T cells expressed low levels of PD-1, TIM-3 and LAG-3; while activated cells significantly increased the expression of PD-1 and slightly increased the expression of TIM-3 and LAG-3 (Figure 1C-D). Due to the high levels of PD-L1 expression on matured CB-DCs and PD-1 expression on activated CB-CD8+ T cells, we primarily focused on these two molecules to optimize the knockout protocol.



## PROTOCOL TO GENETICALLY MODIFY CB-DCS AND CB-CD8+ CELLS USING CRISPR/CAS9 ELECTROPORATION

To delete inhibitory receptors from the surface of CB-DCs and CB-CD8+ T cells, we set up a protocol using the CRISPR-Cas9 technology. A ribonucleoprotein (RNP) complex, containing CRISPR-RNA (crRNA) annealed to the trans-activating crRNA (tracrRNA) and Cas9 endonuclease, is delivered by electroporation (EP). Several protocols were tested for both cell types (data not shown) and showed that 3 pulses of 1600V for 10ms resulted in higher KO efficiency for both CB-DCs and CB-CD8+ T cells. Within the CB-DC culture, the electroporation of the RNP complex was tested both at day 4, during expansion, and day 11, during differentiation (Figure 2A), although PD-L1 surface expression was only detected at the final stage of the culture protocol. Indeed, electroporation on day 11 is most favourable for PD-L1 manipulation when compared to day 4, measured by flow cytometry on day 15 and confirmed, at the genomic level, using Sanger sequencing and TIDE analysis (Supplementary Figure 3). Compared to only electroporated cells (CTRL), RNP complex electroporated cells showed 70-97% KO efficiency (Figure 2A-B). Next to PD-L1, we also tested the protocol for other highly expressed inhibitory receptors on mature DCs, namely ILT4 and CD276, to demonstrate the applicability of the protocol on different targets. In both cases, the KO efficiency was 50% to 75% (Supplementary Figure 2).

CB-CD8+ T cells were activated with the aCD3/CD28 beads for 3 days prior to EP with the RNP complex. Cells were subsequently kept in a resting medium for 3 days and activated again with aCD3/CD28 beads to determine the KO efficiency. On day 7 after EP and activation, cells showed 25-45% of KO efficiency (Figure 2C-D), confirmed at the genomic level using Sanger sequencing and TIDE analysis (KO efficiency 34.675% (±12.84%) – Supplementary Figure 3). It is important to stress that PD-1 expression is strictly dependent on the level of T cell activation; we therefore consider the percentage derived from the genomic analysis to be most relevant and reliable. Viability of both CB-DCs and CB-CD8+ T cells was measured after the electroporation procedure and no significant decrease was detected (Figure 2E-F).

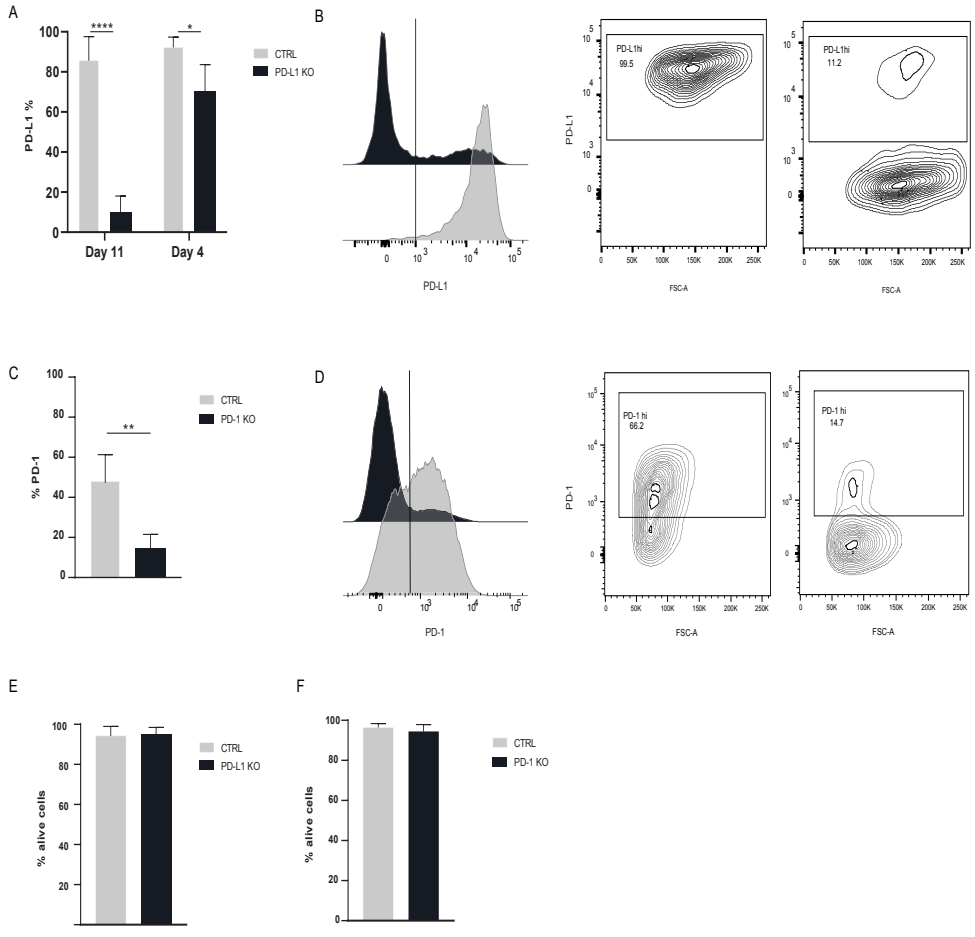



**Figure 2. PD-L1 and PD-1 KO efficiency.** A) Surface PD-L1 expression gated within HLA-DR+CD11c+ DCs. RNP-EP DCs are compared to CTRL (only EP) DCs, measured at day 15, the end of the protocol. On the left, PD-L1 expression within DCs after KO at day 11 of culture, on the right PD-L1 expression within DCs after KO at day 4 of culture. B) Representative flow cytometry plots of PD-L1 expression within DCs. C) Surface PD-1 expression gated within CD3+CD8+ T cells. KO T cells are compared to CTRL (only EP) CD8+ T cells, measured at day 7. D) Representative FACS plots of PD-1 expression within CD8+ T cells. The average of at least 4 experiments is shown. T- test were used for significance; p-values are depicted as * =<0.05; **=<0.01; ***=<0.001 ****=<0.0001

## LACK OF PD-L1/PD-1 EXPRESSION DOES NOT INFLUENCE T-CELL PROLIFERATION AND CYTOKINE PRODUCTION

To study the effect of the CRISPR/Cas9 procedure on the function of CB-CD8+ T cells, T cells were stimulated with aCD3/CD28 beads. The Division Index was calculated, to compare the average number of cell divisions that a cell in the original population has undergone between donors. The PD-1 KO CB-CD8+ T cells showed an increased proliferation when compared to unmanipulated T cells with comparable TNF-α secretion capacity (Figure 3A). To determine the effect of the CRISPR-Cas9 procedure on the function of DCs, a mixed leukocyte reaction (MLR) was performed. The gene-modified DCs showed T cell stimulatory potential to a similar level as unmodified DCs (Figure 3B), both in term of proliferation and TNF-α production.

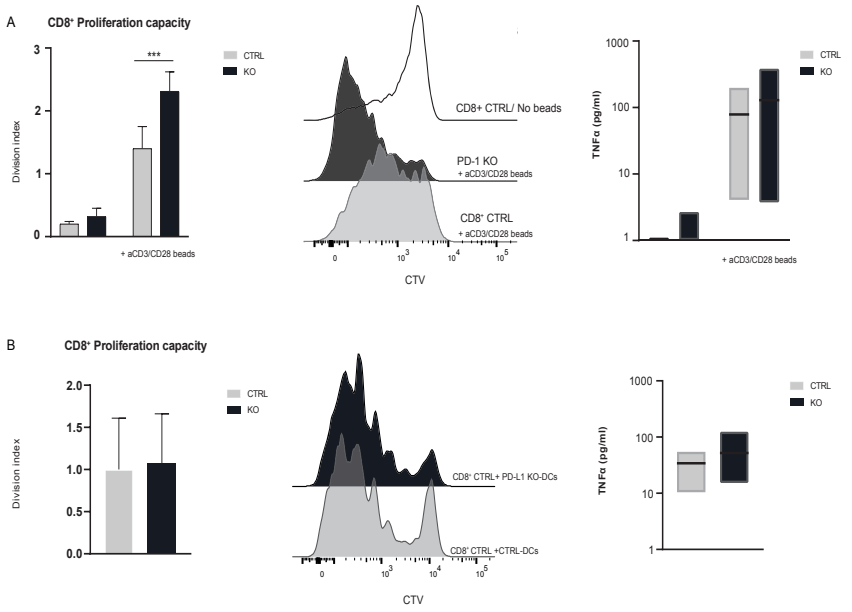



**Figure 3. T-cell proliferation and TNF-α production after CRISPR-Cas9 procedure**

A) Target cells are labelled with cell tracer violet (CTV) to measure proliferation after 4 day stimulation with aCD3/CD28 beads. On the right, the division index of cells CTRL (grey) or PD-1-KO (black) with or without the stimulation with aCD3/CD28 beads. Representative FACS plot of the proliferative capacity of T cells. On the left, TNF-α production after 4 days stimulation with or without aCD3/CD28 beads. B) On the right, division index of CB CD8+ cells when in co-culture with CTRL (grey) or PD-L1-KO (black) DCs. Representative FACS plot of the proliferative capacity of T cells. On the right, TNF-α production by CD3+ T cells when co-cultured with CTRL and PD-L1-KO DCs. The average of at least 4 experiments is shown. T- test were used for statistical analysis; p-values are depicted as * =<0.05; **=<0.01; ***=<0.001 ****=<0.0001

## DISCUSSION

Immune cells are characterized by a series of activating and repressing molecules that, when balanced, allow the response to dangerous insults and the return to steady-state[19]. However, when considering cell therapies approaches, inhibitory signals might hamper efficacy and long lasting immunity[20]. Monoclonal antibodies targeting immune checkpoint inhibitors are currently used in the clinic to treat several types of cancers. However, blocking antibodies have beneficial effects only in a limited number of patients, mostly characterized by a suppressive tumor micro-environment [21]. Combining therapies, such as cell therapeutic products with the use of checkpoint inhibitors, has therefore attracted increasing attention, with hundreds of clinical trials registered to test the efficacy of a combinatorial approach (clinicaltrial.gov; [22]). In the last years, the advent of new gene editing technologies, such as CRISPR-Cas9, has enabled the direct elimination of inhibitory molecules on T cells. This strategy has been tested in clinical trials with the aim to generate a superior and inclusive cell product while avoiding the possible side effects of multiple therapies combined [14,23]. The results from these pilot clinical trials are promising, demonstrating the safety and feasibility of the approach.

However, they solely focus on autologous and patient-derived T cells. In this manuscript, we set out to translate and optimize this protocol in CB-derived immune cells, important actors in the generation of allogeneic cell products. Firstly, the expression of inhibitory receptors was determined in CB-derived DCs and CD8+ T cells. As shown in Figure 1, both CB-DCs and CB-CD8+ T cells express relatively high levels of inhibitory receptors, especially upon maturation or activation, respectively. Interestingly, PD-L1 expression stood out as the most prominent change from immature to mature DCs, and PD-1 expression followed the same pattern in CB-CD8+ T cells. Therefore, as a proof of principle, we focused on removing PD-L1 from the surface of CB-DCs and PD-1 from CB CD8+ cells using the CRISPR-Cas9 methodology. KO efficiency ranged from 70-95% for PD-L1; and 25-45% for PD-1 (Figure 2). The feasibility of the approach was also tested targeting other checkpoint receptors expressed on CB-DCs, confirming KO efficiency ranging from 50 to 75% of edited cells (Supplementary Figure 2). Regarding CB-DCs, the efficiency of CRISPR-Cas9 seems to be most effective during the final stage of differentiation. This could be due to a favorable expansion of wild type cells in the CB culture, or the fact that the PD-L1 gene is more accessible during the final stage of differentiation[24]. These results are in line with the successes achieved for the CRISPR-Cas9 mediated gene editing of human monocyte-derived DCs and peripheral blood T cells [10,11,13,25,26]; and

that the use of the CRISPR-Cas9 tool can further enhance the clinical potential of CB-derived cell therapies; as also recently shown with CB-derived natural killer (NK) cells by Daher & Basar *et al.*[27].

To study the effect of the gene-editing procedure on DC and T cell function, the proliferation and TNF-α production capacity was determined in a short-term functional assay. The elimination of PD-1 resulted in an overall advantage for CB-CD8+ T cells in terms of proliferation; particularly visible when cells were stimulated with aCD3/CD28 beads (Figure 3A). PD-L1 KO CB-DC were able to stimulate effector T cells in an MLR, to the same extent of unmanipulated CB-DCs (Figure 3B). Longer experimental settings, such as exhaustion systems in vitro[28,29] or in vivo, are needed to better determine the impact of genomically deleting ICI molecules.

Nonetheless, we here demonstrate that the procedure is successful and it does not impact viability and functionality of both CB-DCs and CB-CD8+ T cells.

## CONCLUSIONS

In conclusion, the optimization of an efficient CRISPR-Cas9 protocol for CB-DCs and CB-CD8+ T cells sets the stage for further investigation and possible combinations that might increase the efficiency of allogeneic cell therapy products.

## CONFLICT OF INTEREST

The authors do not have any conflict of interest.

## ACKNOWLEDGMENT

The authors also thank the women for donating the CB and the nurses (Wilhelmina's Children Hospital, UMCU, the Netherlands) for collecting it. The work done for the manuscript was supported by the Dutch Cancer Society (KWF; grant number 10474) and by the WKZ foundation (grant number D-19-012573).

## AUTHORSHIP CONFIRMATION STATEMENT

VLP designed and performed experiments, analysed the data and wrote the manuscript. AC, YD and KFM performed experiments and data analysis. ED and DvdB helped performing experiments. AMC helped in the design of experiments and revision of the manuscript. MP and SN designed experiments and contributed in writing and revising the manuscript.

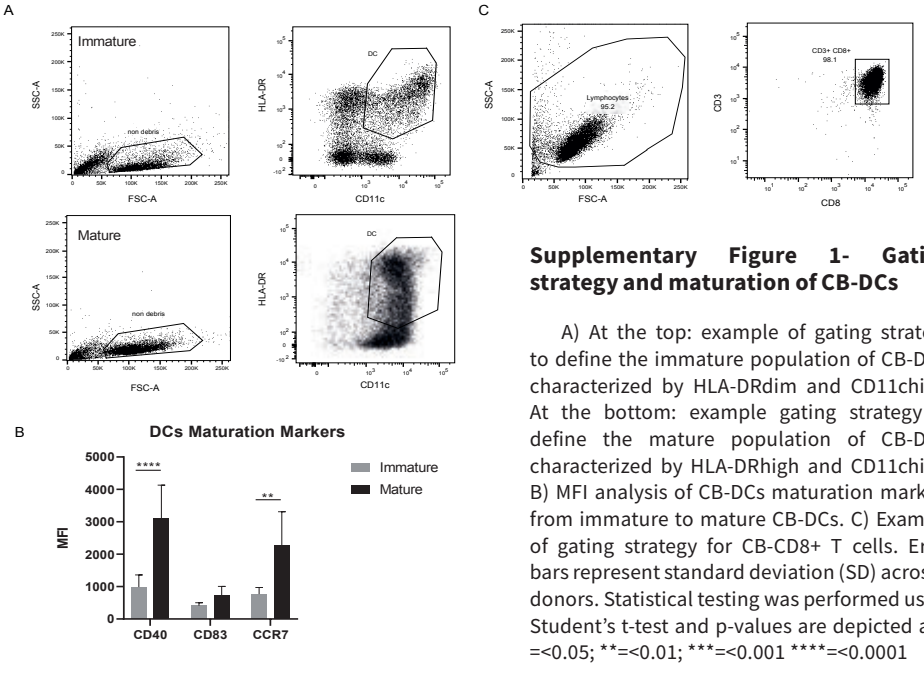
## BIBLIOGRAPHY


1. Kalos M, June CH. Adoptive T Cell Transfer for Cancer Immunotherapy in the Era of Synthetic Biology. Immunity 2013;39:49–60.
2. Levenga H, Schaap N, Maas F, et al. Partial T Cell-Depleted Allogeneic Stem Cell Transplantation following Reduced-Intensity Conditioning Creates a Platform for Immunotherapy with Donor Lymphocyte Infusion and Recipient Dendritic Cell Vaccination in Multiple Myeloma. Biol Blood Marrow Transplant 2010;16:320–332.
3. Santos PM, Butterfield LH. Dendritic cell-based cancer vaccines. J Immunol . Epub ahead of print 2017. DOI: 10.4049/jimmunol.1701024.
4. Guedan S, Ruella M, June CH. Emerging Cellular Therapies for Cancer. Annu Rev Immunol 2019;37:145–171.
5. Dyck L, Mills KHG. Immune checkpoints and their inhibition in cancer and infectious diseases. Eur J Immunol 2017;47:765–779.
6. Jinek M, East A, Cheng A, et al. RNA-programmed genome editing in human cells. Elife 2013;2013:1–9.
7. Vakulskas CA, Dever DP, Rettig GR, et al. A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. Nat Med 2018;24:1216–1224.
8. Yin H, Xue W, Anderson DG. CRISPR–Cas: a tool for cancer research and therapeutics. Nat Rev Clin Oncol 2019;16:281–295.
9. Rupp LJ, Schumann K, Roybal KT, et al. CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. Sci Rep 2017;7:737.
10. Ren J, Liu X, Fang C, et al. Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition. Clin Cancer Res 2017;2255–2267.
11. Choi BD, Yu X, Castano AP, et al. CRISPR-Cas9 disruption of PD-1 enhances activity of universal EGFRvIII CAR T cells in a preclinical model of human glioblastoma. J Immunother Cancer 2019;7:1–8.
12. Su S, Hu B, Shao J, et al. CRISPR-Cas9 mediated efficient PD-1 disruption on human primary T cells from cancer patients. Sci Rep 2016;6:1–14.
13. Hu W, Zi Z, Jin Y, et al. CRISPR/Cas9-mediated PD-1 disruption enhances human mesothelin-targeted CAR T cell effector functions. Cancer Immunol Immunother 2019;68:365–377.
14. Stadtmauer EA, Fraietta JA, Davis MM, et al. CRISPR-engineered T cells in patients with refractory cancer. Science (80- ) 2020;367:1–64.
15. de Haar C, Plantinga M, Blokland NJ, et al. Generation of a cord blood-derived Wilms Tumor 1 dendritic cell vaccine for AML patients treated with allogeneic cord blood transplantation. Oncoimmunology 2015;4:e1023973.
16. Plantinga M, Lo Presti V, de Haar CG, et al. Clinical Grade Production of Wilms' Tumor-1 Loaded Cord Blood-Derived Dendritic Cells to Prevent Relapse in Pediatric AML After Cord Blood Transplantation. Front Immunol;11 . Epub ahead of print 2020. DOI: 10.3389/

fimmu.2020.559152.

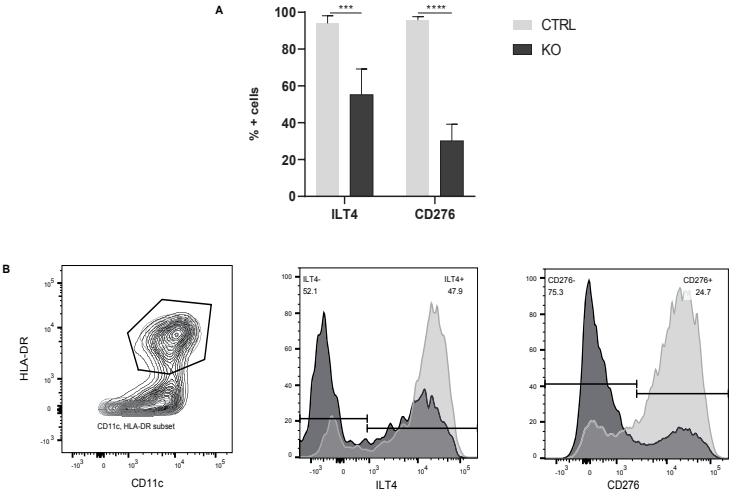
17. Lo Presti V, Cornel AM, Plantinga M, et al. Efficient lentiviral transduction method to gene modify cord blood CD8+ T cells for cancer therapy applications. Mol Ther - Methods Clin Dev 2021;21:357–368.
18. Brinkman EK, Chen T, Amendola M, et al. Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res 2014;42:1–8.
19. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol 2013;13:227–242.
20. Hargadon KM, Johnson CE, Williams CJ. Immune checkpoint blockade therapy for cancer: An overview of FDA-approved immune checkpoint inhibitors. Int Immunopharmacol 2018;62:29–39.
21. Haslam A, Prasad V. Estimation of the percentage of us patients with cancer who are eligible for and respond to checkpoint inhibitor immunotherapy drugs. JAMA Netw Open 2019;2:1–9.
22. Nowicki TS, Berent-Maoz B, Cheung-Lau G, et al. A pilot trial of the combination of transgenic NY-ESO-1–reactive adoptive cellular therapy with dendritic cell vaccination with or without ipilimumab. Clin Cancer Res 2019;25:2096–2108.
23. Lu Y, Xue J, Deng T, et al. Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. Nat Med 2020;26:732–740.
24. Jensen KT, Fløe L, Petersen TS, et al. Chromatin accessibility and guide sequence secondary structure affect CRISPR-Cas9 gene editing efficiency. FEBS Lett 2017;591:1892–1901.
25. Jost M, Jacobson AN, Hussmann JA, et al. Crispr-based functional genomics in human dendritic cells. Elife 2021;10:1–28.
26. Rupp LJ, Schumann K, Roybal KT, et al. CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. Sci Rep 2017;7:737.
27. Daher M, Basar R, Gokdemir E, et al. Targeting a cytokine checkpoint enhances the fitness of armored cord blood CAR-NK cells. Blood 2021;137:624–636.
28. Zhao M, Kiernan CH, Stairiker CJ, et al. Rapid in vitro generation of bona fide exhausted CD8+ T cells is accompanied by Tcf7 promotor methylation. PLoS Pathog 2020;16:1–27.
29. Kwon M, Choi YoJ, Sa M, et al. Two-Round Mixed Lymphocyte Reaction for Evaluation of the Functional Activities of Anti-PD-1 and Immunomodulators. Immune Netw 2018;33:1–11.

## SUPPLEMENTARY INFORMATION



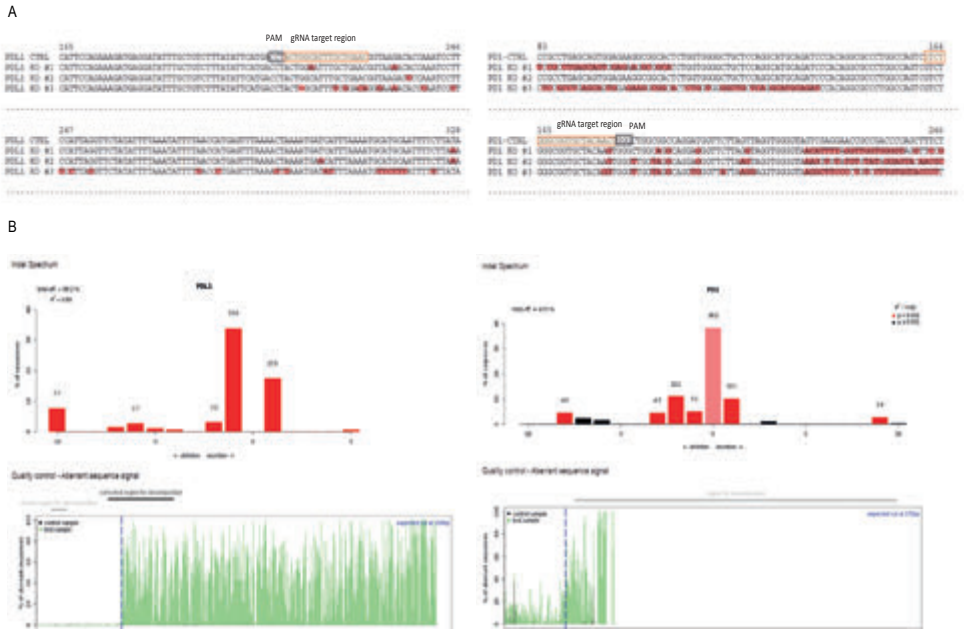

**Supplementary Figure 1- Gating strategy and maturation of CB-DCs**

A) At the top: example of gating strategy to define the immature population of CB-DCs, characterized by HLA-DRdim and CD11chigh. At the bottom: example gating strategy to define the mature population of CB-DCs, characterized by HLA-DRhigh and CD11chigh. B) MFI analysis of CB-DCs maturation markers from immature to mature CB-DCs. C) Example of gating strategy for CB-CD8+ T cells. Error bars represent standard deviation (SD) across 5 donors. Statistical testing was performed using Student's t-test and p-values are depicted as * =<0.05; **=<0.01; ***=<0.001 ****=<0.0001




**Supplementary Figure 2 – KO efficiency for ILT4 and CD276 in CB-DCs**

A) Surface expression of ILT4 and CD276 in CTRL-DCs (mock-electroporated, light grey) compared to KO CB-DCs (black). B) Representative flow cytometry plots of ILT4 and CD276 expression within CTRL-DCs (mock-electroporated, light grey) compared to KO CB-DCs (dark grey). T- test were used for significance; p-values are depicted as * =<0.05; **=<0.01; ***=<0.001 ****=<0.0001




**Supplementary Figure 3 – KO efficiency for PD-L1 and PD-1 at the genomic level**

A) At the top: alignment of PD-L1 CTRL sequences compared to 3 sequences of PD-L1 KO CB-DCs. Highlighted in orange the gRNA target sequence and in a black square the PAM sequence. The mutations in the edited sequences are highlighted in red. At the bottom: a representative graph of the TIDE analysis comparing the CTRL sequence with edited sequences for the frequency of Indels. B) At the top: alignment of PD-1 CTRL sequences compared to 3 sequences of PD-1 KO CB-CD8+ T cells. Highlighted in orange the gRNA target sequence and in a black square the PAM sequence. The mutations in the edited sequences are highlighted in red. At the bottom: a representative graph of the TIDE analysis comparing the CTRL sequence with edited sequences for the frequency of Indels.

# 8

# GENE AUGMENTATION AND EDITING TO IMPROVE TCR ENGINEERED T CELL THERAPY AGAINST SOLID TUMORS

Vania Lo Presti [1], Frank Buitenwerf [1] , Niek P. van Til [1,2] and Stefan Nierkens [1,3]

[1]Center for Translational Immunology, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands;

[2] AVROBIO, Cambridge, MA 02139, USA

[3] Princess Máxima Center for Pediatric Oncology, 3584 CS Utrecht, The Netherlands

*Vaccines, 2020*
*Special Issue- Cancer Immunotherapy: Advances and Future Prospects*

## ABSTRACT

Recent developments in gene engineering technologies have drastically improved the therapeutic treatment options for cancer patients. The use of eective chimeric antigen receptor T (CAR-T) cells and recombinant T cell receptor engineered T (rTCR-T) cells has entered the clinic for treatment of hematological malignancies with promising results. However, further fine-tuning, to improve functionality and safety, is necessary to apply these strategies for the treatment of solid tumors. The immunosuppressive microenvironment, the surrounding stroma, and the tumor heterogeneity often results in poor T cell reactivity, functionality, and a diminished infiltration rates, hampering the ecacy of the treatment. The focus of this review is on recent advances in rTCR-T cell therapy, to improve both functionality and safety, for potential treatment of solid tumors and provides an overview of ongoing clinical trials. Besides selection of the appropriate tumor associated antigen, ecient delivery of an optimized recombinant TCR transgene into the T cells, in combination with gene editing techniques eliminating the endogenous TCR expression anddisrupting specific inhibitory pathways could improve adoptively transferred T cells. Armoring the rTCR-T cells with specific cytokines and/or chemokines and their receptors, or targeting the tumor stroma, can increase the infiltration rate of the immune cells within the solid tumors. On the other hand, clinical “o-tumor/on-target” toxicities are still a major potential risk and can lead to severe adverse events. Incorporation of safety switches in rTCR-T cells can guarantee additional safety. Recent clinical trials provide encouraging data and emphasize the relevance of gene therapy and gene editing tools for potential treatment of solid tumors.

## 1. ADVANTAGES AND DISADVANTAGES OF USING RTCR-T CELLS FOR THE TREATMENT OF SOLID TUMORS: FROM THE BENCH TO THE BEDSIDE

The endogenous immune system is a key regulator in recognizing and controlling tumor growth. However, many cancers are able to modulate the anti-tumor properties of immune cells by preventing recognition by immune cells, counteracting the influx of cells, or actively suppressing their function. Cancer immunotherapy has been one of the most exciting approaches in the fight against cancer over the past several decades. The aim of this type of therapy is to strengthen the patients' immune system to recognize, target, and subsequently eliminate tumor cells[1]. For instance, clinical successes have been obtained by monoclonal antibodies functioning as immune checkpoint inhibitors to increase the anti-tumor effects of T lymphocytes. In 2011, the first checkpoint inhibitor targeting cytotoxic T lymphocyte-associated protein 4 (CTLA-4) was approved by the FDA (Ipilimumab), followed by several other drugs targeting CTLA-4, programmed cell death protein 1 (PD-1) and PD-1 ligand (PD-L1)[2]. However, even if application of checkpoint inhibitors may be durable in some cases and lead to remission of tumor growth, the response rates to date have rarely exceeded 40% [3].

An alternative strategy that has emerged to strengthen the immune system for cancer treatment is adoptive cell transfer (ACT). This technique is based on selection of immune cells to enhance their ability to recognize and eliminate tumor cells [4]. ACT has proven to be effective in several cancer types, especially using the transfer of T lymphocytes [5]. Moreover, gene modification techniques have increased the success rate of ACT against cancer by transferring a specific receptor, either a chimeric antigen receptor (CAR) or a T cell receptor (TCR), able to recognize a tumor associated antigen (TAA). Treatment of hematological tumors has been particularly successful, leading to the first approval of ACT using CD19-CAR (CAR19) T cells for the treatment of acute lymphoblastic leukemia (ALL) in 2017 (Kymriah)[6]. Although many hematological cancer patients have received clinical benefits from CAR-T cell treatment, so far, it has been less effective in the treatment of solid tumors. Several researchers have recently summarized the latest clinical trials involving the use of CAR-T cells in solid tumor treatment, highlighting the potency and limitations of the approach[7,8]. Solid tumors comprise an immunosuppressive tumor microenvironment characterized by a dense tumor stroma and by the presence of immunosuppressive signals, creating both a physical and chemical barrier for immune cells to infiltrate and to act[9]. Moreover, solid tumors are characterized by a heterogeneous antigen expression, which is in part responsible for the treatment

Table 1. Registered clinical trial using rTCR-T cells against solid tumors (clinicaltrial.gov).

| Target | Trial ID Number | Cancer Type | Additional Treatment | Location | Date | Status |
|---|---|---|---|---|---|---|
| NY-ESO-1 | NCT01967823 | Melanoma<br>Meningioma<br>Breast Cancer<br>(and 2 more...) | Lymphodepletion<br>Aldesleukin | United States | 24 October 2013 | Completed |
| | NCT02775292 | Adult Solid Neoplasm<br>Childhood Solid Neoplasm<br>Metastatic Neoplasm | Lymphodepletion<br>Aldesleukin<br>Nivolumab | Unites States | 3 January 2017 | Completed |
| | NCT03029273 | Lung Cancer, Non-small Cell, Recurrent<br>Advanced Fallopian Tube Carcinoma | Lymphodepletion | China | 21 March 2017 | Recruiting |
| | NCT02650986 | Advanced Malignant Solid Neoplasm<br>Advanced Melanoma<br>(and 47 more...) | Lymphodepletion<br>TGF-β blocker | United States | 30 June 2017 | Recruiting |
| | NCT01567891 | Ovarian Cancer | Lymphodepletion<br>Aldesleukin | United States | 3 July 2017 | Completed |
| | NCT03017131 | Recurrent Fallopian Tube Carcinoma<br>Recurrent Ovarian Carcinoma<br>Recurrent Primary Peritoneal Carcinoma<br>Multiple Myeloma | Lymphodepletion<br>Aldesleukin | United States | 8 December 2017 | Recruiting |
| | NCT03638206 | Oesophagus Cancer<br>Lung Cancer<br>(and 13 more…) | Lymphodepletion | China | 1 March 2018 | Recruiting |
| | NCT03462316 | Bone Sarcoma | Lymphodepletion<br>Aldesleukin | China | 21 May 2018 | Recruiting |
| | NCT03709706 | NSCLC | Pembrolizimab | United States | 31December 2018 | Recruiting |
| | NCT03691376 | Platinum-Resistant Fallopian Tube Carcinoma<br>Platinum-Resistant Ovarian Carcinoma<br>Platinum-Resistant Primary Peritoneal Carcinoma<br>(and 9 more...) | Chemotherapy<br>Aldesleukin<br>Cellular Therapy | United States | 24 January 2019 | Recruiting |

| | | | | | | |
|---|---|---|---|---|---|---|
| HPV E7 | NCT03941626 | Oesophagus Cancer<br>Hepatoma<br>Glioma | Lymphodepletion | China | 1 September 2019 | Recruiting |
| | NCT03967223 | Gastric Cancer<br>Neoplasms<br>Papillomavirus Infections | Lymphodepletion | United States | 31 December 2019 | Recruiting |
| | NCT02858310 | Cervical Intraepithelial Neoplasia<br>Carcinoma In Situ<br>(and 2 more...) | Lymphodepletion | United States | 27 January 2017 | Recruiting |
| | NCT03912831 | Human Papillomavirus (HPV) 16+<br>Relapsed/Refractory Cancer | Lymphodepletion | United States | 8 June 2019 | Recruiting |
| | NCT03937791 | Squamous Intraepithelial Lesions of Vulva<br>Neoplasms, Squamous Cell<br>Vulvar HSIL | N/A | United States | 9 October 2019 | Recruiting |
| | NCT04411134 | Cervical Intraepithelial Neoplasia | N/A | United States | 5 June 2020 | Not yet recruiting |
| | NCT04044950 | Papillomavirus Infections<br>Oropharyngeal Neoplasms | N/A | United States | 5 June 2020 | Not yet recruiting |
| | NCT04015336 | Papillomavirus Infections<br>Oropharyngeal Neoplasms | Lymphodepletion<br>Aldesleukin | United States | 5 June 2020 | Recruiting |
| | NCT02280811 | Vaginal Cancer<br>Cervical Cancer<br>Anal Cancer<br>(and 2 more...) | Lymphodepletion<br>Aldesleukin | United States | 14 October 2014 | Completed |
| HPV E6 | NCT03197025 | Human Papillomavirus<br>HPV-16<br>High Grade Squamous Intraepithelial Lesion | Aldesleukin | United States | 9 January 2018 | Completed |
| | NCT03578406 | Cervical Cancer<br>Head and Neck Squamous Cell Carcinoma | PD-1 Antagonist | China | 1 September 2018 | Recruiting |
| | NCT04139057 | Head and Neck Squamous Cell Carcinoma | PD-1 Antagonist | China | 1 March 2019 | Recruiting |

| | | | | | |
|---|---|---|---|---|---|
| MAGE family | NCT02111850 | Cervical Cancer<br>Renal Cancer<br>Urothelial Cancer<br>(and 2 more...) | United States | 7 February 2014 | Recruiting |
| | NCT03139370 | Solid Tumor | United States | 8 May 2017 | Recruiting |
| | NCT03247309 | Solid Tumor<br>Cancer<br>Head and Neck Squamous Cell Carcinoma | United States | 19 December 2018 | Recruiting |
| | NCT03441100 | Non-small Cell Lung Cancer<br>Solid Tumor, Adult<br>Cancer<br>Hepatocellular Carcinoma<br>(and 4 more...) | United States<br>Germany | 2 May 2019 | Recruiting |
| | NCT03412877 | Glioblastoma<br>Non-Small Cell Lung Cancer<br>Ovarian Cancer<br>(and 2 more...) | United States | 6 September 2018 | Recruiting |
| Neoantigens | NCT03970382 | Solid Tumor<br>Glioblastoma | United States | 3 July 2019 | Recruiting |
| | NCT04102436 | Non-Small Cell Lung Cancer<br>Breast Cancer<br>(and 2 more...) | United States | 5 June 2020 | Recruiting |
| MART1 F5 | NCT00509288 | Melanoma<br>Skin Cancer | United States | June 2007 | Completed |
| | NCT00910650 | Metastatic Melanoma | United States | 13 October 2009 | Completed |
| HBV antigen | NCT02719782 | Recurrent Hepatocellular Carcinoma | China | 2 July 2015 | Recruiting |
| | NCT02686372 | Hepatocellular Carcinoma | China | December 2015 | Recruiting |
| EBV antigen | NCT03648697 | Nasopharyngeal Carcinoma | China | 10 October 2018 | Not yet recruiting |

response failure [10]. The identification of an ideal target antigen is a limiting factor for two principal reasons. First, tumor associated antigens (TAA) are often expressed on healthy tissue and cells, raising safety concerns. Second, tumors have a low percentage of cell surface TAA expression (1 to 10% depending on the tumor type). For the latter problem, rTCR-T cells might have advantages for the solid tumor treatment[11].

rTCR-T cells contain TCRs recognizing a specific TAA when presented by a human leukocyte antigen (HLA) molecule. The majority of T cells typically express a TCR comprising of an α and β chain, which can interact with peptides presented on the HLA molecules in association with co-receptors (e.g., CD4 and CD8)[12–14]. rTCR-T cells are restricted to HLA recognition but recognize both extracellular and intracellular proteins, a major advantage compared to CAR T cells, since more than 85% of cellular proteins are located intracellularly (Figure 1) [15].

However, the advantage of recognizing peptides bound to the HLA molecules also increases the possibility to recognize a non-cognate peptide, derived from a different protein, the so-called off-target effect. Unfortunately, this led to an unpredictable outcome in clinical trials, with patients developing strong inflammatory responses[16] and in some cases resulting in patients' death[17,18]. As a result of advanced screening procedures for on-target and off-target side effects, rTCR-T cells with low toxicity have been implemented in recent clinical trials. A list of clinical trials targeting specific antigens for the treatment of solid tumors can be found in Table 1. In cases where solid tumors failed to respond to ACT, complementary treatments to improve efficacy and safety of the adoptively transferred T cells, such as prior lymphodepletion, interleukin 2 (Aldesleukin; IL-2), and PD-1 blockade have been incorporated. Both lymphodepletion and IL-2 administration have been shown to promote survival and proliferation of the infused engineered T cells[19], whereas PD-1 blockade decreases T cell exhaustion[20]. Even if rTCR-T cells can have an advantage over other therapies in the context of solid tumors, improvements are still necessary to increase efficacy and safety. This review summarizes the most recent developments, with a strong focus on genetic approaches, that have been applied in the field of rTCR-T cells and the treatment of solid tumors.



## 2. INCREASING AFFINITY AND FUNCTIONAL AVIDITY OF RTCRS WHILE MAINTAINING A SAFE PROFILE

To obtain an efficacious response by rTCR-T cell therapy, it is necessary to select TCRs with high functional avidity and affinity to TAA. The identification of tumor

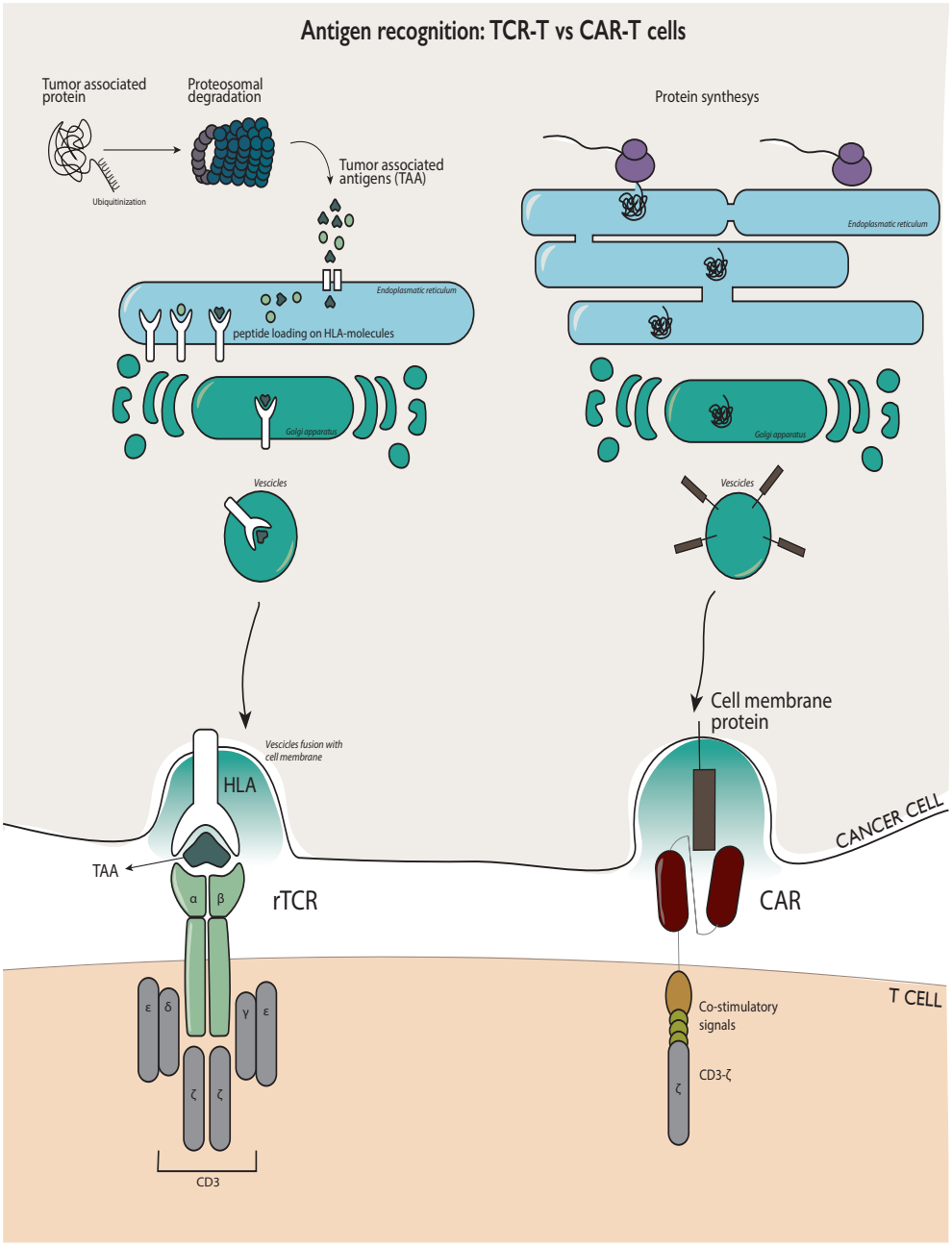



**Figure 1.** Differences in the mode of action of antigen recognition and activation of rTCR-T cells (on the left) and CAR-T cells (on the right). rTCR cells can recognize the presented peptide from both intracellular and extracellular proteins that are proteolytically processed and presented through human leukocyte antigen (HLA) molecules. CAR-T cells can solely recognize extracellular membrane proteins.

infiltrating lymphocytes (TILs) often leads to the isolation of potent TCRs. All TCRs used in clinical trials are derived from T cells isolated from cancer patients or from allogeneic donor cells[21–23]. Genetic modification of key amino acids, identified with the use of phage display and point mutations, has shown the possibility to further increase the TCR affinity, evaluated measuring binding of the recombinant TCRs (rTCRs) to the HLA-bound cognate peptides[24,25]. If the TCR affinity is important at the level of antigen recognition, TCR functional avidity is essential to obtain an actual cytotoxic effect. Enhancing the rTCR expression on the cell surface is one of the strategies to increase the functional avidity. A high level of rTCR expressed on the cell surface can double the production of cytotoxic cytokines, such of IFNɣ, compared to a low expression[26]. Retroviral and lentiviral vectors are the golden standard for delivering rTCRs genes into primary T cells. Over the years, mutations in the envelope of the vectors[27] and culture systems[28,29] have been optimized to specifically increase the transduction efficiency of T cells and optimize a stable expression of the rTCR on the cell surface. Moreover, mutations in the TCR constant regions, such as the introduction of additional cysteine di-sulfide bonds, sequences of murine origin, or genetic bond to the CD3ζ-complex, have been used to increase rTCR expression and reduce human TCR α and β chain mispairing with endogenous TCRs, but often resulted in a partial success [30,31]. More recently, it has been demonstrated that substitutions of 3 amino acid residues in the TCR variable domains consistently increase the expression of rTCRs on the surface of engineered T cells and the efficacy of the final product without acting on its affinity [32].

Intrinsic inhibitory signaling can also play an important role in the expression and functional avidity of the TCR on the cell surface. For instance, the cytokine-inducible SH2-containing (CISH) protein physically interacts with the TCR intermediate phospholipase Cɣ1 (PLC-ɣ1), targeting it for proteasomal degradation after TCR stimulation by the cognate peptide. The depletion of CISH unleashed a TCR-dependent hyperactive program, resulting in the upregulation of pro-functional, proliferative, and survival genes. Moreover, the genetic deletion of CISH, in a mouse model of melanoma, significantly increases CD8+ T cells related cytokines production (IFNɣ, TNFα, and IL-2) and anti-tumor reactivity, improving the survival of mice for more than 60 days. The effect of CISH downregulation was also evaluated using peripheral blood CD8+ T cells expressing a rTCRs. CD8+ T cells, treated with CISH silencing RNA (siRNA), showed a 2 fold increase in cytokine production when co-cultured with target tumor cells[33]. Recently, a Phase I/II clinical trial on metastatic gastrointestinal cancers has started the patients recruiting phase to evaluate the effect of TILs in which the CISH gene is inhibited using gene editing techniques (NCT03538613).

Increasing affinity and avidity of the TCRs have a reflection on the safety of the T cell therapy. This has been demonstrated in clinical trials with a high affinity rTCR-T cells against melanoma antigen recognized by T cells 1 (MART-1) causing severe toxicities of the skin, eye, and ears [34]. On the contrary, in an earlier clinical trial including melanoma patients, low toxicity and respectable toleration were observed when using a TCR with lower affinity for MART-1 [35]. In addition, to preserve the safety of the therapy, an extensive analysis of the expression of the antigen on healthy tissue and the possible cross-reactivity against non-cognate peptide is essential before using rTCR in the clinic. In particular, the recognition of key amino acid residues allows the prediction of possible cross-reactive antigens. Bijen et al. tested the cross-reactivity of a histocompatibility antigen 2 (HA2) specific TCR using a 9-mer combinatorial peptide library (CPL) screening. This technique was able to recognize cross-reactivity toward a Cadherin 13 (CDH13)-derived peptide, not detected using the most frequent test (alanine scanning mutagenesis). Further experiments proved the ability from the HA2 specific rTCR-T cells to recognize healthy cells, such as fibroblasts and keratinocytes, known as CDH13 expressing cells [36].

Several techniques to better predict cross-reactivity of rTCRs have been explored in the last few years, extensively illustrated in a recent review by Bentzen and Hadrup[37]. The cross-reactivity prediction tools in vitro help minimize the onset of unexpected serious side effects that have been reported in previous clinical trials. In 2013, two clinical trials reported unexpected toxicities derived from the administration of melanoma associated antigen (MAGE)-A3-rTCR-T cells in melanoma patients. The first trial showed serious neurological reaction in 3 out of 9 patients treated. Post mortem evaluation of patients' brain showed positivity for MAGE-A12, one of the recognized epitopes by the TCR used in this study[17]. The other clinical trial reported that two treated patients developed cardiogenic shock and died within a few days upon the MAGE-A3-rTCR-T cells. Only afterwards the rTCR-T cells were found to be cross-reactive to a similar epitope derived from the striated muscle-specific protein titin, expressed by cardiac cells [18].



## 3. GENETIC ELIMINATION OF ENDOGENOUS TCR TO IMPROVE EFFICACY AND SAFETY

Preferably, rTCR-T cells intended for ACT should only express the rTCR α and β chain of interest. However, conventional gene modification techniques often use viral systems to introduce the rTCR, without eliminating the expression of the endogenous TCR (eTCR). The presence of the eTCR α and β chains can lead to mispairing with the rTCR subunits[38], increasing the chances of creating novel

peptide recognition that, as shown in a lymphopenic mouse model, can lead to lethal graft versus host disease[39]. Additionally, TCR heterodimer mispairing reduces the formation of the correct rTCRs and compete in the formation of complexes with co-receptors, reducing the functionality of the T cell product [40]. As discussed in the previous paragraph, molecular techniques can be used on the sequence of the rTCR to decrease mispairing with the eTCR.

On the other hand, genome editing techniques have been developed to completely eliminate the expression of the eTCR targeting the constant region of TCR α and β chains. Zinc-finger nucleases (ZFN) and meganucleases have initially been used, but these are cumbersome to develop, and have resulted in only 7% of gene-editing frequencies in primary T cells[41]. More recently, transcription activator-like effector nuclease (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 endonuclease technologies have been developed, which are more attractive due to an easier design and high efficiency gene-editing results. As recently summarized by Zhang *et al.*, the use of CRISPR/Cas9 technology in primary T cells increased the success rate of gene editing compared to ZFN and TALENs[42] and has, in some cases, reached up to 90% of target gene deletion[43]. With these techniques, gene editing multiple genes at the same time (multiplexing) is feasible; for example, CRISPR/Cas9 multiple guide RNAs (gRNAs) can be delivered simultaneously[43–46]. In 2017, a pioneer clinical administration of CAR19 T cells, gene edited with TALEN to simultaneously deplete eTCR and CD52, a target of the serotherapeutic molecule alemtuzumab, showed the successful induction of molecular tumor remission ahead of allogeneic stem cell transplantation in two pediatric B-ALL patients. Additionally to the beneficial effects of the eTCR disruption, the genetic disruption of CD52 expression protected the infused cells from the depleting effect of alemtuzumab. Analysis of gene edited T cells, pre-infusion, showed expression of CAR19 in 85% of cells and depletion of both eTCR α and β chains and CD52 in more than 64% of the cells[45].

More recently, a first-in-human phase I clinical trial has started to test the safety and efficacy of rTCR-T cells, in which the constant regions of eTCR α and β chains (TRAC and TRBC) and PD-1 genes were knocked out (KO) using CRISPR/Cas9. The frequency of editing varied according to the gRNAs and was approximately 45% for TRAC, 15% for TRBC, and 20% for PD-1. It was demonstrated that this multiplexing approach, in conjunction with lentiviral delivery of a TCR recognizing an epitope of NY-ESO-1 and LAGE-1 cancer testis antigens, was feasible to generate triple knockout T cells with recombinant TCR and with initial favorable safety profiling. In one patient analyzed at depth, a frequency of 30% of di-genic and tri-genic editing was

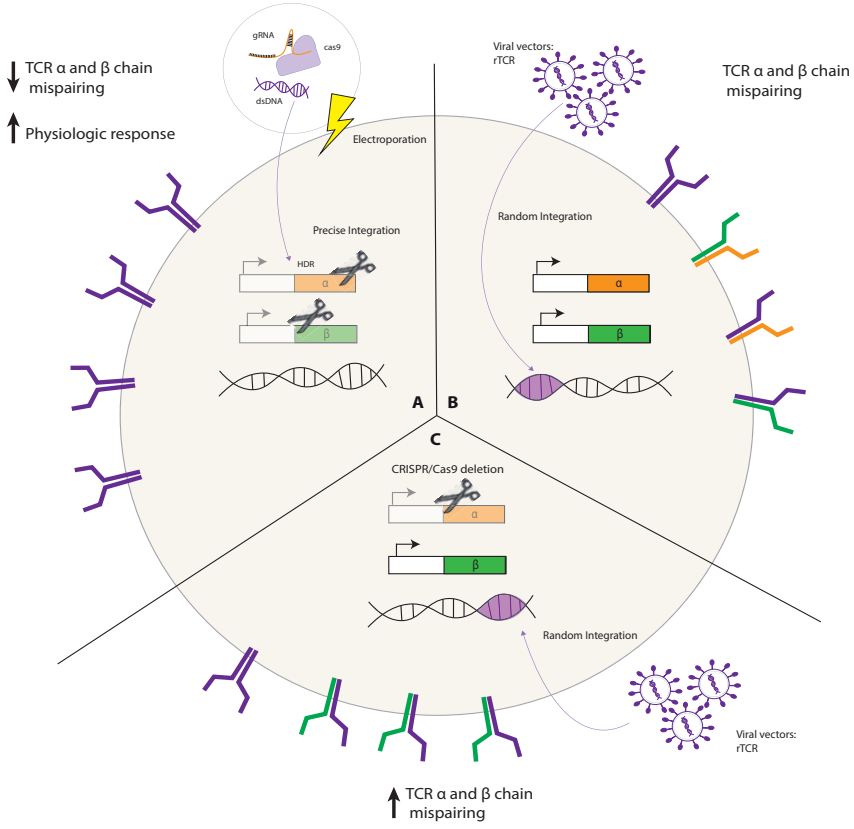



**Figure 2. Options to combine gene-editing with rTCR gene augmentation, with precise or random integration** (A) Precise integration of rTCR in the TRAC locus with simultaneous disruption of TRBC locus decreases the possibility of TCR mispairing and is characterized by a physiological and endogenously controlled expression of the rTCR. KO only the TRAC locus when using random integration techniques decrease the chance of TCR molecule mispairing (C) compared to a not-gene editing approach (B). Notably knocking-in the rTCR into the TRAC locus increased the rate of TCR mispairing on the cell surface (D). (Schematic representation of data obtained by Schober et al. [55]).

achieved in the infused cell population. Infusion of the T cell product in two patients with advanced refractory myeloma and one with metastatic sarcoma demonstrated an on-target effect, with no clinical toxicities. After 100 days, two out of the three patients had a stable disease, cytokine release syndrome did not occur and neither did any other infusion related side effects. Unfortunately, tumor progression was observed after 300 days in all patients, of which two received additional salvage chemotherapy agents and one died from advanced stage myeloma[47].

One of the main challenges of this multistep and multiplex approach is to generate the therapeutic product under GMP compliant conditions. Therefore, reducing the complexity of the process by limiting the number of the necessary components should be beneficial for large-scale clinical transition. A relevant approach is to disrupt the eTCR locus while simultaneously integrating the rTCR in the TCR locus of T cells using CRISPR/Cas9 mediated homologous recombination (HR). Adeno associated viral (AAV) vectors have been used as DNA donor sequence delivery system, especially serotype AAV6[48], particularly efficient to provide HR compared to other viral systems, such as integration deficient lentiviral vectors[49]. The use of AAV6 and HR has been reported for the successful generation of CAR19 T cells. Three studies published in 2017 showed a targeted integration of the CAR19 gene in the TRAC locus in 38 to 45% of primary T cells, inducing a simultaneous loss of TCR expression and proficient expression of the CAR. The administration of TRAC KO-CAR19 T cells in the tumor mice model showed greater responses and prolonged median survival at every T-cell dose compared to only transduced CAR19 T cells [50–52].

Other groups evaluated the possibility to deliver the HR donor sequence without the use of viral vectors to avoid immunogenicity, due to the presence of AAV derived proteins that can activate the pre-existing humoral response in vivo[53], and to accelerate clinical application, considering that non-viral materials typically can be more easily adapted to good manufacturing practices for clinical use. Roth et al. were the first to describe a protocol in which the electroporation of the CRISPR/Cas9 ribonucleoprotein complex (RNP) and a dsDNA template coding for an NY-ESO specific TCR was successfully integrated in 12% of target T cells via HR[54]. Furthermore, major advantages of a targeted integration are that the transcriptional regulation would be physiological, under the control of the endogenous promoter, and the risk of vector-induced insertional mutagenesis is minimized. In a later

study, Schober *et al.* demonstrated the importance of simultaneously targeting both TRAC and TRBC genes, as only knocking in the rTCR into the TRAC leads to an even increased mispairing between the recombinant α-chain and endogenous β-chain, compared to TRACKO viral transduced rTCR-T cells and not gene-edited viral transduced rTCR-T cells. Notably, inserting the desirable rTCR in the TRAC locus while concurrently knocking out the TRBC gene leads to a harmonized expression of the recombinant TCR on the cell surface (Figure 2), ultimately increasing the efficiency of the response against tumor cells in vitro, with an increased production of IFNɣ upon antigen recognition[55].

In conclusion, disruption of both TCR α and β genes can diminish mispairing and can thereby increase efficacy and safety with respect to potential off-target autoimmunity. Clinical benefits have yet to be elucidated in more human clinical trials to provide solid evidence of this technique to improve rTCR-T cell therapy against solid tumors.

## 4. DISRUPTING INHIBITORY PATHWAYS TO PREVENT EXHAUSTION

Immune checkpoint receptors on infused rTCR-T cells are differentially expressed compared to naturally circulating T lymphocytes, and that exhaustion markers are often rapidly upregulated after infusion in vivo [56]. There are many checkpoint receptors, but PD-1, LAG-3, and TIM-3 are commonly modulated by the tumor microenvironment to lead to exhaustion, endogenous T cells as well as gene modified cells[57,58]. The inhibitory immune checkpoint molecule PD-1 has been reported to be overexpressed in rTCR-T cells, especially after infusion, resulting in a diminished IFN- ɣ production and thereby a decreased immune response [56]. Hence, it was hypothesized that the disruption of the PD-1/PDL-1 axis could lead to less T cell exhaustion and improved persistency, and thereby an enhanced immune response. It has been shown that the use of anti-PD1 antibody augments the efficacy of NY-ESO engineered T cells both in vitro and in vivo model of human lung cancer. Repeated intraperitoneal injection of anti-PD1 antibody, perhaps, was able to halve the tumor growth compared to the injection of only rTCR-T cells [59].

Two clinical trials are now recruiting to test the effect of this combination in patients (NCT03578406; NCT04139057), in which it was already reported that two out of four treated patients displayed evidence of tumor regression. Especially in the context of solid tumor, a pre-clinical study, in a mouse model of pleural mesothelioma, showed that the administration of either PD-1 antibody checkpoint blockade, cell-intrinsic PD-1 shRNA blockade, or a PD-1 dominant negative receptor together with CAR

T-cells drastically enhanced tumor burden control and prolonged median survival[60]. The increased general availability of gene engineering techniques has also skewed the focus on knocking-out the checkpoint receptors genes, such as PD-1, resulting in permanent deletion of checkpoint inhibitory signaling. Pre-clinical studies have showed how the knock-out (KO) of PD-1 can increase the efficiency and response persistency of TILs and CAR-T cells in solid tumor settings, such as mouse models of glioblastoma and fibrosarcoma[61–64].

TILs and CAR-T cells with additional KO of PD-1 have been registered for many clinical trials (summarized by Mc Gowan *et al.* [65]); however, to the best of our knowledge, there are still no pre-clinical and clinical studies testing the benefits of PD-1 KO rTCR-T cells in solid tumor clearance; however, recently, a clinical trial including two myeloma patients and one sarcoma patient has shown promising results. PD-1 knockout NY-ESO rTCR-T cells appeared to have a half-life of 83.9 days, the average of the three treated subjects, compared to the half-life of seven days registered in previous clinical trials with NY-ESO rTCR-T cells without the KO[47,66].

Concurrently targeting multiple immune checkpoint receptors could be key in optimizing rTCR-T cell function, and it has already been demonstrated to be possible in pre-clinical studies in CAR T cells, with the one-shot generation of a dual inhibitory resistant universal CAR T cells deficient for TCR, HLA-I, PD-1, and CTLA-4[43]. Specific KO of checkpoint receptors in the transferred cells can be a strategy to decrease the toxicity derived from the use of systemic monoclonal antibodies; however, suppressing the inhibitory pathways that induce exhaustion and cell death can increase the possibility of generating over-activated T cells with enhanced autoimmunity that cannot be controlled. Interestingly, Stadtmauer et al. showed that the percentage of cells with edits in the PD-1 locus decreased to ~5% of the cells expressing the transgenic TCR at four months after infusion, giving a positive note to the safe use of this strategy in humans[47]. However, to assess if this approach has a preferred safety profile specific attention, would be required in a long-term follow-up.



## 5. RISKS OF USING GENE-EDITING TECHNIQUES

The use of gene editing techniques can raise concerns about the safety of rTCR-T cells, such as immunity against Cas9 protein, on target and off-target effects (reviewed by Ghosh *et al.* [67]). A major potential consequence of using gene targeting techniques is off-target double strand breaks, the recognition by the gene editing machinery of a similar genomic sequence. Cleavage at off-target sites can result in

chromosomal rearrangements, including insertion, deletion, translocations, and disruption of important genes and genotoxicity[68].

Genome wide detection techniques, such as Digenome–seq, SITE–seq, and CIRCLE–seq, have been developed to understand the extent of off-target events in an unbiased way [69]. Furthermore, several approaches are now being used to minimize the off-target effect, especially focusing on the CRISPR/Cas9 technology, with optimization of the gRNAs design and the Cas proteins [70]. Point mutations in the sequence of the Cas9 protein have dramatically increased the specificity of the system and generated the so-called high fidelity Cas9 proteins (HiFi Cas9)[71,72]. Outstanding results were obtained with a particular point mutation R691A that led to reduced low off-target effect, down to 1%, while maintaining an efficient on-target effect, which was not always the case for other HiFiCas9 proteins tested[73]. Remarkably, in the three reports of the clinical administration of gene edited T cells (NCT03399448, NCT02793856)[45], off-target events were detected on a relatively low percentage of cells, which decreased over time, suggesting that these translocations did not confer a growth advantage over the other infused gene modified T cells. In the study evaluating the safety and feasibility of PD-1 gene editing autologous T cells using CRISPR/Cas9, 18 predicted off-target sites were analyzed with next generation sequencing (NGS) before the two cycles of T cells infusion. The median mutation frequency of all off-target sites was 0.05%. To further minimize the bias of analyzing only a small number of off target sites, seven samples were also evaluated using whole genome sequencing (WGS) at 100× coverage. Using the prediction tool Cas-OFFinder, not limited by the amount of variety in the protospacer-adjacent motif (PAM), mismatches, and gRNA length; 2086 potential off-target sites were predicted. Indel events were not detected within 15 base pairs (bp) up- and downstream of the sites. When each site was broadened to 200 bp up- and downstream, 84 indel events in 53 sites were detected, but all of them comprised 1-bp length variances on nucleotide repeats and are therefore not considered to be true off-target events[74].

Multiplexing approaches, in which multiple genes are targeted simultaneously, further increase the probability of genomic alterations, more specifically of large chromosomal translocations. In detail, in the clinical report from Qasim et al., using a dual-gene editing strategy with TALENs, chromosomal rearrangements were observed in 4% of infused T cells[45]. Stadtmauer et al. published in their clinical report the development of a qualified qPCR assay to assess the 12 potential translocations that could occur with the simultaneous editing of four loci: TRAC, TRBC1, TRBC2, and PD1. The TRBC1:TRBC2 chromosomal rearrangement was the most frequent, leading to a 9.3 kB deletion. Overall, the percentage of T cells with translocations was

less than 10% of cells in all the three patients before infusion, and at days 30, 150, and 170 chromosomal translocations were at the limits of detection[47]. Furthermore, the most recent development of the CRISPR/Cas9 base editor technique, which allows for a targeted base substitution instead of a double strand break, can further minimize safety concerns[75]. To support the generation of T cell products with a multiplexing approach, a recent study showed that multiplex base edited T cells exhibit improved expansion and lack double strand break-induced translocations observed in T cells edited with Cas9 nuclease[76].

## 6. INCORPORATING CYTOKINES TO ENHANCE T CELL PROLIFERATION

Cytokines are immunomodulatory molecules that influence proliferation of B and T lymphocytes and have been applied in several cancer immunotherapeutic treatments.

Cytokines of the ɣ-chain family, including IL-2, are important for T cell memory and proliferation. These cytokines exert many functions on T cells, such as enhanced proliferation, persistence, and improved antigen recognition[77]. In ACT, high dose of IL-2 is intravenously injected in concurrence with rTCR-T cells, in order to support their functionality and proliferation, but often results in multi-organ toxicities for patients[78]. Local delivery and the generation of mutated protein increased the specificity of the therapy and reduced systemic toxicity[79]. Moreover, expressing the IL-2 transgene simultaneously with the rTCR could further improve the engineered T cells with reduced systemic toxicity. In support of this, in a study in 2001, it was shown that complementary IL-2 expression could remarkably enhance melanoma specific survival of CD8+ T cells in vitro [80]. This was also supported by a later study, showing that IL-2 gene modified TILs could secrete sufficient amounts of IL-2 to prolong their own in vitro survival for up to six months. However, in vivo results were less encouraging. Although the IL-2 transduced T cells were well-tolerated, no significant response rates were observed in this clinical trial compared to non-IL-2-transduced T lymphocytes. The reason for the poor in vivo response in this application has not been elucidated and requires further studies[81].

Gene addition of other cytokines, such as IL-12 and IL-18, has been investigated, in order to increase functionality and maturation of the T cells. rTCR-T cells specific for gp100 with inducible constructs of IL-12 (iIL-12) and IL-18 (iIL-18) have shown elevated IFNy production after encountering their specific antigen and can therefore enhance the anti-tumor response. Administration of iIL-18 rTCR-T cells to mice bearing gp100 positive melanoma tumor cells resulted in a more persistent anti-

tumor response with no detectable toxicity. Administration of iIL-12 rTCR-T cells has been shown to result in increased levels of IFNy and TNFα. However, under control of a nuclear factor of activated T cell (NFAT) sensitive promoter, iIL-12 correlated with compromised T cell persistence, enhanced plasma levels of inflammatory cytokines, and decreased survival in the gp100+ mouse model of melanoma[82]. In a later study, the use of an inducible Tet-On promoter, transiently activated by the antibiotic doxycycline, showed the same benefits in tumor control, in a mouse model of melanoma, obtained with iIL-18 without showing toxicities. Transient expression of iIL-12 showed to be sufficient to inhibit the growth of B16F10 melanoma tumors and to increase the number of tumor-infiltrating rTCR-T cells, without showing toxicity compared with the use of the NFAT-promoter[83].

Additionally, another means of reducing the systemic toxicity of IL-12 could be anchoring iIL-12 to the plasma membrane. In a recent study of tumor mouse models, the toxicity of IL-12 was reduced significantly when applying this approach[84]. IL-12 genetically engineered TILs have been tested in a clinical trial with patients suffering from metastatic melanoma. Administration of doses between 0.3 and 3 × 109 cells showed an objective clinical response in 63% of the treated patients. The administered IL-12 gene engineered TILs were found in circulation for less than a month. More importantly, increasing cell doses was associated with high serum levels of IL-12 and IFNɣ as well as clinical toxicities, including liver dysfunction, high fevers, and sporadic life-threatening hemodynamic instability[85]. A phase I clinical trial protocol was also published for recruiting patients with recurrent ovarian cancer and defined a safe and efficient dose of CAR-T cells gene engineered to over express IL-12 [86].



## 7. INTRODUCTION OF CHEMOKINES RECEPTORS TO PROMOTE MIGRATION AND INFILTRATION

Migration, infiltration, and homing of T cells into solid tumors are often hampered by the presence of a suppressive environment. This poses a major problem, since the absence of T lymphocytes within most solid tumors correlates with a worse prognosis. Additionally, patients with a higher infiltration rate of immune cells often have a better prognosis. Post infusion tracking of T cells in animal models, with biomedical imaging techniques, has shown localization of the infused T cells primarily in the liver, spleen, and lungs, often more prominent than in the tumor site[87,88]. These preclinical models have underlined that migration and localization to the tumor remains a challenge for gene engineered T cells in the treatment of solid tumors.

Chemokines play a major role in the migration and homing of T cells, and are also important for T cell survival and proliferation. There are many chemokines and chemokine receptors axes involved in tumor immunology and T cell responses, comprehensively reviewed by Nagarsheth et al., that have been explored in order to further enhance the effectiveness of ACT[89]. For example, cancers such as metastatic melanoma and ovarian cancer have been associated with proficient homing of tumor infiltrating T cells (TILs), due to a high production of pro-inflammatory chemokines, such as CXCL9 and CXCL10, recognized by the TILs[90]. However, other solid tumor types create a suppressive tumor microenvironment (TME) that express pro-tumoral chemokines to induce metastasis and recruit suppressive immune cells, such as the described effect of CCL12 in recruiting Treg cells[91].

Naturally, T cells only express a restricted set of chemokine receptors on their cell surface limiting their ability to always be attracted by chemokines expressed by tumor cells. Therefore, it was suggested that incorporation of chemokine receptors within T cells could possibly augment recruitment and trafficking to the tumor site. For instance, it was observed that many melanomas had a high production of the chemokine CXCL1, but that its receptor CXCR2 was not profoundly expressed on T lymphocytes. It is noteworthy that CXCL1 was also not expressed by T cells. As a proof of concept, Kershaw et al. were among the first ones to demonstrate that the introduction of CXCR2, via retroviral vector transduction of peripheral blood derived T cells, was a feasible technique to redirect the cells towards the tumor, resulting in proper IFN-ɣ response when activated by their cognate chemokine[92]. In the context of rTCR-T cells, it was found that co-expression of CXCR2 with MAGE-A3 specific TCRs could significantly enhance migration to the tumor site in a mice model of melanoma, resulting in reduced tumor growth [93]. Moreover, the introduction of CXCR2 receptor in CAR-T cells targeting CD70 greatly improved the tumor control and enhanced in pre-clinical models of aggressive tumors such as glioblastoma, ovarian, and pancreatic cancer [94]. This pre-clinical data demonstrate that exploiting chemokines is feasible to improve recruitment of engineered T cells to the tumor-site in a large spectrum of solid tumors. To translate this application to the clinic, a Phase I/II clinical trial is testing the safety and efficiency of TILs transduced with CXCR2 in treating patients with stage III melanoma and metastatic melanoma (NCT01740557).

## 8. TARGETING THE TUMOR SURROUNDING STROMA

The abovementioned strategies focus on improving the functionality, migration, and persistence of rTCR-T cells by additional modification in the genome of the T cells. However, there is the possibility to switch the focus on the tumor itself and its microenvironment. This can be of particular interest to further personalize new therapeutic approaches based on the patient tumor characteristic.

Infiltrating T lymphocytes often accumulate in the stroma surrounding the tumor, comprising non-malignant cells and extracellular matrix (ECM). Cancer associated fibroblasts (CAFs) play an important role in tumor support by secreting pro-tumoral cytokines and by producing an excessive amount of ECM [95]. However, CAFs frequently express fibroblast activation protein alpha (FAPα), making them distinguishable from healthy cells and a potential target for cancer treatment [96]. It has been shown in a mouse model of lung cancer that treatment with FAPα-specific CAR T cells in concurrence with T cells targeting a tumor specific TAA, namely ephrin type-A receptor 2, could significantly enhance the anti-tumor effect compared to separate administration of these gene-engineered T cells[97].

Targeting the tumor vasculature could be an additional way to enhance tumor infiltration of rTCR-T cells in solid tumors. The abnormal tumor vasculature creates a hypoxic and acidic environment, and impedes the infiltration and function of anti-tumoral immune cells including T cells [98]. Additionally, tumor endothelial cells (ECs) have a strong suppressive function on T cells, and can directly block T cells to enter the tumor through downregulation of adhesion molecules, upregulation of inhibitory receptors, and production of FasL [99]. By adapting the tumor vasculature, a path could be paved for T cells to enter. A possible targetable molecule in this approach would be vascular endothelial growth factor receptor 2 (VEGFR-2), constitutively expressed by the tumoral endothelial cells. The use of CAR T cells targeting VEGFR-2 increases infiltration of T cells into the tumor, by means of destroying the endothelial cells in the stroma. However, the overall induced anti-tumor effect was only modest [100]. Concurrent administration of TAA specific rTCR-T cells, for gp100 or TRP-1, with VEGFR-2 specific T cells appeared to increase the anti-tumor effect in vivo significantly compared to administration of either alone. A strong persistence and increased infiltration was seen of the adoptively transferred T cells resulting in increased anti-tumor efficacy[101]. These examples demonstrated that combined therapies, targeting both the tumor microenvironment and the tumor cells, are a promising approach to improve rTCR-T cell therapy in solid tumors.

## 9. INCORPORATION OF SUICIDE GENES TO SAFEGUARD OFF-TARGET TOXICITIES

Mechanisms to eliminate the gene engineered T cell product can have utility in the case of on-target/off-tumor or oncogenic mutations events. Suicide genes have originally been used to modify T cells and decrease the onset of Graft versus Host Disease (GvHD). Integration of inducible suicide genes, resulting in cell death of the gene engineered T cells, allows a better control of side effect and give the possibility to eliminate the product post-infusion. A well-defined suicide gene is the herpes simplex virus thymidine kinase (HSV-tk), which has already been used to restrain graft versus host disease after applying ACT in a variety of malignancies. Activation of HSV-tk is achieved after applying ganciclovir (GCV), resulting in elimination of construct-engineered T cells. HSV-tk has already been included in some CAR T cell therapies, targeting the overexpressed protein CD44 isoform variant 6 (CD44v6), in clinical trials for the treatment of AML and MM patients (NCT04097301)[102]. Recently, the same CAR-T cells were able to reach, infiltrate, and proliferate at tumor sites in an adenocarcinoma tumor model[103]. In all those studies, effective elimination of the infused CAR T cells could be observed upon administration of GCV. Moreover, the use of HSV-tk is also beneficial for tracing the infused cells in the patients, being a valuable PET reporter gene. Using this imaging technique, in a mouse model of sarcoma, CAR-T cells were traced to the tumor, evaluating the efficiency of the therapy and the ablation upon GCV administration[104]. These results could be well translatable to rTCR-T cell therapies.

However, there are some disadvantages related to the use of HSV-Tk genes, such as the immunogenicity of a viral derived gene-product, that can lead to immune activation and removal of therapeutic T cells; the amount of time needed before the T cells are eliminated by HSV-tk; and the need for the first line therapeutic agent for cytomegalovirus (CMV) infections[105]. The immunogenicity of a viral derived protein was overcome with the use of the inducible human caspase 9 (iCasp9) suicide gene. iCasp9 is a fusion protein of the catalytic domain of the pro-apoptotic protein caspase-9 and a domain of FKBP12 and can be activated after the introduction of a chemical inducer of dimerization (CID), such as AP1903 (Rimiducid) and AP20187. The CID can only bind the mutated domain of FKBP12 fused with iCASP9, but not the wild-type domain. Apoptosis of the infused T cells containing the construct is achieved after dimerization of the FKBP12 domains of iCasp9 upon binding of the CID, resulting in activation of the caspase molecules[106]. Robust elimination of donor T lymphocytes has already been shown after hematopoietic stem cell transplantation, underlying the great potential in rTCR-T cell therapy as well. More

than 90% of T cells expressing the construct were eliminated within half an hour after delivery of AP20187, resulting in a diminished graft versus host reaction[107]. Further modification in the iCas9 system substitutes the use of AP1903 (Rimiducid) and AP20187 with Rapamycin. Rapamycin is involved in the inhibition of mTORC1, with FKBP12 as a co-factor, and is used as a well-tolerated immunosuppressive drug. Binding of rapamycin to FKBP12 increases the affinity for the unique FRB domain of TOR. It has been demonstrated that iRC9, a construct comprised of the catalytic domain of caspase 9 and both the FRB and FKBP12 domains, could functionally be activated by rapamycin, both in vitro and in vivo, in a mouse model of leukemia[108].

## 10. CONCLUSIONS

Over the past few decades, immunotherapy has made tremendous progress in improving cancer treatment options. In particular, patients suffering from hematological malignancies have gained clinical benefits, in some cases resulting in complete remission. However, solid tumors remain a greater challenge in this field to overcome. Genetic modification of T cells could address some of the current constraints. In this review, we focused on the use of rTCR-T cell therapy for solid tumors, from ongoing clinical trials to the future applications using cutting-edge genetic strategies to improve both potency and safety (Figure 3).

To be efficacious and safe, rTCR-T cell products have to specifically and effectively target tumor cells and retain functionality within the suppressive tumor environment. Recently developed engineering techniques can help to address these limitations, by combining multiple gene addition approaches into a single treatment, such as gene addition of chemokine receptors and recombinant cytokines. Gene-editing techniques using CRISPR/Cas9 are highly efficient to knock out the endogenous TCR which significantly enhances the safety profile with respect to off-target peptide recognition, and to disrupt inhibitory signaling, which increase the persistency and functionality of the cells. In the context of rTCR-T cell therapy for solid tumors, the potential of gene augmentation and gene editing can be largely applied, especially to improve migration and infiltration into the tumor and T cell persistency after infusion.

We discussed some of the possible strategies to target multiple pathways involved in the effectiveness of the T cell therapy, but we expect that many more approaches, targeting different pathways/signals, will be explored and might be beneficial to the final T cell product.

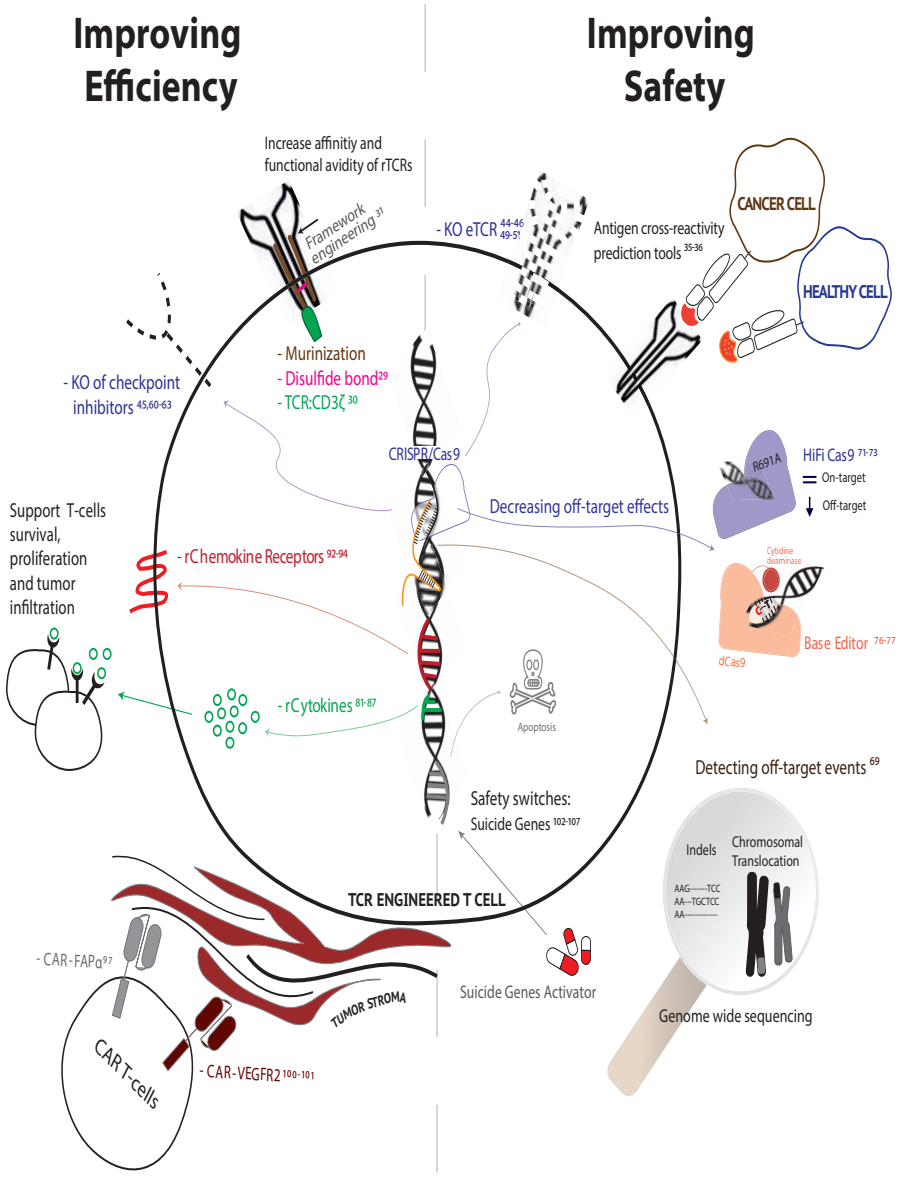



**Figure 3-** Summary of gene-augmentation and gene-editing strategies to improve efficiency (on the left) and safety (on the right) to treat solid tumors.

It is important to move towards more specific personalized treatment regimens, in which multiplex combinations generate a tumor specific cell therapy product. We speculate that, in the future, combining deep, patient-individualized knowledge of solid tumors, with gene augmentation and gene editing techniques applied to rTCR-T cells, is the key to a successful and broadly applicable therapy.

Moreover, it would be beneficial to have more accessibility to GMP-grade available components for gene-editing and to options of automation to standardize the production of multiplexed T cell therapy products in enclosed systems. Although gene augmentation and gene editing methods showed promising pre-clinical benefits, possible major side effects using these novel techniques should be addressed, but initial results indicate that the off-target gene-editing risk in T cells is low. Encouraging results have been presented in clinical trials in which cancer patients were infused with multiplexed genetically modified cells, proving initial safety of the drug product. In conclusion, the success of rTCR-T cells for solid tumor treatment could lie in the complexity of tools to genetically modify these cells on multiple levels creating a balance between efficacy and safety. The general use of these highly advanced rTCR-T cell therapies is coming closer to being a potential treatment option for solid tumors and is highly likely to be implemented in future T cell products for clinical application.

## FUNDING

This research received external funding from the Dutch Cancer Society (KWF) project 10474.



## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

## BIBLIOGRAPHY


1. Yang, Y. Cancer immunotherapy : Harnessing the immune system to battle cancer Find the latest version : Cancer immunotherapy : Harnessing the immune system to battle cancer. J. Clin. Investig. 2015, 125, 3335–3337, doi:10.1172/JCI83871.It.
2. Hargadon, K.M.; Johnson, C.E.; Williams, C.J. Immune checkpoint blockade therapy for cancer: An overview of FDA-approved immune checkpoint inhibitors. Int. Immunopharmacol. 2018, 62, 29–39, doi:10.1016/j.intimp.2018.06.001.
3. Pitt, J.M.; Vétizou, M.; Daillère, R.; Roberti, M.P.; Yamazaki, T.; Routy, B.; Lepage, P.; Boneca, I.G.; Chamaillard, M.; Kroemer, G.; et al. Resistance Mechanisms to Immune-Checkpoint Blockade in Cancer: Tumor-Intrinsic and -Extrinsic Factors. Immunity 2016, 44, 1255–1269, doi:10.1016/j.immuni.2016.06.001.
4. Dudley, M.E.; Rosenberg, S.A. Adoptive-cell-transfer therapy for the treatment of patients with cancer. Nat. Rev. Cancer 2003, 3, 666–675, doi:10.1038/nrc1167.
5. Dudley, M.E.; Wunderlich, J.R.; Robbins, P.F.; Yang, J.C.; Hwu, P.; Schwartzentruber, D.J.; Topalian, S.L.; Sherry, R.; Restifo, N.P.; Hubicki, A.M.; et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science. 2002, 298, 850–854, doi:10.1126/science.1076514.
6. Li, D.; Li, X.; Zhou, W.L.; Huang, Y.; Liang, X.; Jiang, L.; Yang, X.; Sun, J.; Li, Z.; Han, W.D.; et al. Genetically engineered t cells for cancer immunotherapy. Signal. Transduct. Target. Ther. 2019, 4, doi:10.1038/s41392-019-0070-9.
7. Martinez, M.; Moon, E.K. CAR T cells for solid tumors: New strategies for finding, infiltrating, and surviving in the tumor microenvironment. Front. Immunol. 2019, 10, 1–21, doi:10.3389/fimmu.2019.00128.
8. Bagley, S.J.; O'Rourke, D.M. Clinical investigation of CAR T cells for solid tumors: Lessons learned and future directions. Pharmacol. Ther. 2020, 205, doi:10.1016/j.pharmthera.2019.107419.
9. Binnewies, M.; Roberts, E.W.; Kersten, K.; Chan, V.; Fearon, D.F.; Merad, M.; Coussens, L.M.; Gabrilovich, D.I.; Ostrand-rosenberg, S.; Hedrick, C.C.; et al. Understanding the tumor iimune microenvironment ( TIME ) for effective therapy. Nat. Med. 2018, 24, 541–550, doi:10.1038/s41591-018-0014-x.
10. Chen, N.; Li, X.; Chintala, N.K.; Tano, Z.E.; Adusumilli, P.S. Driving CARs on the uneven road of antigen heterogeneity in solid tumors. Curr. Opin. Immunol. 2018, 51, 103–110, doi:10.1016/j.coi.2018.03.002.
11. Garber, K. Driving T-cell immunotherapy to solid tumors. Nat. Biotechnol. 2018, 36, 215–219, doi:10.1038/nbt.4090.
12. 12. Ping, Y.; Liu, C.; Zhang, Y. T-cell receptor-engineered T cells for cancer treatment: Current status and future directions. Protein Cell 2018, 9, 254–266, doi:10.1007/s13238-016-0367-1.
13. Debets, R.; Donnadieu, E.; Chouaib, S.; Coukos, G. TCR-engineered T cells to treat tumors: Seeing but not touching? Semin. Immunol. 2016, 28, 10–21, doi:10.1016/j.smim.2016.03.002.
14. Fesnak, A.D.; June, C.H.; Levine, B.L. Engineered T cells: The promise and challenges of cancer immunotherapy. Nat. Rev. Cancer 2016, 16, 566–581, doi:10.1038/nrc.2016.97.
15. He, Q.; Jiang, X.; Zhou, X.; Weng, J. Targeting cancers through TCR-peptide/MHC interactions. J. Hematol. Oncol. 2019, 12, 1–17, doi:10.1186/s13045-019-0812-8.
16. Parkhurst, M.R.; Yang, J.C.; Langan, R.C.; Dudley, M.E.; Nathan, D.-A.N.; Feldman, S.A.; Davis, J.L.;

Morgan, R. a; Merino, M.J.; Sherry, R.M.; et al. T Cells Targeting Carcinoembryonic Antigen Can Mediate Regression of Metastatic Colorectal Cancer but Induce Severe Transient Colitis. Mol. Ther. 2011, 19, 620–626, doi:10.1038/mt.2010.272.

17. Morgan, R.A.; Chinnasamy, N.; Abate-daga, D.D.; Gros, A.; Robbins, F.; Zheng, Z.; Feldman, S.A.; Yang, J.C.; Sherry, R.M.; Phan, Q.; et al. Cancer regression and neurologic toxicity following anti-MAGE- A3 TCR gene therapy Richard. J. Immunother. 2014, 36, 133–151, doi:10.1097/CJI.0b013e3182829903.
18. Linette, G.P.; Stadtmauer, E.A.; Maus, M.V.; Rapoport, A.P.; Levine, B.L.; Emery, L.; Litzky, L.; Bagg, A.; Carreno, B.M.; Cimino, P.J.; et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. Blood 2013, 122, 863–871, doi:10.1182/blood-2013-03-490565.
19. Klebanoff, C.A.; Khong, H.T.; Antony, P.A.; Palmer, D.C.; Restifo, N.P. Sinks, suppressors and antigen presenters: How lymphodepletion enhances T cell-mediated tumor immunotherapy. Trends Immunol. 2005, 26, 111–117, doi:10.1016/j.it.2004.12.003.
20. Seliger, B. Combinatorial Approaches with Checkpoint Inhibitors to Enhance Anti-tumor Immunity. Front. Immunol. 2019, 10, 1–10, doi:10.3389/fimmu.2019.00999.
21. Inderberg-Suso, E.M.; Trachsel, S.; Lislerud, K.; Rasmussen, A.M.; Gaudernack, G. Widespread CD4+ T-cell reactivity to novel hTERT epitopes following vaccination of cancer patients with a single hTERT peptide GV1001. Oncoimmunology 2012, 1, 670–686, doi:10.4161/onci.20426.
22. Cohen, C.J.; Gartner, J.J.; Horovitz-Fried, M.; Shamalov, K.; Trebska-McGowan, K.; Bliskovsky, V.V.; Parkhurst, M.R.; Ankri, C.; Prickett, T.D.; Crystal, J.S.; et al. Isolation of neoantigen-specific T cells from tumor and peripheral lymphocytes. J. Clin. Investig. 2015, 125, 3981–3991, doi:10.1172/JCI82416.
23. Linnemann, C.; Heemskerk, B.; Kvistborg, P.; Kluin, R.J.C.; Bolotin, D.A.; Chen, X.; Bresser, K.; Nieuwland, M.; Schotte, R.; Michels, S.; et al. Technical Reports High-throughput identification of antigen-specific TCRs by TCR gene capture. Nat. Med. 2013, 19, 1534–1541, doi:10.1038/nm.3359.
24. Cole, D.K.; Sami, M.; Scott, D.R.; Rizkallah, P.J.; Borbulevych, O.Y.; Todorov, P.T.; Moysey, R.K.; Jakobsen, B.K.; Boulter, J.M.; Baker, B.M.; et al. Increased peptide contacts govern high affinity binding of a modified TCR whilst maintaining a native pMHC docking mode. Front. Immunol. 2013, 4, 1–8, doi:10.3389/fimmu.2013.00168.
25. Malecek, K.; Grigoryan, A.; Zhong, S.; Gu, W.J.; Johnson, L.A.; Rosenberg, S.A.; Cardozo, T.; Krogsgaard, M. Specific Increase in Potency via Structure-Based Design of a TCR. J. Immunol. 2014, 193, 2587–2599, doi:10.4049/jimmunol.1302344.
26. Spear, T.T.; Wang, Y.; Foley, K.C.; Murray, D.C.; Scurti, G.M.; Simms, P.E.; Garrett-Mayer, E.; Hellman, L.M.; Baker, B.M.; Nishimura, M.I. Critical biological parameters modulate affinity as a determinant of function in T-cell receptor gene-modified T-cells. Cancer Immunol. Immunother. 2017, 66, 1411–1424, doi:10.1007/s00262-017-2032-9.
27. Costa, C.; Hypolite, G.; Bernadin, O.; Lévy, C.; Cosset, F.-L.; Asnafi, V.; Macintyre, E.; Verhoeyen, E.; Tesio, M. Baboon envelope pseudotyped lentiviral vectors: A highly efficient new tool to genetically manipulate T-cell acute lymphoblastic leukaemia-initiating cells. Leukemia 2017, 31, 977–980, doi:10.1038/leu.2016.372.
28. Cavalieri, S.; Cazzaniga, S.; Geuna, M.; Magnani, Z.; Bordignon, C.; Naldini, L.; Bonini, C. Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. Blood 2003, 102, 497–505, doi:10.1182/blood-2003-01-0297.
29. Circosta, P.; Granziero, L.; Follenzi, A.; Vigna, E.; Stella, S.; Vallario, A.; Elia, A.R.; Gammaitoni,

L.; Vitaggio, K.; Orso, F.; et al. T Cell Receptor (TCR) Gene Transfer with Lentiviral Vectors Allows Efficient Redirection of Tumor Specificity in Naive and Memory T Cells Without Prior Stimulation of Endogenous TCR. Hum. Gene Ther. 2009, 20, 1576–1588, doi:10.1089/hum.2009.117.

30. Kuball, J.; Dossett, M.L.; Wolfl, M.; Ho, W.Y.; Voss, R.H.; Fowler, C.; Greenberg, P.D. Facilitating matched pairing and expression of TCR chains introduced into human T cells. Blood 2007, 109, 2331–2338, doi:10.1182/blood-2006-05-023069.
31. Sebestyén, Z.; Schooten, E.; Sals, T.; Zaldivar, I.; José, E.S.; Alarcón, B.; Bobisse, S.; Szöllosi, J.; Gratama, J.W.; Ralph, A.; et al. Human TCR That Incorporate CD3 ζ Induce Highly Preferred Pairing between TCR α and β Chains following Gene Transfer. J. Immunol. 2020, doi:10.4049/jimmunol.180.11.7736.
32. Thomas, S.; Mohammed, F.; Reijmers, R.M.; Woolston, A.; Stauss, T.; Kennedy, A.; Stirling, D.; Holler, A.; Green, L.; Jones, D.; et al. Framework engineering to produce dominant T cell receptors with enhanced antigen-specific function. Nat. Commun. 2019, 10, 1–15, doi:10.1038/s41467-019-12441-w.
33. Palmer, D.C.; Guittard, G.C.; Franco, Z.; Crompton, J.G.; Eil, R.L.; Patel, S.J.; Ji, Y.; Panhuys, N. Van; Klebanoff, C.A.; Sukumar, M.; et al. Cish actively silences TCR signaling in CD8 + T cells to maintain tumor tolerance. J. Exp. Med. 2013, 2095–2113, doi:10.1084/jem.20150304.
34. Duinkerken, C.W.; Rohaan, M.W.; De Weger, V.A.; Lohuis, P.J.F.M.; Latenstein, M.N.; Theunissen, E.A.R.; Balm, A.J.M.; Dreschler, W.A.; Haanen, J.B.A.G.; Zuur, C.L. Sensorineural Hearing Loss after Adoptive Cell Immunotherapy for Melanoma Using MART-1 Specific T Cells: A Case Report and Its Pathophysiology. Otol. Neurotol. 2019, 40, e674–e678, doi:10.1097/MAO.0000000000002332.
35. Morgan, R. a.; Dudley, M.E.; Wunderlich, J.R.; Hughes, M.S.; Yang, J.C.; Sherry, R.M.; Royal, R.E.; Topalian, S.L.; Kammula, U.S.; Restifo, N.P.; et al. Cancer Regression in Patients After Transfer of Genetically Engineered Lymphocytes. Science 2006, 314, 126–129, doi:10.1126/science.1129003.
36. Bijen, H.M.; van der Steen, D.M.; Hagedoorn, R.S.; Wouters, A.K.; Wooldridge, L.; Falkenburg, J.H.F.; Heemskerk, M.H.M. Preclinical Strategies to Identify Off-Target Toxicity of High-Affinity TCRs. Mol. Ther. 2018, 26, 1206–1214, doi:10.1016/j.ymthe.2018.02.017.
37. Bentzen, A.K.; Hadrup, S.R. Evolution of MHC-based technologies used for detection of antigen-responsive T cells. Cancer Immunol. Immunother. 2017, 66, 657–666, doi:10.1007/s00262-017-1971-5.
38. Shao, H.; Zhang, W.; Hu, Q.; Wu, F.; Shen, H.; Huang, S. TCR mispairing in genetically modified T cells was detected by fluorescence resonance energy transfer. Mol. Biol. Rep. 2010, 37, 3951–3956, doi:10.1007/s11033-010-0053-y.
39. Bendle, G.M.; Linnemann, C.; Hooijkaas, A.I.; Bies, L.; de Witte, M.A.; Jorritsma, A.; Kaiser, A.D.M.; Pouw, N.; Debets, R.; Kieback, E.; et al. Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. Nat. Med. 2010, 16, 565–570, doi:10.1038/nm.2128.
40. Legut, M.; Dolton, G.; Mian, A.A.; Ottmann, O.G.; Sewell, A.K. CRISPR-mediated TCR replacement generates superior anticancer transgenic t cells. Blood 2018, 131, 311–322, doi:10.1182/blood-2017-05-787598.
41. Provasi, E.; Genovese, P.; Lombardo, A.; Magnani, Z.; Liu, P.-Q.; Reik, A.; Chu, V.; Paschon, D.E.; Zhang, L.; Kuball, J.; et al. Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. Nat. Med. 2012, 18, 807–815, doi:10.1038/nm.2700.
42. Zhang, X.; Cheng, C.; Sun, W.; Wang, H. Engineering T Cells Using CRISPR/Cas9 for Cancer Therapy. In RNA Interference and CRISPR Technologies; Springer: Berlin, Germany 2020; Volume 2115, pp.

503–509.

43. Ren, J.; Zhang, X.; Liu, X.; Fang, C.; Jiang, S. A versatile system for rapid multiplex genome-edited CAR T cell generation. Oncotarget 2017, 8, 17002–17011.
44. Morton, L.T.; Reijmers, R.M.; Wouters, A.K.; Kweekel, C.; Remst, D.F.G.; Pothast, C.R.; Falkenburg, J.H.F.; Heemskerk, M.H.M. Simultaneous Deletion of Endogenous TCRαβ for TCR Gene Therapy Creates an Improved and Safe Cellular Therapeutic. Mol. Ther. 2020, 28, 64–74, doi:10.1016/j.ymthe.2019.10.001.
45. Qasim, W.; Zhan, H.; Samarasinghe, S.; Adams, S.; Amrolia, P.; Stafford, S.; Butler, K.; Rivat, C.; Wright, G.; Somana, K.; et al. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. Sci. Transl. Med. 2017, 9, eaaj2013, doi:10.1126/scitranslmed.aaj2013.
46. Liu, X.; Zhang, Y.; Cheng, C.; Cheng, A.W.; Zhang, X.; Li, N.; Xia, C.; Wei, X.; Liu, X.; Wang, H. CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells. Cell Res. 2017, 27, 154–157, doi:10.1038/cr.2016.142.
47. Stadtmauer, E.A.; Fraietta, J.A.; Davis, M.M.; Cohen, A.D.; Weber, K.L.; Lancaster, E.; Mangan, P.A.; Kulikovskaya, I.; Gupta, M.; Chen, F.; et al. CRISPR-engineered T cells in patients with refractory cancer. Science 2020, 367, 1–64, doi:10.1126/science.aba7365.
48. Wang, J.; DeClercq, J.J.; Hayward, S.B.; Li, P.W.L.; Shivak, D.A.; Gregory, P.D.; Lee, G.; Holmes, M.C. Highly efficient homology-driven genome editing in human T cells by combining zinc-finger nuclease mRNA and AAV6 donor delivery. Nucleic Acids Res. 2016, 44, 1–9, doi:10.1093/nar/gkv1121.
49. Ortinski, P.I.; O'Donovan, B.; Dong, X.; Kantor, B. Integrase-Deficient Lentiviral Vector as an All-in-One Platform for Highly Efficient CRISPR/Cas9-Mediated Gene Editing. Mol. Ther. Methods Clin. Dev. 2017, 5, doi:10.1016/j.omtm.2017.04.002.
50. Hale, M.; Lee, B.; Honaker, Y.; Leung, W.H.; Grier, A.E.; Jacobs, H.M.; Sommer, K.; Sahni, J.; Jackson, S.W.; Scharenberg, A.M.; et al. Homology-Directed Recombination for Enhanced Engineering of Chimeric Antigen Receptor T Cells. Mol. Ther. Methods Clin. Dev. 2017, doi:10.1016/j.omtm.2016.12.008.
51. Eyquem, J.; Mansilla-soto, J.; Giavridis, T.; Stegen, S.J.C. Van Der; Hamieh, M.; Cunanan, K.M.; Odak, A.; Gönen, M.; Sadelain, M. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nat. Publ. Gr. 2017, 543, 113–117, doi:10.1038/nature21405.
52. MacLeod, D.T.; Antony, J.; Martin, A.J.; Moser, R.J.; Hekele, A.; Wetzel, K.J.; Brown, A.E.; Triggiano, M.A.; Hux, J.A.; Pham, C.D.; et al. Integration of a CD19 CAR into the TCR Alpha Chain Locus Streamlines Production of Allogeneic Gene-Edited CAR T Cells. Mol. Ther. 2017, doi:10.1016/j.ymthe.2017.02.005.
53. Louis Jeune, V.; Joergensen, J.A.; Hajjar, R.J.; Weber, T. Pre-existing anti-adeno-associated virus antibodies as a challenge in AAV gene therapy. Hum. Gene Ther. Methods 2013, 24, 59–67, doi:10.1089/hgtb.2012.243.
54. Roth, T.L.; Puig-saus, C.; Yu, R.; Shifrut, E.; Carnevale, J.; Li, J.; Hiatt, J.; Saco, J.; Krystofinski, P.; Li, H.; et al. Repromming human T cell function and specificity with non-viral genome targeting. Nature 2018, doi:10.1038/s41586-018-0326-5.
55. Schober, K.; Müller, T.R.; Busch, D.H. Orthotopic T-Cell Receptor Replacement-An "Enabler" for TCR-Based Therapies. Cells 2020, 9, 1–20, doi:10.3390/cells9061367.
56. Abate-Daga, D.; Hanada, K.I.; Davis, J.L.; Yang, J.C.; Rosenberg, S.A.; Morgan, R.A. Expression profiling of TCR-engineered T cells demonstrates overexpression of multiple inhibitory receptors

in persisting lymphocytes. Blood 2013, 122, 1399–1410, doi:10.1182/blood-2013-04-495531.

57. Datar, I.; Sanmamed, M.F.; Wang, J.; Henick, B.S.; Choi, J.; Badri, T.; Dong, W.; Mani, N.; Toki, M.; Mejías, L.D.; et al. Expression analysis and significance of PD-1, LAG-3, and TIM-3 in human non-small cell lung cancer using spatially resolved and multiparametric single-cell analysis. Clin. Cancer Res. 2019, 25, 4663–4673, doi:10.1158/1078-0432.CCR-18-4142.
58. Li, X.; Wang, R.; Fan, P.; Yao, X.; Qin, L.; Peng, Y.; Ma, M.; Asley, N.; Chang, X.; Feng, Y.; et al. A comprehensive analysis of key immune checkpoint receptors on tumor-infiltrating t cells from multiple types of cancer. Front. Oncol. 2019, 9, doi:10.3389/fonc.2019.01066.
59. Moon, E.K.; Ranganathan, R.; Eruslanov, E.; Kim, S.; Newick, K.; O'Brien, S.; Lo, A.; Liu, X.; Zhao, Y.; Albelda, S.M. Blockade of programmed death 1 augments the ability of human T cells engineered to target NY-ESO-1 to control tumor growth after adoptive transfer. Clin. Cancer Res. 2016, 22, 436–447, doi:10.1158/1078-0432.CCR-15-1070.
60. Cherkassky, L.; Morello, A.; Villena-Vargas, J.; Feng, Y.; Dimitrov, D.S.; Jones, D.R.; Sadelain, M.; Adusumilli, P.S. Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition. J. Clin. Investig. 2016, 126, 3130–3144, doi:10.1172/JCI83092.
61. Rupp, L.J.; Schumann, K.; Roybal, K.T.; Gate, R.E.; Ye, C.J.; Lim, W.A.; Marson, A. CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. Sci. Rep. 2017, 7, 737, doi:10.1038/s41598-017-00462-8.
62. Su, S.; Hu, B.; Shao, J.; Shen, B.; Du, J.; Du, Y.; Zhou, J.; Yu, L. CRISPR-Cas9 mediated efficient PD-1 disruption on human primary T cells from cancer patients. Nat. Publ. Gr. 2016, 1–14, doi:10.1038/srep20070.
63. Menger, L.; Sledzinska, A.; Bergerhoff, K.; Varga, F.A.; Smith, J.; Poirot, L.; Pule, M.; Hererro, J.; Peggs, K.S.; Quezada, S.A. TALEN-Mediated Inactivation of PD-1 in Tumor-Reactive Lymphocytes Promotes Intratumoral T-cell Persistence and Rejection of Established Tumors. Cancer Res. 2016, 76, 2087–2093, doi:10.1158/0008-5472.CAN-15-3352.
64. Choi, B.D.; Yu, X.; Castano, A.P.; Darr, H.; Henderson, D.B.; Bouffard, A.A.; Larson, R.C.; Scarfò, I.; Bailey, S.R.; Gerhard, G.M.; et al. CRISPR-Cas9 disruption of PD-1 enhances activity of universal EGFRvIII CAR T cells in a preclinical model of human glioblastoma. J. Immunother. Cancer 2019, 7, 1–8, doi:10.1186/s40425-019-0806-7.
65. McGowan, E.; Lin, Q.; Ma, G.; Yin, H.; Chen, S.; Lin, Y. PD-1 disrupted CAR-T cells in the treatment of solid tumors: Promises and challenges. Biomed. Pharmacother. 2020, 121, 109625, doi:10.1016/j.biopha.2019.109625.
66. Rapoport, A.P.; Stadtmauer, E.A.; Binder-Scholl, G.K.; Goloubeva, O.; Vogl, D.T.; Lacey, S.F.; Badros, A.Z.; Garfall, A.; Weiss, B.; Finklestein, J.; et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. Nat. Med. 2015, 21, 914–921, doi:10.1038/nm.3910.
67. Ghosh, D.; Venkataramani, P.; Nandi, S.; Bhattacharjee, S. CRISPR–Cas9 a boon or bane : The bumpy road ahead to cancer therapeutics. Cancer Cell Int. 2019, 1–10, doi:10.1186/s12935-019-0726-0.
68. Weber, J.; Öllinger, R.; Friedrich, M.; Ehmer, U.; Barenboim, M.; Steiger, K.; Heid, I. CRISPR/Cas9 somatic multiplex-mutagenesis for high-throughput functional cancer genomics in mice. PNAS 2015, 112, doi:10.1073/pnas.1512392112.
69. Li, J.; Hong, S.; Chen, W.; Zuo, E.; Yang, H. Advances in detecting and reducing off-target effects generated by CRISPR-mediated genome editing. J. Genet. Genom. 2019, 46, 513–521, doi:10.1016/j.jgg.2019.11.002.

70. Naeem, M.; Majeed, S.; Hoque, M.Z.; Ahmad, I. Latest Developed Strategies to Minimize the Off-Target Effects in CRISPR-Cas-Mediated Genome Editing. Cells 2020, 9, 1–23, doi:10.3390/cells9071608.

71. Slaymaker, I.M.; Gao, L.; Zetsche, B.; Scott, D.A.; Yan, W.X.; Zhang, F. Rationally engineered Cas9 nucleases with improved specificity. Science 2016, 351, 84–88, doi:10.1126/science.aad5227.

72. Kleinstiver, B.P.; Pattanayak, V.; Prew, M.S.; Tsai, S.Q.; Nguyen, N.T.; Zheng, Z.; Joung, J.K. High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. Nature 2016, 529, 490–495, doi:10.1038/nature16526.

73. Vakulskas, C.A.; Dever, D.P.; Rettig, G.R.; Turk, R.; Jacobi, A.M.; Collingwood, M.A.; Bode, N.M.; McNeill, M.S.; Yan, S.; Camarena, J.; et al. A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. Nat. Med. 2018, 24, 1216–1224, doi:10.1038/s41591-018-0137-0.

74. Lu, Y.; Xue, J.; Deng, T.; Zhou, X.; Yu, K.; Deng, L.; Huang, M.; Yi, X.; Liang, M.; Wang, Y.; et al. Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. Nat. Med. 2020, 26, 732–740, doi:10.1038/s41591-020-0840-5.

75. Gaudelli, N.M.; Komor, A.C.; Rees, H.A.; Packer, M.S.; Badran, A.H.; Bryson, D.I.; Liu, D.R. Programmable base editing of A-T to G-C in genomic DNA without DNA cleavage. Nature 2017, 551, 464–471, doi:10.1038/nature24644.

76. Webber, B.R.; Lonetree, C. lin; Kluesner, M.G.; Johnson, M.J.; Pomeroy, E.J.; Diers, M.D.; Lahr, W.S.; Draper, G.M.; Slipek, N.J.; Smeester, B.S.; et al. Highly efficient multiplex human T cell engineering without double-strand breaks using Cas9 base editors. Nat. Commun. 2019, 10, doi:10.1038/s41467-019-13007-6.

77. Dwyer, C.J.; Knochelmann, H.M.; Smith, A.S.; Wyatt, M.M.; Rivera, G.O.R.; Arhontoulis, D.C.; Bartee, E.; Li, Z.; Rubinstein, M.P.; Paulos, C.M. Fueling Cancer Immunotherapy With Common Gamma Chain Cytokines. Front. Immmunology 2019, 10, 1–18, doi:10.3389/fimmu.2019.00263.

78. White, D.E.; Rosenberg, S.A. Trends in the Safety of High Dose Bolus Interleukin-2 Administration in Patients with Metastatic Cancer. Cancer 1998, 2, 797–805.

79. Tang, A.; Harding, F. The challenges and molecular approaches surrounding interleukin-2-based therapeutics in cancer. Cytokine X 2019, 1, 100001, doi:10.1016/j.cytox.2018.100001.

80. Liu, K.; Rosenberg, S.A. Transduction of an IL-2 Gene into Human Melanoma-Reactive Lymphocytes Results in Their Continued Growth in the Absence of Exogenous IL-2 and Maintenance of Specific Antitumor Activity. J. Immunol. 2001, doi:10.4049/jimmunol.167.11.6356.

81. Heemskerk, B.; Liu, K.; Dudley, M.E.; Johnson, L.A.; Kaiser, A.; Downey, S.; Zheng, Z.; Shelton, T.E.; Matsuda, K.; Robbins, P.F.; et al. Adoptive cell therapy for patients with melanoma, using tumor-infiltrating lymphocytes genetically engineered to secrete interleukin-2. Hum. Gene Ther. 2008, 19, 496–510, doi:10.1089/hum.2007.0171.

82. 82. Kunert, A.; Chmielewski, M.; Wijers, R.; Berrevoets, C.; Abken, H.; Debets, R. Intra-tumoral production of IL18, but not IL12, by TCR-engineered T cells is non-toxic and counteracts immune evasion of solid tumors. Oncoimmunology 2018, 7, 1–12, doi:10.1080/2162402X.2017.1378842.

83. Alsaieedi, A.; Holler, A.; Velica, P.; Bendle, G.; Stauss, H.J. Safety and efficacy of Tet-regulated IL-12 expression in cancer-specific T cells. Oncoimmunology 2019, 8, 1–11, doi:10.1080/2162402X.2018.1542917.

84. Zhang, L.; Davies, J.S.; Serna, C.; Yu, Z.; Restifo, N.P.; Rosenberg, S.A.; Morgan, R.A.; Hinrichs, C.S. Enhanced efficacy and limited systemic cytokine exposure with membrane- anchored

interleukin-12 T—Cell therapy in murine tumor models. J. Immunother. Cancer 2019, 1–12, doi:10.1136/jitc-2019-000210.

85. Zhang, L.; Morgan, R.A.; Beane, J.D.; Zheng, Z.; Dudley, M.E.; Kassim, S.H.; Nahvi, A.V.; Ngo, L.T.; Sherry, R.M.; Phan, G.Q.; et al. Tumor-infiltrating lymphocytes genetically engineered with an inducible gene encoding interleukin-12 for the immunotherapy of metastatic melanoma. Clin. Cancer Res. 2015, 21, 2278–2288, doi:10.1158/1078-0432.CCR-14-2085.

86. Koneru, M.; O'Cearbhaill, R.; Pendharkar, S.; Spriggs, D.R.; Brentjens, R.J. A phase I clinical trial of adoptive T cell therapy using IL-12 secreting MUC-16ecto directed chimeric antigen receptors for recurrent ovarian cancer. J. Transl. Med. 2015, 13, 1–11, doi:10.1186/s12967-015-0460-x.

87. Gonzales, C.; Yoshihara, H.A.I.; Dilek, N.; Leignadier, J.; Irving, M.; Mieville, P.; Helm, L.; Michielin, O.; Schwitter, J. In-vivo detection and tracking of T cells in various organs in a melanoma tumor model by 19F-fluorine MRS/MRI. PLoS ONE 2016, 11, 1–18, doi:10.1371/journal.pone.0164557.

88. Boschi, F.; De Sanctis, F.; Ugel, S.; Spinelli, A.E. T-cell tracking using Cerenkov and radioluminescence imaging. J. Biophotonics 2018, 11, 1–11, doi:10.1002/jbio.201800093.

89. Nagarsheth, N.; Wicha, M.S.; Zou, W. Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy. Nat. Publ. Gr. 2017, 17, 559–572, doi:10.1038/nri.2017.49.

90. Bronger, H.; Singer, J.; Windmu, C.; Reuning, U.; Zech, D.; Delbridge, C. CXCL9 and CXCL10 predict survival and are regulated by cyclooxygenase inhibition in advanced serous ovarian cancer. Br. J. Cancer 2016, 553–563, doi:10.1038/bjc.2016.172.

91. Curiel, T.J.; Coukos, G.; Zou, L.; Alvarez, X.; Cheng, P.; Mottram, P.; Evdemon-Hogan, M.; Conejo-Garcia, J.R.; Zhang, L.; Burow, M.; et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat. Med. 2004, 10, 942–949, doi:10.1038/nm1093.

92. Kershaw, M.H.; Wang, G.; Westwood, J.A.; Pachynski, R.K.; Tiffany, H.L.; Marincola, F.M.; Wang, E.; Young, H.A.; Murphy, P.M.; Hwu, P. Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. Hum. Gene Ther. 2002, 13, 1971–1980, doi:10.1089/10430340260355374.

93. Idorn, M.; Skadborg, S.K.; Kellermann, L.; Halldórsdóttir, H.R.; Holmen Olofsson, G.; Met, Ö.; Thor Straten, P. Chemokine receptor engineering of T cells with CXCR2 improves homing towards subcutaneous human melanomas in xenograft mouse model. Oncoimmunology 2018, 7, doi:10.1080/2162402X.2018.1450715.

94. Jin, L.; Tao, H.; Karachi, A.; Long, Y.; Hou, A.Y.; Na, M.; Dyson, K.A.; Grippin, A.J.; Deleyrolle, L.P.; Zhang, W.; et al. CXCR1- or CXCR2-modified CAR T cells co-opt IL- 8 for maximal antitumor efficacy in solid tumors. Nat. Commun. 2019, 1–13, doi:10.1038/s41467-019-11869-4.

95. Bremnes, R.M.; Dønnem, T.; Al-Saad, S.; Al-Shibli, K.; Andersen, S.; Sirera, R.; Camps, C.; Marinez, I.; Busund, L.T. The role of tumor stroma in cancer progression and prognosis: Emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer. J. Thorac. Oncol. 2011, 6, 209–217, doi:10.1097/JTO.0b013e3181f8a1bd.

96. Liu, R.; Li, H.; Liu, L.; Yu, J.; Ren, X. Fibroblast activation protein: A potential therapeutic target in cancer. Cancer Biol. Ther. 2012, 4047, doi:10.4161/cbt.13.3.18696.

97. Wang, L.S.; Lo, A.; Scholler, J.; Sun, J.; Majumdar, R.S.; Kapoor, V.; Antzis, M.; Cotner, C.E.; Johnson, L.A.; Durham, A.C.; et al. Targeting Fibroblast Activation Protein in Tumor Stroma with Chimeric Antigen Receptor T Cells Can Inhibit Tumor Growth and Augment Host Immunity without Severe Toxicity. Cancer Immunol. Res. 2014, 2, 154–167, doi:10.1158/2326-6066.CIR-13-0027.

98. Mpekris, F.; Voutouri, C.; Baish, J.W.; Duda, D.G.; Munn, L.L. Combining microenvironment normalization strategies to improve cancer immunotherapy. PNAS 2020, 117, doi:10.1073/pnas.1919764117.

99. Lanitis, E.; Irving, M.; Coukos, G. Targeting the tumor vasculature to enhance T cell activity. Curr. Opin. Immunol. 2015, 33, 55–63, doi:10.1016/j.coi.2015.01.011.

100. Chinnasamy, D.; Yu, Z.; Theoret, M.R.; Zhao, Y.; Shrimali, R., R.K.; Morgan, R.A.; Feldman, S.A.; Restifo, N.P.; Rosenberg, S.A. Gene therapy using genetically modified lymphocytes targeting VEGFR-2 inhibits the growth of vascularized syngenic tumors in mice. J. Clin. Investig. 2010, 120, 3953–3968, doi:10.1172/JCI43490.

101. Chinnasamy, D.; Tran, E.; Yu, Z.; Morgan, R.A.; Restifo, N.P.; Rosenberg, S.A. Simultaneous targeting of tumor antigens and the tumor vasculature using t lymphocyte transfer synergize to induce regression of established tumors in mice. Cancer Res. 2013, 73, 3371–3380, doi:10.1158/0008-5472.CAN-12-3913.

102. Casucci, M.; Di Robilant, B.N.; Falcone, L.; Camisa, B.; Norelli, M.; Genovese, P.; Gentner, B.; Gullotta, F.; Ponzoni, M.; Bernardi, M.; et al. CD44v6-targeted T cells mediate potent antitumor effects against acute myeloid leukemia and multiple myeloma. Blood 2013, 122, 3461–3472, doi:10.1182/blood-2013-04-493361.

103. Porcellini, S.; Asperti, C.; Corna, S.; Cicoria, E.; Valtolina, V.; Stornaiuolo, A.; Valentinis, B.; Bordignon, C.; Traversari, C. CAR T Cells Redirected to CD44v6 Control Tumor Growth in Lung and Ovary Adenocarcinoma Bearing Mice. Front. Immunol. 2020, 11, 1–14, doi:10.3389/fimmu.2020.00099.

104. Murty, S.; Labanieh, L.; Murty, T.; Gowrishankar, G.; Haywood, T.; Alam, I.S.; Beinat, C.; Robinson, E.; Aalipour, A.; Klysz, D.D.; et al. PET reporter gene imaging and ganciclovir-mediated ablation of chimeric antigen receptor T-cells in solid tumors. Cancer Res. 2020, canres.3579.2019, doi:10.1158/0008-5472.can-19-3579.

105. Tiberghien, P.; Ferrand, C.; Lioure, B.; Milpied, N.; Angonin, R.; Deconinck, E.; Certoux, J.M.; Robinet, E.; Saas, P.; Petracca, B.; et al. Administration of herpes simplex-thymidine kinase-expressing donor T cells with a T-cell-depleted allogeneic marrow graft. Blood 2001, 97, 63–72, doi:10.1182/blood.V97.1.63.

106. Straathof, K.C.; Pulè, M.A.; Yotnda, P.; Dotti, G.; Vanin, E.F.; Brenner, M.K.; Heslop, H.E.; Spencer, D.M.; Rooney, C.M. An inducible caspase 9 safety switch for T-cell therapy. Blood 2005, 105, 4247–4254, doi:10.1182/blood-2004-11-4564.

107. Di Stasi, A.; Tey, S.K.; Dotti, G.; Fujita, Y.; Kennedy-Nasser, A.; Martinez, C.; Straathof, K.; Liu, E.; Durett, A.G.; Grilley, B.; et al. Inducible apoptosis as a safety switch for adoptive cell therapy. N. Engl. J. Med. 2011, 365, 1673–1683, doi:10.1056/NEJMoa1106152.

108. Stavrou, M.; Philip, B.; Traynor-White, C.; Davis, C.G.; Onuoha, S.; Cordoba, S.; Thomas, S.; Pule, M. A Rapamycin-Activated Caspase 9-Based Suicide Gene. Mol. Ther. 2018, 26, 1266–1276, doi:10.1016/j.ymthe.2018.03.001.

# 9

# GENERAL DISCUSSION AND FUTURE PERSPECTIVE

*PERSONAL NOTE*

My entire family has studied humanistic topics: law, philosophy, languages, politics and so on. I am the youngest member of the family and also the only one that decided to explore the scientific path. Every time we gather together (the few times that I am able to go to the deepest south of Italy, Calabria); they always ask what the progresses in my research are. When I start to talk about gene-engineering, gene-editing, cut and paste of the DNA, isolation of immune cells; they constantly look at me with doubtful and confused eyes. At that moment, there is only one sentence that allows me to engage them in the discussion "We are trying to improve survival of pediatric cancer patients with new and more specific treatments". That's it, my family is looking at me and ask for more details! You might ask yourself: why? The reason is that (almost) everyone has experienced, directly or indirectly, the consequences of cancer treatment, the heavy side-effects, the hope that the treatment will work even though it is not specific.

Those discussions often remind me of the actual aim of this thesis, and that you don't always need to use difficult words to explain it. After all, what this project was all about is exactly what I say to my family: "Improve survival of pediatric cancer patients". How? I guess you know my answer at this point: ARCOBALENO - Advancing Research on COrd Blood ALlogeneic, ENgineered and Off-the-shelf cell therapy products.

In this thesis we showed several ways to use cord blood as a source of immune cells for the treatment of pediatric cancer patients, but many questions remain unanswered: how far are those findings from being used in medical practice? What do we still need and what should we consider to finally succeed in improving pediatric patients' lives? How do we make those therapies accessible for most of the patients?

The answer to these questions, the main findings of this thesis and the perspective in the field are discussed in this last chapter.

## THE ALLOGENEIC REVOLUTION: FROM BIOLOGY TO MANUFACTURE

The use of autologous immune cells for the generation of cellular therapies can be challenging in certain situations, especially in heavily pre-treated patients or in disease types that affect the number and fitness of immune cells. A range of 7 to 13% of patients fail to obtain the treatment due to low lymphocyte count and related product failure issues [1]. Therefore, taking in consideration the use of an allogeneic source can result in a life-saving solution. As summarized in **Chapter 2** the availability of CB units, the enriched presence of CD34+ hematopoietic stem cells, the expansion and anti-tumor capacity of CB-T cells are general advantages when opting for an allogeneic source. However, the amount of registered clinical trials using CB derived allogeneic cells for the generation of advanced cell therapy products (ACTPs), remained low over the past 5 years. At the moment, there are only four registered clinical trials using CB derived cells, and mostly focusing on CAR or CAR-NK cells (NCT03881774, NCT0509245, NCT05110742, NCT04796675). It is relevant to mention that the use of an allogeneic source is not limited to CB. In the last years, increasing attention is directed at the use of peripheral blood (PB) cells from healthy donors. Similarly to the allogeneic HCT setting, and depending on the type of selected cell therapy products, HLA matching remains an important consideration for the safety and efficacy of the treatment. However, differently from the HCT, the possibility to gene-edit cell therapy products to become invisible to the recipient immune system (universal cells) has put grounds for the future of off-the-shelf cell therapy products.

Contrary to CB, there are more than 25 registered clinical trials based on the use of allogeneic PB immune cells from healthy donors. However, even if PB seems to be the favorite choice at the moment, some considerations might still favor the use of CB. For instance, in terms of cell composition, immune cells derived from an healthy donor present a complex immune phenotype, determined by the immune life-related experiences that characterize different individuals; while CB, due to its immunological inexperience, is characterized by a naïve and homogenous cell composition[2]. As shown in **Chapter 3** the immune-phenotype of cells in these sources shows the presence of a totally naïve population of T cells in CB; while memory, effector and naïve subpopulations are present in the PB. In addition, CB T cells show very low expression of checkpoint receptors, an important feature to avoid lack of efficiency in vivo. Cell therapy products derived from naïve T cells can be, with the help of specific cytokines (e.g. IL7, IL15), pushed towards a T stem cell memory phenotype (Tscm), a phenotype that has shown persistency and efficacy in in vivo models of hematological tumors[3,4].

When using healthy donor PB immune cells, it is possible to select an optimal subpopulation, such as naïve T cells, but this choice will decrease the amount of available starting material and requires several enrichment steps to achieve an adequate therapeutic cell number. The use of CB derived T cells is optimal for this purpose, since its homogenous naïve phenotype, does not require any additional enrichment steps for obtaining high numbers of cells. The data presented in the manuscript shows that CB CD8+ T cells are transduced efficiently by LV vectors, achieving even superior transduction efficiency when comparing to PB CD8+ T cells. As a proof of principle for therapeutic application, CB CD8+ T cells transduced with a relevant TCR recognizing an AML related peptide, specifically WT1, proved to be activated and able to kill cells presenting the target peptide.

Allogeneic sources do not only have biological advantages but also a favorable impact on the manufacture process of ACTPs. Time and resources necessary for the generation of ACTPs are limiting factors for the broad application of those therapies. At the moment, the manufacturing process is strictly dependent on the patient-derived starting material and the production cycle is conducted for a single patient at a time, increasing the costs and decreasing the scalability of the procedure. The generation of individualized cell products requires two different clinical procedures, isolation and re-infusion, divided by several weeks in between due to complex logistics involved for the production. In addition, every production is intrinsically subjected to variability and Quality Control (QC) tests need to be performed for multiple steps of the production line[5]. The use of an allogeneic source, such as CB, can significantly improve the manufacturing process. In fact, the large availability of starting material, independent from a single patient, allows the production of multiple batches of ACTPs with standardize procedures and generalized quality control tests in one single production line.

The production of allogeneic derived ACTPs aligns more to a regular biopharmaceutical work line and it can, therefore, decrease the time between the eligibility for treatment and the treatment itself, as well as the production costs. With allogeneic derived products the focus shifts from a fully personalized production to a fully personalized treatment with a generalized production. The possibility to create an ACTPs biobank, ranging from HLA-matched to universal cell products, that is qualitatively controlled and assured, means that personalized requests can be readily accommodated.

In **Chapter 4** we show an example of an optimized and validated GMP production performed in our academic center for the generation of a CB derived DC vaccine. Importantly the manuscript shows the possibility of expanding low amounts of CD34+ cells in a closed bag system to sufficient DCs for at least three rounds of vaccinations in the patient. The final product was characterized by high expression of maturation markers (CD80 and CD83) and was able to prime naïve T cells, essential pre-requisite for this cell therapy approach. The proposed settings are specific for the possibility of vaccination after HCT, however, the procedure can easily be applied to off-the-shelf production. The possibility to generate a biobank of pre-made products was also supported by the results that show the maintenance of a viable, phenotypically unaltered and functional product after 2 years from the freezing procedure.

As the first part of the thesis focuses on the use of CB derived T cells and DCs, it is relevant to mention that there is growing interest to use different types of CB derived immune cells, such as natural killer (NK) cells. CB contains a higher percentage of NK cells when compared to the same fraction in PB, and they are characterized by a homologous immature phenotype [6]. The use of cytokines and feeder cells is essential to achieve maturation and proliferation of CB-NK cells and to stimulate susceptibility towards viral transduction to express a specific CAR[7]. Pre-clinical results have shown the efficacy and feasibility of targeting lymphoid tumors with this strategy, broadening the possibilities to use CB derived immune cells for therapeutic applications.

## GENE-EDITING TECHNIQUES AS A TOOL TO IMPROVE THE NEW GENERATION OF ADVANCED CELL THERAPY PRODUCTS

The recent discoveries and accessibility of tools to gene-edit mammalian cells, in a short time and with relatively low costs, ha definitely increased the expectation to find and optimize the best cure for everyone. The possibility to generate all the CRISPR components in GMP grade has shortened the time from the discovery of this technique to its clinical application[8]. Nowadays, CRISPR is involved in 37 clinical trials, ranging from hemoglobinopathies to cancer therapy; and even touching upon treatment for COVID-19 patients (clinicaltrial.gov on 1/12/2021). The use of gene editing techniques for ACTPs is mainly focusing on increasing the safety and the efficacy of the final products[9].

## INCREASING SAFETY

When generating T cell therapies from an allogeneic source, it is essential to consider the presence of an endogenous TCR (eTCR), which could possibly trigger a GvHD-like reaction, due to mismatched recognition of HLA. For some application, such as CAR-T cells, matching the HLA-type of donor and recipient might be sufficient to decrease the risks; but in the case TCR-engineered T cells, it is not sufficient. In fact, the presence of the eTCR and a recombinant TCR (rTCR) can generate TCRs mispairing, for which the downstream conseqeunces are difficult to predict[10].

Therefore, eliminating the expression of the endogenous TCR, with the use of CRISPR, can decrease the chance to develop GvHD symptoms. The first FDA approved clinical trial using this approach demonstrated how TCR engineered T cells, gene-edited to eliminate the expression of the eTCR, were safe and feasible to produce for clinical application[11].

Even if, as mentioned before, the CB derived T cells are known to be less immunogenic and cause less GvHD reactions; the safest choice remains to completely abrogate the possibility of side effects. Therefore, in **Chapter 5** we show an efficient method to achieve, in CB derived TCR engineered T cells, the complete elimination of eTCR expression; increasing the safety and efficiency of the final product. In line with recent discovery on the importance to eliminate the expression of both alpha and beta chain of the eTCR[12,13], the manuscript shows an optimized method to use the CRICPR-Cas9 platform for multiplex gene-editing of CB derived CD8+ T cells. Importantly, the procedure did not affect the proliferation capacity nor the phenotype of the cells; while it increased their cytotoxic capacity when in culture with target tumor cell lines.

To best resemble the pediatric tumor microenvironment, eTCR$^{-/-}$-WT1-TCR T cells were tested for safety and efficacy in a 3D model which allows primary, pediatric patients derived, AML blasts to survive and proliferate in vitro. The proposed ACTP showed active killing of target tumor cells and T cell proliferation upon target recognition, contrary to untransduced T cells. Related to the safety, the final product did not show any toxicity against stromal cells present in the 3D model, supporting the idea that eliminating the eTCR leads to an increased safety profile. The use of this in vitro 3D model gave us the opportunity to have a clinically relevant and quick evaluation of efficiency and safety of the ACTP before performing in vivo studies. Most of the pre-clinical mouse models used in the field of AML, to test the efficacy of T cell therapies targeting WT1, are based on the injection of cell lines in NOD/LtSz-scid with IL2ɣc null (NSG) mice[14,15].

However, the clinically relevance of those models has for long been debated. Patient derived xenograft (PDX) models have been used to improve the relevance of AML animal models, especially consequently to the generation of several new mouse models that increase the engraftment of AML primary blasts [16,17]. However, the long time necessary to generate the PDX models (12-16 weeks for engraftment) and the variability in engraftment success, due to inter-patients' differences, are still limiting factors especially when considering screening methods for ACTPs. Certainly, a living organism gives a lot more information regarding the behavior and safety of allogeneic T cell products outside the in vitro conditions; however performing initial efficacy screening on innovative 3D in vitro models can minimize the use of animal models for the final steps of the process.

Combining the elimination of eTCR with expression of a rTCR has shown clear advantages for clinical applications; however, this approach has its own limitations. In primis the cells are modified in two separated procedures, potentially affecting more the final product compared to a one-step approach[18]. Moreover, the semi-random integration of viral vectors, used to express relevant receptors, has a heterogeneous number of integrations and can have insertional mutagenesis consequences; especially when using ɣ-retroviral vectors[19].

One strategy to overcome those problems is to integrate the donor sequence during the CRISPR mediated editing, taking advantage of the HDR mechanism that cells initiate after a DSB. In **Chapter 6** we demonstrated how this can be achieved using IDLV, a form of LV vector modified to eliminate its capacity to independently integrate into the genome of target cells. However, as in other cases [12] the number of cells that are successfully edited (around 5% of the total cells) are still too low to think of clinical applications. In the last couple of years, however, the use of AAV6 reinvigorated the potential of this approach, obtaining from 50 to 70% of cells that integrated the CD19-CAR sequence in the TRAC locus, eliminating the expression of the eTCR [18,20,21]. Even more recently new approaches, completely abrogating the use of viral delivery systems, draw attention in the field. Roth *et al.* showed a successful 12% of precisely gene-edited T cells by simply adding, during the electroporation of the RNP complex, a dsDNA molecule expressing a rTCR and designed to target the TRAC locus [22]. The same group showed how the percentage of edited T cells can further be increased to 50% using polymer-stabilized Cas9 nanoparticles and modified repair templates containing the CRISPR-Cas9 targeted sequence[23], suggesting that precise modifications, in CRISPR's platform components, can increase the number of edited cells to be clinically relevant in the near future.

Overall, the most important question left to address regards the safety of introducing gene-editing approaches in the clinical practice. Due to the novelty of the technique, it is of extreme importance to standardize and set universally accepted safety requirements to protect the patients from having long-term undesired effects. Most common concerns, considering the fast move towards clinical application, remain the lack of extensive knowledge regarding the consequences of creating DSB in the genome of cells. Many studies have highlighted the peril of those approaches due to the on-target and off-target effects [24–27]; and required caution before running into the same mistakes committed with the first human gene therapy studies. Continuous research is involved in further understanding and limiting the side effects of this technique and only the future will determine how quick the translation to the clinic will be.

Moreover, new uses of the CRISPR platform are proving their effectiveness without the risks of creating a DSB, such as the use of the base editor technique. However, at the moment genomic editing of the TRAC locus using advanced base-editor techniques resulted in only moderately reduced trafficking of the T-cell receptor to the cell surface, which is not optimal yet[28]. Surely, the use of gene-editing tools *ex vivo*, in somatic cells, has lower safety concerns compared to the use of these techniques *in vivo* or *ex vivo* in germinal cells; but it is important to not underestimate the risks. As of today, the approved clinical trials using gene-edited T cells have shown a safe profile and no heavy side-effects have been reported in treated patients[11,29]. When referring to gene-editing techniques we mainly think of the CRISPR platform, but it is relevant to mention that it is not only limited to that. Innovative and alternative approaches, such as the Sleeping Beauty transposon system, are being considered for the modification of T cells. CD19 CAR-T cells generated with this system have been tested recently in a clinical trial showing the feasibility, safety, and anti-myeloma efficacy of this product[30].



### Increasing efficacy

Apart from increasing safety, the use of gene-editing techniques can be an essential tool to also increase the efficacy in difficult-to-treat tumors. Intrinsic mechanisms such lack of persistency, high exhaustion levels and low proliferation rates are often at the base of a scarce success rate of cell therapies applications, especially for the treatment of solid tumors [31].

**Chapter 7** shows an efficient method, using the CRISPR-Cas9 platform, to eliminate "breaks" interfering with the efficiency of the proposed cell therapies in this thesis, CB derived CD8+ T cells and DCs vaccines. The manuscript focuses mainly on the elimination of PD-1 on T cells and PD-L1 on DCs; however, many other targets can be considered for future studies. Gene-deletion of inhibitory receptors is especially useful to avoid combinatorial treatment with monoclonal antibodies, that have a much more broad and systemic effect.

A current challenge is to understand which molecules will be beneficial to eliminate, and which tumor's specific characteristics will have to be taken in consideration. It is also important to mention that the upregulation of checkpoint receptors correlates with cell activation, and usually only chronic and multiple expression is a sign of exhaustion. Eliminating at the genomic level those receptors might interfere with activation and, therefore, has a detrimental effect on proliferation and persistency of the therapeutic product in the patients [32]. At the moment, there are two clinical trials that have published their reports on the use of PD-1 KO T cells for the treatment of patients affected by melanoma, synovial sarcoma, multiple myeloma and non-small-cell lung cancer, proving the safety and feasibility of the approach; however the data are still inconclusive in terms of therapeutic advantages[11,29].

Additional molecules, unrelated to the checkpoint receptors family, can be targeted with gene-editing tools, especially when traditional pharmaceutical target is difficult to achieve. An example is proposed by Tang *et al*. that efficiently eliminated the expression of TGFβ receptor from CAR-T cells, increasing the long term efficacy of the final product in preclinical models[33]. Genetic elimination of inhibitory molecules, and other recent strategies, to improve TCR engineered T cells for the treatment of solid tumors, are summarized in **Chapter 8**. Some of the mentioned strategies have been initially proposed for the treatment of hematological tumors, such as the KO of the eTCR, while others are more specific for solid tumors, such as KO of checkpoint receptors and disruption of the surrounding stroma. Moreover, recently, it is becoming clear that CD4+ T cells play a bigger role than previously thought, especially for immunologically-cold and aggressive solid tumors. CD4+ T cells proved to have a critical supportive role in enhancing the anti-tumor effects of CD8+ T cells, via the production of cytokines and chemokines, but also the ability to acquire independent cytotoxic capacity, fully contributing to the elimination of tumor cells[34]. The advantages of using TCR engineered T cells for the treatment of solid tumors will need to be further evaluated, but the number of active clinical trials gives hope for the future.

## ALIGNING RESEARCH DEVELOPMENT WITH CLINICAL AND SOCIAL IMPACT

We are living throughout a revolutionary moment in medicine, which means that some concepts that were valuable for approving new treatments in the clinic are becoming obsolete. The standard process of drug approval is based on the discovery and development of small molecules. Potential candidates with optimal preliminary results in pre-clinical models can be tested on a large number of patients to determine kinetics, efficacy and safety of the proposed treatment regimen. However, the introduction of living cells as therapeutic agents has exposed the incompleteness or inadequacy of the approval system. Especially when considering treatment strategies based on autologous or allogeneic ex-vivo gene-modified cells it is impossible to obtain information on parameters considered essential for molecular compounds, for example bio-distribution and clearance.

Around 70% of all FDA drugs approved in the last five years are small molecules, a percentage that corroborates the reality of the problem. Therefore, it is essential that an agile and fast adaptation of the system will start as soon as possible. Moving toward a personalized form of medicine outruns the possibility to perform old-style clinical trials, therefore new ways to define safety and efficacy need to be proposed. Another limiting factor for the broad distribution of ACTPs is related to the elevated cost of the therapy. To give an example, the generation of an autologous CAR-T cell product range between 373.000$ and 475.000$; while it appeared that for being cost effective the price should set around 150.000$ [35]. The use of allogeneic sources, such as CB, can decrease the manufacturing costs and increase the accessibility. In the future, the possibility to generate ready to use, off-the-shelf products might benefit also the delivery of advanced therapies to developing countries, usually excluded due to the difficulty and the costs of production.

In conclusion it is clear that the field of cancer therapy is facing a big challenge: finding the balance between an extremely personalized medicine (n=1) and a “one-size fits all” (n=all the patients) type of approach. In both cases the future will depend on how well we are able to look outside the box of known targets and immunological standard pathways. For instance, taking advantage of bioinformatic and machine learning is essential to develop superior predictive tools to fully personalize new therapies and characterize every patient. On the other hand, recent findings have shed a light on the possibility of going back to generalized, but precise, treatment. Two elegant studies have recently proposed new molecules as a possible immunotherapy target, cluster differentiation 1c (CD1c)[36] or MHC class

I-related molecule 1 (MR1)[37]; both classified as antigen presenting molecules with limited or absent polymorphism and highly expressed in cancer cells.

In addition, the use of conventional T cells expressing a recombinant γδ TCR (TEGs) have further broadened the spectrum of cell therapy products, with encouraging pre-clinical results[38] that led to use of this technology in two clinical trials (NTR6541; NCT04688853). TEGs cells recognize metabolic changes and increased expression of phosphoantigens, without targeting a specific tumor associated antigen. The generation of rTCR targeting those type of molecules eliminates the need to generate HLA-matching TCR engineered T cells; valorizing the chance to produce off-the-shelf, broadly applicable, ACTPs.

In the last decade advances in cancer treatment have been tremendous, and it is difficult to imagine how many more discoveries will lead to better treatments, especially in the field of immunotherapy. What remains clear, at least to me, is that the future of cancer patients is starting to be a bit brighter than before, a bit more colorful.

## BIBLIOGRAPHY


1. Graham, C.; Jozwik, A.; Pepper, A.; Benjamin, R. Allogeneic CAR-T Cells : More than Ease of Access ? Cells 2018, 1–11, doi:10.3390/cells7100155.
2. Yun, H.D.; Varma, A.; Hussain, M.J.; Nathan, S.; Brunstein, C. Clinical relevance of immunobiology in umbilical cord blood transplantation. J. Clin. Med. 2019, 8, 1–16, doi:10.3390/jcm8111968.
3. Sabatino, M.; Hu, J.; Sommariva, M.; Gautam, S.; Fellowes, V.; Hocker, J.D.; Dougherty, S.; Qin, H.; Klebanoff, C.A.; Fry, T.J.; et al. Generation of clinical-grade CD19-specific CAR-modified CD81 memory stem cells for the treatment of human B-cell malignancies. Blood 2016, 128, 519–528, doi:10.1182/blood-2015-11-683847.
4. Cieri, N.; Camisa, B.; Cocchiarella, F.; FOrcato, M.; Oliviera, G.; Provasi, E.; Bondanza, A.; Bordignon, C.; Peccatori, J.; Ciceri, F.; et al. IL-7 and IL-15 instruct the generation of human memory stemTcells from naive precursors. Blood 2013, 121, 573–585, doi:10.1182/blood-2012-05-431718.There.
5. Kaiser, a D.; Assenmacher, M.; Schröder, B.; Meyer, M.; Orentas, R.; Bethke, U.; Dropulic, B. Towards a commercial process for the manufacture of genetically modified T cells for therapy. Cancer Gene Ther. 2015, 22, 72–78, doi:10.1038/cgt.2014.78.
6. Luevano, M.; Daryouzeh, M.; Alnabhan, R.; Querol, S.; Khakoo, S.; Madrigal, A.; Saudemont, A. The unique profile of cord blood natural killer cells balances incomplete maturation and effective killing function upon activation. Hum. Immunol. 2012, 73, 248–257, doi:10.1016/j.humimm.2011.12.015.
7. Liu, E.; Ang, S.O.T.; Kerbauy, L.; Basar, R.; Kaur, I.; Kaplan, M.; Li, L.; Tong, Y.; Daher, M.; Ensley, E.L.; et al. GMP-Compliant Universal Antigen Presenting Cells (uAPC) Promote the Metabolic Fitness and Antitumor Activity of Armored Cord Blood CAR-NK Cells. Front. Immunol. 2021, 12, 1–14, doi:10.3389/fimmu.2021.626098.
8. Uddin, F.; Rudin, C.M.; Sen, T. CRISPR Gene Therapy: Applications, Limitations, and Implications for the Future. Front. Oncol. 2020, 10, doi:10.3389/fonc.2020.01387.
9. Ghaffari, S.; Khalili, N.; Rezaei, N. CRISPR/Cas9 revitalizes adoptive T-cell therapy for cancer immunotherapy. J. Exp. Clin. Cancer Res. 2021, 40, 1–18, doi:10.1186/s13046-021-02076-5.
10. Shao, H.; Zhang, W.; Hu, Q.; Wu, F.; Shen, H.; Huang, S. TCR mispairing in genetically modified T cells was detected by fluorescence resonance energy transfer. Mol. Biol. Rep. 2010, 37, 3951–3956, doi:10.1007/s11033-010-0053-y.
11. Stadtmauer, E.A.; Fraietta, J.A.; Davis, M.M.; Cohen, A.D.; Weber, K.L.; Lancaster, E.; Mangan, P.A.; Kulikovskaya, I.; Gupta, M.; Chen, F.; et al. CRISPR-engineered T cells in patients with refractory cancer. Science (80-. ). 2020, 367, 1–64, doi:10.1126/science.aba7365.
12. Schober, K.; Müller, T.R.; Gökmen, F.; Grassmann, S.; Effenberger, M.; Poltorak, M.; Stemberger, C.; Schumann, K.; Roth, T.L.; Marson, A.; et al. Orthotopic replacement of T-cell receptor α- and β-chains with preservation of near-physiological T-cell function. Nat. Biomed. Eng. 2019, doi:10.1038/s41551-019-0409-0.
13. Morton, L.T.; Reijmers, R.M.; Wouters, A.K.; Kweekel, C.; Remst, D.F.G.; Pothast, C.R.; Falkenburg, J.H.F.; Heemskerk, M.H.M. Simultaneous Deletion of Endogenous TCRαβ for TCR Gene Therapy Creates an Improved and Safe Cellular Therapeutic. Mol. Ther. 2020, 28, 64–74, doi:10.1016/j.ymthe.2019.10.001.

14. Xue, S.A.; Gao, L.; Hart, D.; Gillmore, R.; Qasim, W.; Thrasher, A.; Apperley, J.; Engels, B.; Uckert, W.; Morris, E.; et al. Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. Blood 2005, doi:10.1182/blood-2005-01-0146.

15. Stauss, H.J.; Thomas, S.; Cesco-Gaspere, M.; Hart, D.P.; Xue, S.A.; Holler, A.; King, J.; Wright, G.; Perro, M.; Pospori, C.; et al. WT1-specific T cell receptor gene therapy: Improving TCR function in transduced T cells. Blood Cells, Mol. Dis. 2008, doi:10.1016/j.bcmd.2007.06.018.

16. Gopalakrishnapillai, A.; Kolb, E.A.; Dhanan, P.; Bojja, A.S.; Mason, R.W.; Corao, D.; Barwe, S.P. Generation of pediatric leukemia xenograft models in NSG-B2m mice: Comparison with NOD/SCID mice. Front. Oncol. 2016, 6, 1–11, doi:10.3389/fonc.2016.00162.

17. Wunderlich, M.; Chou, F.-S.; Link, K.A.; Mizukawa, B.; Perry, R.L.; Carroll, M.; Mulloy, J.C. AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. Leukemia 2010, 24, 1785–1788, doi:10.1038/leu.2010.158.

18. Eyquem, J.; Mansilla-soto, J.; Giavridis, T.; Stegen, S.J.C. Van Der; Hamieh, M.; Cunanan, K.M.; Odak, A.; Gönen, M.; Sadelain, M. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nat. Publ. Gr. 2017, 543, 113–117, doi:10.1038/nature21405.

19. Modlich, U.; Navarro, S.; Zychlinski, D.; Maetzig, T.; Knoess, S.; Brugman, M.H.; Schambach, A.; Charrier, S.; Galy, A.; Thrasher, A.J.; et al. Insertional transformation of hematopoietic cells by self-inactivating lentiviral and gammaretroviral vectors. Mol. Ther. 2009, 17, 1919–1928, doi:10.1038/mt.2009.179.

20. MacLeod, D.T.; Antony, J.; Martin, A.J.; Moser, R.J.; Hekele, A.; Wetzel, K.J.; Brown, A.E.; Triggiano, M.A.; Hux, J.A.; Pham, C.D.; et al. Integration of a CD19 CAR into the TCR Alpha Chain Locus Streamlines Production of Allogeneic Gene-Edited CAR T Cells. Mol. Ther. 2017, doi:10.1016/j.ymthe.2017.02.005.

21. Wiebking, V.; Lee, C.M.; Mostrel, N.; Lahiri, P.; Bak, R.; Bao, G.; Roncarolo, M.G.; Bertaina, A.; Porteus, M.H. Genome editing of donor-derived T-cells to generate allogenic chimeric antigen receptor-modified T cells: Optimizing αβ T cell-depleted haploidentical hematopoietic stem cell transplantation. Haematologica 2020, haematol.2019.233882, doi:10.3324/haematol.2019.233882.

22. Roth, T.L.; Puig-saus, C.; Yu, R.; Shifrut, E.; Carnevale, J.; Li, J.; Hiatt, J.; Saco, J.; Krystofinski, P.; Li, H.; et al. Repromming human T cell function and specificity with non-viral genome targeting. Nature 2018, doi:10.1038/s41586-018-0326-5.

23. Nguyen, D.N.; Roth, T.L.; Li, P.J.; Chen, P.A.; Apathy, R.; Mamedov, M.R.; Vo, L.T.; Tobin, V.R.; Goodman, D.; Shifrut, E.; et al. Polymer-stabilized Cas9 nanoparticles and modified repair templates increase genome editing efficiency. Nat. Biotechnol. 2020, 38, 44–49, doi:10.1038/s41587-019-0325-6.

24. Kosicki, M.; Tomberg, K.; Bradley, A. Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. Nat. Biotechnol. 2018, 36, doi:10.1038/nbt.4192.

25. Cullot, G.; Boutin, J.; Toutain, J.; Prat, F.; Pennamen, P.; Rooryck, C.; Teichmann, M.; Rousseau, E.; Lamrissi-Garcia, I.; Guyonnet-Duperat, V.; et al. CRISPR-Cas9 genome editing induces megabase-scale chromosomal truncations. Nat. Commun. 2019, 10, 1–14, doi:10.1038/s41467-019-09006-2.

26. Fu, Y.; Foden, J.A.; Khayter, C.; Maeder, M.L.; Reyon, D.; Joung, J.K.; Sander, J.D. High-

frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat. Biotechnol. 2013, 31, 822–826, doi:10.1038/nbt.2623.

27. Sheridan, C. CRISPR therapies march into clinic, but genotoxicity concerns linger. Nat. Biotechnol. 2021, 39, 897–899, doi:10.1038/d41587-021-00017-3.
28. Gaudelli, N.M.; Lam, D.K.; Rees, H.A.; Solá-Esteves, N.M.; Barrera, L.A.; Born, D.A.; Edwards, A.; Gehrke, J.M.; Lee, S.J.; Liquori, A.J.; et al. Directed evolution of adenine base editors with increased activity and therapeutic application. Nat. Biotechnol. 2020, 38, 892–900, doi:10.1038/s41587-020-0491-6.
29. Lu, Y.; Xue, J.; Deng, T.; Zhou, X.; Yu, K.; Deng, L.; Huang, M.; Yi, X.; Liang, M.; Wang, Y.; et al. Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. Nat. Med. 2020, 26, 732–740, doi:10.1038/s41591-020-0840-5.
30. Prommersberger, S.; Reiser, M.; Beckmann, J.; Danhof, S.; Amberger, M.; Quade-Lyssy, P.; Einsele, H.; Hudecek, M.; Bonig, H.; Ivics, Z. CARAMBA: a first-in-human clinical trial with SLAMF7 CAR-T cells prepared by virus-free Sleeping Beauty gene transfer to treat multiple myeloma. Gene Ther. 2021, 28, 560–571, doi:10.1038/s41434-021-00254-w.
31. Yeku, O.; Li, X.; Brentjens, R.J. Adoptive T-Cell Therapy for Solid Tumors. 2020, 193–204.
32. Legat, A.; Speiser, D.E.; Pircher, H.; Zehn, D.; Fuertes Marraco, S.A. Inhibitory receptor expression depends more dominantly on differentiation and activation than “exhaustion” of human CD8 T cells. Front. Immunol. 2013, 4, 1–15, doi:10.3389/fimmu.2013.00455.
33. Tang, N.; Cheng, C.; Zhang, X.; Qiao, M.; Li, N.; Mu, W.; Wei, X.F.; Han, W.; Wang, H. TGF-β inhibition via CRISPR promotes the long-term efficacy of CAR T cells against solid tumors. JCI Insight 2020, 5, doi:10.1172/jci.insight.133977.
34. Tay, R.E.; Richardson, E.K.; Toh, H.C. Revisiting the role of CD4+ T cells in cancer immunotherapy—new insights into old paradigms. Cancer Gene Ther. 2021, 28, 5–17, doi:10.1038/s41417-020-0183-x.
35. Fiorenza, S.; Ritchie, D.S.; Ramsey, S.D.; Turtle, C.J.; Roth, J.A. Value and affordability of CAR T-cell therapy in the United States. Bone Marrow Transplant. 2020, 55, 1706–1715, doi:10.1038/s41409-020-0956-8.
36. Consonni, M.; Garavaglia, C.; Grilli, A.; de Lalla, C.; Mancino, A.; Mori, L.; De Libero, G.; Montagna, D.; Casucci, M.; Serafini, M.; et al. Human T cells engineered with a leukemia lipid-specific TCR enables donor-unrestricted recognition of CD1c-expressing leukemia. Nat. Commun. 2021, 12, doi:10.1038/s41467-021-25223-0.
37. Crowther, M.D.; Dolton, G.; Legut, M.; Caillaud, M.E.; Lloyd, A.; Attaf, M.; Galloway, S.A.E.; Rius, C.; Farrell, C.P.; Szomolay, B.; et al. Genome-wide CRISPR–Cas9 screening reveals ubiquitous T cell cancer targeting via the monomorphic MHC class I-related protein MR1. Nat. Immunol. 2020, 21, 178–185, doi:10.1038/s41590-019-0578-8.
38. Johanna, I.; Straetemans, T.; Heijhuurs, S.; Aarts-Riemens, T.; Norell, H.; Bongiovanni, L.; De Bruin, A.; Sebestyen, Z.; Kuball, J. Evaluating in vivo efficacy - Toxicity profile of TEG001 in humanized mice xenografts against primary human AML disease and healthy hematopoietic cells. J. Immunother. Cancer 2019, 7, 1–13, doi:10.1186/s40425-019-0558-4.

# APPENDICES

# ENGLISH SUMMARY

Immunotherapy has been considered a breakthrough in cancer treatment in the past decade. Immune checkpoint inhibitors and Chimeric Antigen Receptor T cells (CAR-T cells) have entered clinical practice and achieved promising success rates in some tumor type, such as melanoma and acute lymphoblastic leukemia, and are under clinical tests in many other cancer types. Immune checkpoint inhibitors relies on the direct activation of the patient immune system, via the use of monoclonal antibodies directed against checkpoint receptors, while CAR-T cells account for ex-vivo modification of patients-derived immune cells to induce specificity towards tumor cells. As the second strategy is characterized by the use of living cells, many limiting factors, not only related to the method of action but also to the production of the therapy, are holding back the application of this approach.

First of all, a fully personalized production is required for every patient, stretching the time from availability for treatment to the possibility to receive it. Moreover, the number and fitness of cancer patient-derived immune cells, does not always meet the quality control requirements essentials to produce cell therapy products. Those two production hurdles cause delay and decrease the chances for a successful administration of a potentially curable treatment. As explained in **Chapter 1**, the aim of this thesis is to shed a light on the use of an alternative allogeneic source of immune cells for the generation of cell therapy product, and overcome some of the problems that have obscured the potential of cell therapy. More in detail, the use of umbilical cord blood (CB) derived immune cells, and the possibility to modify them with novel gene augmentation and gene-editing techniques, is the core of this dissertation.

**Chapter 2** defines the advantages of the use of CB derived immune cells, mainly related to the high availability of this source and its known low immunogenicity in the context of hematopoietic stem cell transplantation. The manuscript focuses specifically on the use of CB derived T cells for the generation of T cell therapy to be used as an adjuvant treatment after hematopoietic cell transplantation or in combination with other treatments, such as dendritic cell vaccines. CB derived T cells are characterized by a naïve immune phenotype, a great expansion potential and anti-tumor capacity. Even if undervalued, the use of CB derived T cells can dimmish cost and time of production, while maintaining biological advantages against tumor cells.

As fresh CB derived T cells are characterized by an endogenous repertoire of T cell receptors (TCRs), specifically targeting the tumor cells might remain elusive. Therefore **Chapter 3** focuses on optimizing a method of gene-transfer, using lentiviral vectors, to confer specificity towards cancer in CB CD8+ T cells. Method optimization was performed using a lentiviral vector expressing GFP as gene of interest, achieving more than 90% of transduction efficiency with the use of a new generation transduction enhancer. The proposed method was confirmed successful using a recombinant TCR and a CAR targeting a relevant tumor associated antigen. Moreover, it is shown that the method does not affect viability and expansion capacity of T cells, highlighting the clinical applicability.

Cell therapy strategies are often referring to T cell-based products but are not limited to them. Other immune cell type autologous and allogeneic, can be produced in a good manufacturing practice (GMPs) setting and therefore can be used in clinical practice. **Chapter 4** describes a method to generate GMP-grade dendritic cell vaccine from CB derived CD34+ cells. This method allows the generation of a sufficient number of therapeutic DCs, from a rather low amount of CD34+, characterized by the upregulation of co-stimulatory molecules and migration capacity. Loading the DCs with a tumor associated peptide induce activation of T cells and subsequent killing of tumor cell lines and primary cells.

The second part of the thesis focuses on gene-editing techniques used to further improve safety and efficacy of CB derived cell therapy products. **Chapter 5** shows the generation of a CB CD8+ T cell product expressing a Wilm's Tumor 1 (WT1) specific TCR, while knocked out for the expression of the endogenous TCR. Targeting of both the alpha and beta chain of the TCR via the CRISPR-Cas9 editing system demonstrated a complete elimination of the endogenous TCR, decreasing the probability of generating mis-paired TCRs. When compared to only transduced cells, the gene-edited product showed a similar immune phenotype and a trend of increased activation and cytokines production. Gene-edited cells showed an increased killing capacity against tumor cell lines naturally expressing WT1. Finally, gene-edited cells proved a substantial killing capacity of primary AML cells embedded in a 3D model resembling the bone marrow niche of relapsed patients.

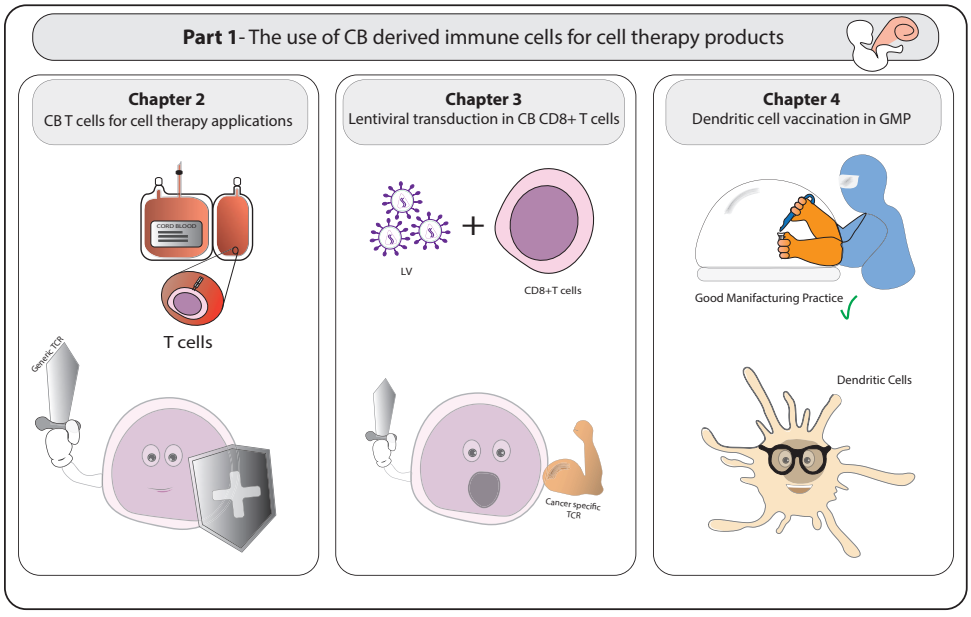
**Chapter 6** focuses on the further optimization of the strategy presented in Chapter 5, exploring how to take advantage of the homologous directed repair (HDR) system that cells can initiate after CRISPR mediated editing. Contrary to a random integration in the target genome associated with using lentiviral vectors, CRISPR mediated was designed to insert the gene of interest precisely in the constant region

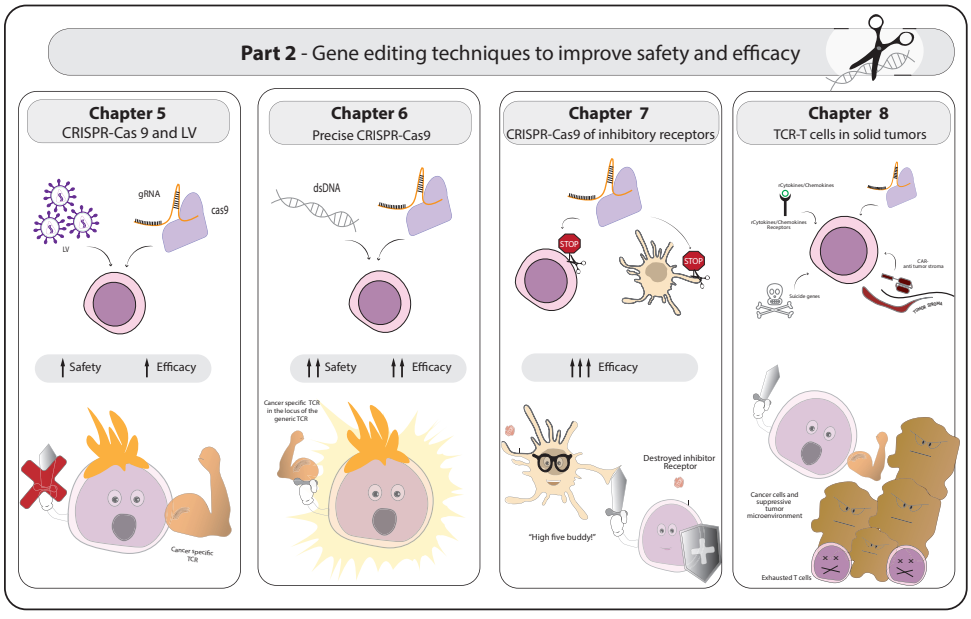
of the TCR alpha chain. This method disrupts the expression of the endogenous TCR while, at the same time, induces the expression of the gene of interest. As proof of principle the chapter explores the possibility to use Integration deficiency lentiviral vector (IDLV) as a delivery system of the genetic information needed for the HDR; achieving up to 5% of successful HDR in primary CB CD8+ T cells.

Eliminating the expression of the endogenous TCR influences, mostly, the safety of the cell therapy product; while eliminating the expression of inhibitory receptors can influence the potency and effectiveness of the products. For this reason, **Chapter 7** proposes an optimized method to eliminate several inhibitory receptors on the surface of CB DCs and CB CD8+ T cells. This method showed an editing efficacy up to 95%, in CB DCs, and did not affect viability and basic functionality of both cell types, opening the ground to more extensive studies on the generation of more potent CB derived cell therapy products.

As their efficacy in solid tumors has been long debated, this thesis and the majority of cell therapy application rely on their use in hematological malignancies **Chapter 8** focuses on presenting the scientific literature on the use of TCR T cell therapy in the context of solid tumors, starting with the enumeration of the current registered clinical trials and further evaluating the explorative possibilities that can give a second opportunity to these cell therapy for the treatment of solid malignancies.

Finally, **Chapter 9** integrates the finding of this thesis in the scientific and social context. Critically assess the advantages and the limitations of the approach and discuss future perspective in the field.


Part 1- The use of CB derived immune cells for cell therapy products
Chapter 2
CB T cells for cell therapy applications
CORD BLOOD
T cells
Generic TCR
Chapter 3
Lentiviral transduction in CB CD8+ T cells
LV
CD8+T cells
Cancer specific TCR
Chapter 4
Dendritic cell vaccination in GMP
Good Manufacturing Practice
Dendritic Cells


Part 2 - Gene editing techniques to improve safety and efficacy
Chapter 5
CRISPR-Cas 9 and LV
LV
gRNA
cas9
Safety
Efficacy
Cancer specific TCR
Chapter 6
Precise CRISPR-Cas9
dsDNA
Safety
Efficacy
Cancer specific TCR in the locus of the generic TCR
Chapter 7
CRISPR-Cas9 of inhibitory receptors
STOP
STOP
Efficacy
Destroyed inhibitor Receptor
"High five buddy!"
Chapter 8
TCR-T cells in solid tumors
Cytokines/Chemokines
Cytokines/Chemokines Receptors
CAR-
and tumor stroma
Suicide genes
Cancer cells and suppressive tumor microenvironment
Exhausted T cells

# NEDERLANDS SAMENVATTING

Immunotherapie wordt de afgelopen tien jaar beschouwd als een doorbraak in de behandeling van kanker. Checkpointremmers en chimere antigeenreceptor T-cellen (CAR-T-cellen) zijn de kliniek ingegaan en hebben veelbelovende successen behaald bij sommige tumortypes, zoals melanoom en acute lymfoblastische leukemie, en worden klinisch getest bij veel andere kankertypes. De eerste strategie is gebaseerd op de directe activering van het immuunsysteem van de patiënt, via het gebruik van monoklonale antilichamen gericht tegen checkpoint-receptoren, terwijl de tweede strategie rekening houdt met ex vivo modificatie van de van de patiënt afgeleide immuuncellen om specificiteit voor tumorcellen te induceren. Aangezien de tweede strategie wordt gekenmerkt door het gebruik van levende cellen, geeft dat een aantal belemmerende factoren, niet alleen met betrekking tot de werkwijze maar ook met de productie van de therapie. Allereerst is een volledig gepersonaliseerde productie vereist voor elke patiënt, waarbij de tijd wordt gespannen van beschikbaarheid voor behandeling tot de mogelijkheid om deze te ontvangen. Bovendien voldoet het aantal en de geschiktheid van van kankerpatiënten afkomstige immuuncellen niet altijd aan de kwaliteitscontrole-eisen die essentieel zijn om celtherapieproducten te produceren. Die twee productiehindernissen veroorzaken vertraging en verkleinen de kans op een succesvolle toediening van een mogelijk geneesbare behandeling. Zoals uitgelegd in **Hoofdstuk 1**, is het doel van dit proefschrift om een licht te werpen op het gebruik van een alternatieve allogene bron van immuuncellen voor het genereren van celtherapieproducten, en om enkele van de problemen op te lossen die het potentieel van celtherapie hebben verdoezeld. Meer in detail vormt het gebruik van van navelstrengbloed (CB) afgeleide immuuncellen, en de mogelijkheid om ze te modificeren met nieuwe technieken voor genmanipulatie, de kern van dit proefschrift.

In **Hoofdstuk 2** worden de voordelige redenen voor het gebruik van CB-afgeleide immuuncellen gedefinieerd, voornamelijk gerelateerd aan de hoge beschikbaarheid van deze bron en de bekende lage immunogeniciteit in de context van hematopoëtische stamceltransplantatie. Het manuscript richt zich specifiek op het gebruik van CB-afgeleide T-cellen voor het genereren van T-celtherapie om een adjuvante behandeling te gebruiken na hematopoëtische celtransplantatie of in combinatie met andere behandelingen, zoals dendritische celvaccins. Van CB afgeleide T-cellen worden gekenmerkt door een naïef immuunfenotype, een groot expansiepotentieel en antitumorcapaciteit. Zelfs als het ondergewaardeerd is, kan het gebruik van van CB afgeleide T-cellen de kosten en productietijd verminderen;

met behoud van biologische voordelen tegen tumorcellen.

Aangezien verse van CB afgeleide T-cellen worden gekenmerkt door een endogeen repertoire van T-celreceptoren (TCR's), kan het moeilijk zijn om specifiek op de tumorcellen te richten. Daarom richt **Hoofdstuk 3** zich op het optimaliseren van een methode van genoverdracht, met behulp van lentivirale vectoren, om specificiteit te verlenen aan kanker in CB CD8+ T-cellen. Methode-optimalisatie werd uitgevoerd met behulp van een lentivirale vector die GFP tot expressie brengt als gen van belang, waarbij meer dan 90% van de transductie-efficiëntie werd bereikt met het gebruik van een nieuwe generatie transductieversterker. De voorgestelde methode werd succesvol bevestigd met behulp van een recombinante TCR en een CAR gericht op een relevant tumor-geassocieerd antigeen. Bovendien is aangetoond dat de methode geen invloed heeft op de levensvatbaarheid en uitbreidingscapaciteit van T-cellen, daarom toepasbaar in de klinische instellingen.

Celtherapiestrategieën verwijzen vaak naar op T-cellen gebaseerde producten, maar zijn daar niet toe beperkt. Andere autologe en allogene immuunceltypes kunnen worden geproduceerd in een goede fabricagepraktijk (GMP's) en in de klinische praktijk worden gebruikt. **Hoofdstuk 4** beschrijft een methode om GMP-grade dendritische celvaccins te genereren uit CB-afgeleide CD34+-cellen. Deze methode maakt het mogelijk een voldoende aantal therapeutische DC's te genereren, uit een vrij lage hoeveelheid CD34+, gekenmerkt door de opregulatie van co-stimulerende moleculen en migratiecapaciteit. Het laden van de DC's met een tumor-geassocieerd peptide induceert activering van T-cellen en het daaropvolgende doden van tumorcellijnen en primaire cellen.

Het tweede deel van het proefschrift richt zich op technieken voor het bewerken van genen die worden gebruikt om de veiligheid en werkzaamheid van van CB afgeleide celtherapieproducten verder te verbeteren. **Hoofdstuk 5** toont het genereren van een CB CD8+ T-celproduct dat een Wilm's Tumor 1 (WT1)-specifieke TCR tot expressie brengt, terwijl het wordt uitgeschakeld voor de expressie van de endogene TCR. Door zowel de alfa- als de bètaketen van de TCR te targeten, via het CRISPR-Cas9-bewerkingssysteem, werd de endogene TCR geelimineerd, waardoor de kans op het genereren van verkeerd gepaarde TCR's tot een minimum werd teruggebracht. In vergelijking met alleen getransduceerde cellen vertoonde het gen-bewerkte product een vergelijkbaar immuunfenotype en een trend van verhoogde activering en cytokinenproductie. Gemanipuleerde cellen vertoonden een verhoogd dodend vermogen tegen tumorcellijnen die van nature WT1 tot expressie brengen. Ten slotte bewezen genetisch gemanipuleerde cellen een aanhoudende dodingscapaciteit

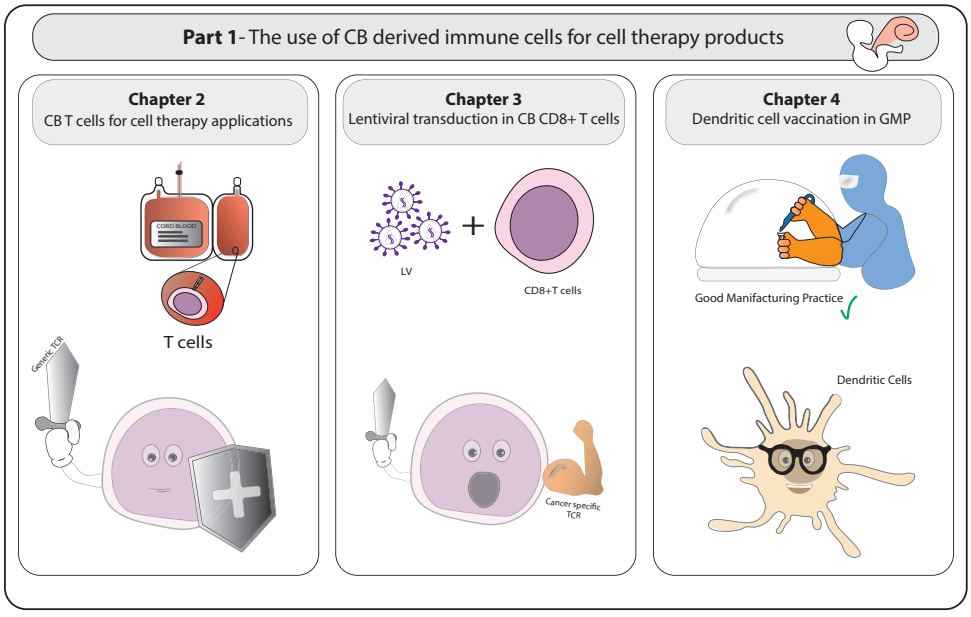
van primaire AML-cellen ingebed in een 3D-model dat lijkt op de beenmergniche van relapse patiënten.

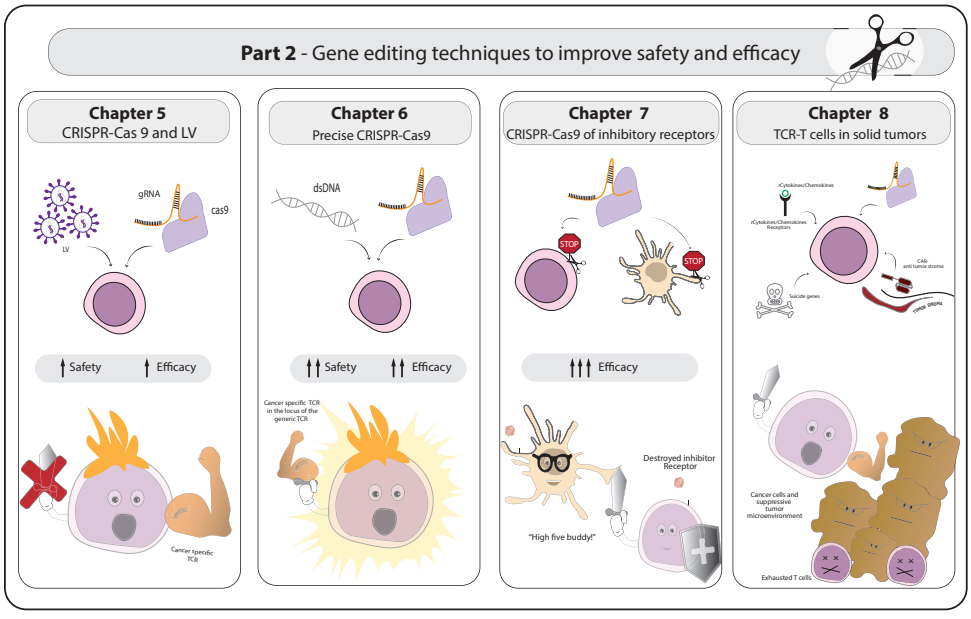
**Hoofdstuk 6** richt zich op de verdere optimalisatie van de strategie gepresenteerd in Hoofdstuk 5, waarbij wordt onderzocht hoe gebruik kan worden gemaakt van het homologe gerichte reparatie (HDR)-systeem dat cellen kunnen initiëren na CRISPR-gemedieerde bewerking. In tegenstelling tot een willekeurige integratie in het doelgenoom, met behulp van lentivirale vectoren, werd deze methode ontworpen om het gen van belang precies in het constante gebied van de TCR-alfaketen in te voegen; het verstoren van de expressie van de endogene TCR en tegelijkertijd het induceren van de expressie van het gen van belang. Als bewijs van het principe onderzoekt het hoofdstuk de mogelijkheid om Integration deficiency lentiviral vector (IDLV) te gebruiken als een leveringssysteem van de genetische informatie die nodig is voor de HDR; het behalen van tot 5% succesvolle HDR in primaire CB CD8+ T-cellen.

Terwijl de expressie van de endogene TCR-invloed wordt geëlimineerd, voornamelijk de veiligheid van het celtherapieproduct; het elimineren van de expressie van remmende receptoren op het oppervlak van cellen kan de uiteindelijke potentie en effectiviteit van het product beïnvloeden. Om deze reden stelt **Hoofdstuk 7** een geoptimaliseerde methode voor om verschillende remmende receptoren op het oppervlak van CB DCs en CB CD8+ T-cellen te elimineren. Deze methode vertoonde een bewerkingsefficiëntie tot 95%, in CB DC's, en had geen invloed op de levensvatbaarheid en basisfunctionaliteit van beide celtypen; de weg vrijmaken voor uitgebreidere studies over het genereren van krachtiger van CB afgeleid product voor celtherapie. Evenals in dit proefschrift berust het merendeel van de toepassingen van celtherapie op hun gebruik bij hematologische maligniteiten; terwijl hun werkzaamheid bij solide tumoren al lang ter discussie staat.

**Hoofdstuk 8** richt zich op het presenteren van de wetenschappelijke literatuur over het gebruik van TCR T-celtherapie in de context van solide tumoren, te beginnen met de opsomming van de huidige geregistreerde klinische studies en het verder evalueren van de verkennende mogelijkheden die een tweede kans kunnen geven aan deze celtherapie voor de behandeling van solide maligniteiten.

Ten slotte integreert **Hoofdstuk 9** de bevinding van dit proefschrift in de wetenschappelijke en sociale context. Beoordeel de voordelen en de beperkingen van de aanpak kritisch en bespreek toekomstperspectief in het veld.


Part 1- The use of CB derived immune cells for cell therapy products
Chapter 2
CB T cells for cell therapy applications
CORD BLOOD
T cells
Generic TCR
Chapter 3
Lentiviral transduction in CB CD8+ T cells
LV
CD8+T cells
Cancer specific TCR
Chapter 4
Dendritic cell vaccination in GMP
Good Manufacturing Practice
Dendritic Cells


Part 2 - Gene editing techniques to improve safety and efficacy
Chapter 5
CRISPR-Cas 9 and LV
LV
gRNA
cas9
Safety
Efficacy
Cancer specific TCR
Chapter 6
Precise CRISPR-Cas9
dsDNA
Safety
Efficacy
Cancer specific TCR in the locus of the generic TCR
Chapter 7
CRISPR-Cas9 of inhibitory receptors
STOP
STOP
Efficacy
Destroyed inhibitor Receptor
"High five buddy!"
Chapter 8
TCR-T cells in solid tumors
Cytokines/Chemokines
Cytokines/Chemokines Receptors
CAR- and tumor stroma
Suicide genes
Cancer cells and suppressive tumor microenvironment
Exhausted T cells

# RIASSUNTO IN ITALIANO

L'immunoterapia è stata considerata una svolta nel trattamento del cancro degli ultimi dieci anni. Principali esempi di questi nuovi tipi di trattamenti sono l'inibizione dei checkpoint immunitari e le cellule T modificati con un *chimeric antigen receptor* (cellule CAR-T). Entrambe queste strategie sono entrate nella pratica clinica, hanno raggiunto promettenti tassi di successo in alcuni tipi di tumore, come il melanoma e la leucemia linfoblastica acuta, e sono in valutazione clinica per molti altri tipi di tumore. La prima strategia si basa sull'attivazione diretta del sistema immunitario del paziente, tramite l'uso di anticorpi monoclonali diretti ad inibire recettori di checkpoint immunitari. Al contrario, la seconda strategia modifica ex vivo le cellule T derivate dal paziente per indurre un'immunità spontanea specifica verso le cellule tumorali. Poiché la seconda strategia è caratterizzata dall'uso di cellule viventi, molti fattori limitanti, non solo legati al metodo di azione ma anche alla sua produzione, ne stanno frenando l'applicazione come terapia clinica.

Innanzitutto, l'approccio terapeutico con cellule CAR-T richiede una produzione personalizzata per ogni paziente, estendendo i tempi tra l'assegnazione di eligibilitá e l'effettivo trattamento. Inoltre, la quantitá e la qualitá delle cellule immunitarie derivate da pazienti oncologici non soddisfano sempre i requisiti necessari per prodotti di terapia cellulare. Queste difficoltá di produzione causano ritardi e riducono le possibilità di una corretta somministrazione di un trattamento potenzialmente curativo. Come esposto nel **Capitolo 1**, lo scopo di questa tesi è di presentare una fonte alternativa di cellule immunitarie allogeniche per terapia cellulare, superando alcuni dei problemi che hanno ridotto il potenziale di questa terapia fino ad oggi. Più in dettaglio, viene analizzato l'uso di cellule immunitarie derivate da sangue del cordone ombelicale (CB) e la possibilità di utilizzare nuove tecniche di ingegneria genetica per modificarne l'efficiacia e la sicurezza.

Nel **Capitolo 2** sono definiti i vantaggi dell'uso di cellule immunitarie derivate da CB: l'elevata disponibilità e bassa immunogenicità. Il manoscritto si concentra, in particolare, sull'uso di cellule T derivate da CB per la generazione di un trattamento adiuvante al trapianto di cellule ematopoietiche o ad altri trattamenti come i vaccini a base di cellule dendritiche. Il manoscritto illustra che le cellule T derivati da CB sono caratterizzati da un fenotipo immunitario naïve, un grande potenziale di espansione e un'elevate capacità antitumorale. L'uso di cellule T derivate da CB può diminuire i costi e i tempi di produzione, mantenendo delle caratteristiche biologiche vantaggiose contro le cellule tumorali.

Poiché le cellule T derivate da CB sono caratterizzate da un ampio repertorio endogeno di recettori delle cellule T (TCR), con la potenzialità di riconoscere vari antigeni, il targeting specifico delle cellule tumorali potrebbe essere difficile. Pertanto il **Capitolo 3** si concentra sull'utilizzo e ottimizzazione di vettori lentivirali, per conferire specificità verso il tumore alle cellule T citotossiche (CD8+) derivate da CB. L'ottimizzazione del metodo, verificata con costrutto che esprime GFP, è risultata in oltre il 90% di efficienza di trasduzione grazie all'uso di un potenziatore di trasduzione di nuova generazione. Il metodo proposto è stato confermato con successo utilizzando un TCR ricombinante e un CAR, entrambi mirati a riconoscere un antigene associato al tumore. Inoltre, è stato dimostrato che il metodo non pregiudica la vitalità e la capacità di replicazione delle cellule T, rendendolo quindi adatto all' ambito clinico.

Le strategie di terapia cellulare, come quelle discusse finora, si riferiscono spesso a prodotti a base di cellule T, ma non si limitano ad essi. Infatti, anche altre cellule immunitarie autologhe e allogeniche possono essere prodotte secondo le norme di buona fabbricazione (GMP) e di conseguenza essere utilizzati nella pratica clinica. Il **Capitolo 4** descrive un metodo per generare un vaccino a cellule dendritiche di grado GMP da cellule staminali CD34+ derivate da CB. Il metodo da noi proposto consente la generazione di un numero sufficiente di DCs terapeutiche, a partire da una quantità piuttosto moderata di CD34+, e produce DCs caratterizzate da sovraregolazione delle molecole costimolatorie e da capacità di migrazione. Dimostriamo anche che la somministrazione di un peptide associato al tumore alle DCs induce l'attivazione delle cellule T e la successiva uccisione di linee cellulari tumorali e di cellule tumorali primarie.

La seconda parte della tesi si concentra sulle tecniche di editing genetico utilizzate per migliorare ulteriormente la sicurezza e l'efficacia dei prodotti di terapia cellulare derivati dal CB. Il **Capitolo 5** mostra la possibilitá di combinare un efficiente transduzione con vettori lentivirali e la tecnica di editiging genetico CRISPR-Cas9. Questa combinazione permette di un ottenere un prodotto a base di cellule T CD8+ derivati da CB che esprimono un TCR specifico per la leucemia mieloide acuta (AML), Wilm's Tumor 1 (WT1), mentre viene eliminata l'espressione del TCR endogeno. Il targeting della catena alfa e beta del TCR, tramite il sistema di editing CRISPR-Cas9, determina una completa eliminazione del TCR endogeno, comunemente conosciuto come knock-out (KO), diminuendo al minimo la probabilità di generare TCR accoppiati in modo errato.

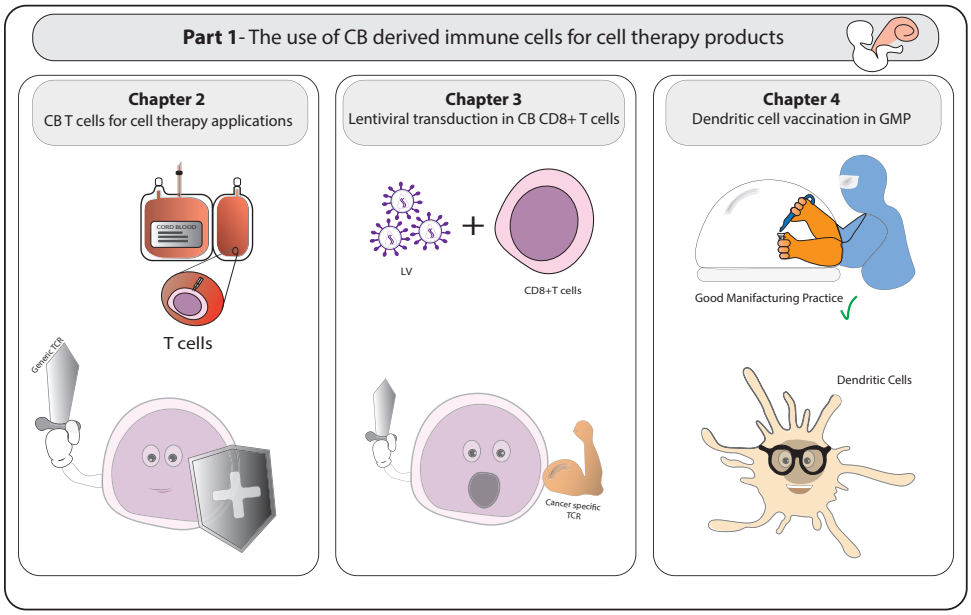
Rispetto alle sole cellule trasdotte con vettori lentivirali per esprimenre il WT1-TCR, il prodotto con supplementare editing genetico ha mostrato un invariato immunofenotipo e una tendenza all'aumento dell'attivazione e della produzione di citochine. Le cellule così modificate hanno mostrato una maggiore efficienza di uccisione delle linee cellulari tumorali che esprimono naturalmente WT1. Infine, il prodotto qui proposto, quando inserito in un modello 3D riproducente la nicchia del midollo osseo di pazienti in recidiva di leucemia mieloide acuta, dimostra una capacità di uccisione significativa delle cellule AML primarie.

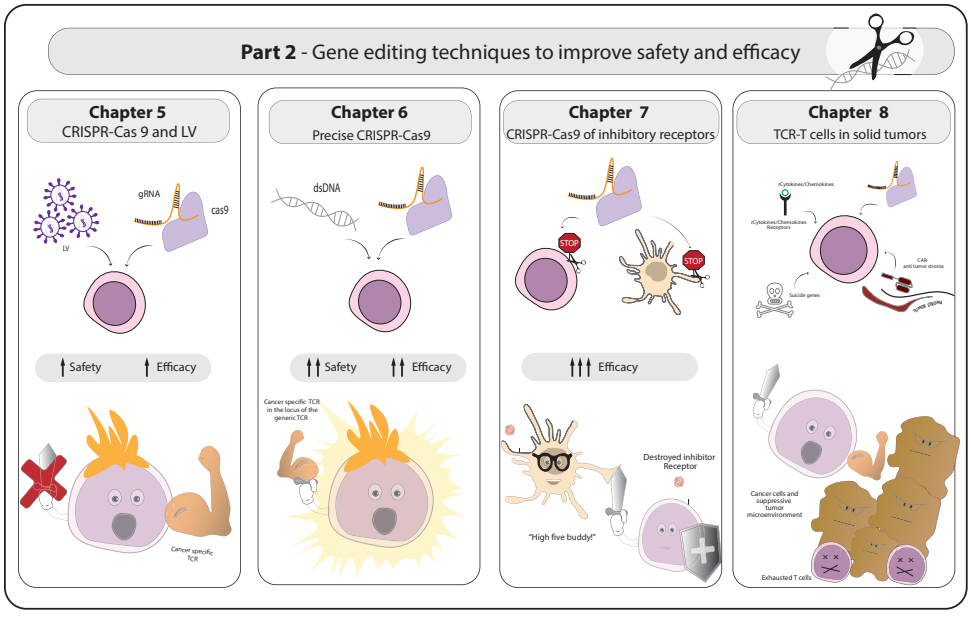
Il **Capitolo 6** si concentra sull'ulteriore ottimizzazione della strategia presentata nel Capitolo 5 tramite sfruttamento del meccanismo di riparazione omologa diretta (HDR) del DNA che le cellule possono avviare dopo l'editing mediato da CRISPR. Contrariamente all'integrazione casuale nel genoma bersaglio generata da vettori lentivirali, l'editing genetico con CRISPR-Cas9 è stato progettato per inserire il gene di interesse proprio nella regione costante della catena alfa del TCR. L'integrazione regione-specifica interrompe l'espressione del TCR endogeno e, allo stesso tempo, induce l'espressione del gene di interesse. Per dimostrare la fattibilità di questo approccio, il capitolo esplora la possibilità di utilizzare i vettori lentivirali con carenza di integrazione (IDLV) come sistema di consegna delle informazioni genetiche necessarie per l'HDR. Dopo varie fasi di ottimizzazioni, questa strategia ha dimostrato la possibilitá di poter raggiungere fino al 5% di HDR nelle cellule T CD8+ derivate da CB.

Eliminare l'espressione del TCR endogeno influenza, principalmente, la sicurezza del prodotto di terapia cellulare; mentre l'eliminazione dell'espressione di checkpoint immunitari può influenzare la potenza e l'efficacia del prodotto. Per questo motivo, il **Capitolo 7** propone un metodo ottimizzato per eliminare l'espressione di diversi checkpoint immunitari dalla superficie delle CB DC e dei CB linfociti T CD8+. Questo metodo ha mostrato un'efficacia di editing fino al 95% nelle CB DC e al 50% nelle cellule T CD8+, senza influenzare né la vitalità né le funzionalità di entrambi i tipi cellulari, fornendo le basi per studi più ampi sulla generazione di prodotti più potenti per la terapia cellulare derivati da CB. Similmente a questa tesi, la maggior parte delle terapie cellulari é limitata al trattamento delle neoplasie ematologiche; mentre la loro efficacia nei tumori solidi è stata a lungo dibattuta.

Il **Capitolo 8** presenta la letteratura scientifica riguardo l'applicazione della terapia a base di cellule T modificate con TCR recombinanti specifici per antigeni espressi nel contesto dei tumori solidi, partendo dall'enumerazione degli attuali studi clinici registrati e proseguendo con la valutazione delle future possibilità per queste terapie nel trattamento di neoplasie solide.

Infine, il **Capitolo 9** integra i risultati di questa tesi nel contesto scientifico e sociale. Valuta criticamente i vantaggi e i limiti dell'approccio e discute le prospettive future nel campo.



# LIST OF PUBLICATIONS

**Lo Presti V**, Nierkens S, Boelens JJ, van Til NP. Use of cord blood derived T-cells in cancer immunotherapy: milestones achieved and future perspectives.

*Expert Rev Hematol. 2018 Mar*; 11(3):209-218. doi:10.1080/17474086.2018.1431119. Epub 2018 Feb 1. PMID: 29359983.

Plantinga M, **Lo Presti V**, de Haar CG, Dünnebach E, Madrigal A, Lindemans CA, Boelens JJ, Nierkens S. Clinical Grade Production of Wilms' Tumor-1 Loaded Cord Blood-Derived Dendritic Cells to Prevent Relapse in Pediatric AML After Cord Blood Transplantation.

*Front Immunol. 2020 Sep 25*; 11:559152. doi: 10.3389/fimmu.2020.559152. PMID: 33101274; PMCID: PMC7546401.

**Lo Presti V**, Cornel AM, Plantinga M, Dünnebach E, Kuball J, Boelens JJ, Nierkens S, van Til NP. Efficient lentiviral transduction method to gene modify cord blood CD8+ T cells for cancer therapy applications.

*Mol Ther Methods Clin Dev. 2021 Mar 23*;21:357-368.doi:10.1016/j.omtm.2021.03.015. PMID: 33898633; PMCID: PMC8056177.

**Lo Presti V**, Buitenwerf F, van Til NP, Nierkens S. Gene Augmentation and Editing to Improve TCR Engineered T Cell Therapy against Solid Tumors.

*Vaccines (Basel). 2020 Dec 3*; 8(4):733. doi: 10.3390/vaccines8040733. PMID: 33287413; PMCID: PMC7761868.

*UNDER REVIEW*

**Lo Presti V**, Cutilli A, Dogariu Y, Müskens K, Dünnebach E, van de Beemt D, Cornel A, Plantinga M and Nierkens S. Gene editing of checkpoint molecules in cord blood-derived dendritic cells and CD8+ T cells using CRISPR-Cas9.

*The CRISPR Journal*. Submitted 2021

# CURRICULUM VITAE

Vania was born on the 17th of May of 1993 in Reggio Calabria, Italy. In 2011 she enrolled in the "Medical and Pharmaceutical Biotechnologies" bachelor course at the San Raffaele University, in Milan. She graduated with the highest grade in 2014, writing a thesis on gene-editing techniques for the treatment of β-thalassemia, under the supervision of prof. C. Camaschella and dr. L. Silvestri. She continued her studies at San Raffaele University enrolling in the Master degree "Molecular and Cellular Medical Biotechnologies". During her master, she joined the laboratory of prof. S. Rivella at the Children's Hospital of Philadelphia for her major master internship. Her project focused on unraveling the role of TNFα in a mouse model of Anemia of Inflammation. She graduated in November 2016 with honors (*cum laude*). After her graduation, she remained in the group of prof. Rivella as a visiting scientist. In August 2017, she started her PhD journey at the UMC Utrecht, in the Center for Translational Immunology, under the supervision of dr. Stefan Nierkens (promotor), prof. Jaap Jan Boelens, prof. Jurgen Kuball (promotor) and dr. Niek P. van Til (co-promotor). The results of her projects, focusing on the generation of cell therapy products using cord blood derived immune cells, are presented in this thesis. Currently she works in the Amsterdam UMC as coordinator of the ImmunoTherapy Center, supporting the creation of a centralized hub for expertise in the field of cancer immunology and immunotherapy.

# AKNOLEDGMENTS/ DANKWOORD/RINGRAZIAMENTI

*"Try to be the rainbow in somebody else's clouds"*
Maya Angelou

Almost 5 years later, with a book in my hand and with so many memories, I can finally write one of the most important chapters of my PhD thesis. **Why is it so important?** Because it shows my personal story and growth, it shows the adaptation in a foreign country, it shows the good moments and bad ones, and especially it talks about all the people that contributed (scientifically and not) to the successful finish of this book, all the people that were "the rainbow in my clouds".

First things first, a big thanks to the people that made this journey possible, my supervisors:

**Stefan**, thank you for being my mentor throughout this important journey. You were the solid rock that never moved, and as a perfect rock you supported me in good and bad times. I have absorbed way more knowledge on immunology from you that from the many books that I have read in university. But you didn't transfer only knowledge, you transferred a passion for immunology that will always stay with me. Thank you for caring about our personal development and for investing time to understand us besides the science. A mentor doesn't only help you performing the best experiment, it helps you developing as a scientist and you greatly did that for me. Thank you also for all the laughs, the joyful times, the coffee breaks and the discussion about the latest books we were reading. It was a great pleasure to be part of your group.

**Niek**, thank you for your having the idea of this project that fulfilled my scientific life in the last five years. I still remember our first video-call to discuss about the project. You were so calm and kind that it made me feel it would have been a good match for my PhD. Thank you also for trusting me and considering me independent from the very beginning. Special thanks for guiding my writing throughout the years, I have learned a lot.

**Jaap Jan**, thank you for being such a charismatic and enthusiastic boss! Our shared time in the UMCU was a bit short, but I happily remember our discussions and your help with moving the project forward in the first year.

**Jurgen**, thank you for being my promotor and a good mentor. You were there when I needed it but you also supported my independence. Thanks for the many scientific suggestions on the TCRs and the animal studies, your contribution was always very helpful.

**Zsolt**, you are not officially one of my supervisors but you have acted like one, so I would like to thank you. Thanks for devolving always some time to discuss with me

about the project, even if you didn't have to. After our talks, I always felt full of new ideas and more motivated than before, and I think that's what a good mentor is.

Thanks also to all the people with whom I have scientifically collaborate those years. **Anja, Aida, Angelo, and Inez** you were extremely nice and available and part of this thesis could have not been possible without your help.

And now a bit more about my life…

When I firstly arrived in Utrecht. Everything was new around me, bikes were crossing my way from every angle, canals were tossing me a sparkle of magic, rain was pouring from the sky almost everyday and people around me were saying words like "Lekker, gezellig, dankewel, bitterballen" that sounded impossible to pronounce and understand. But luckily, there were two big group of people that made this new life a lot easier: the UMC-U and the "Biltstraat".

**THE UMC-U**

Throughout those years I have met and enjoyed life with so many people in the UMC-U, from different background, different departments, different ages. Some of those people stayed in my life for a short chat, some others for a few months, but many are still here and I know they will stay for a long time ahead.

Some special thanks in the big UMC-U family:

**The BoNi**: Funny enough in Italian this means "beautiful people" (in a very colloquial way); andI believe there is no better explanation for what they are. I have seen this group changing completely at least a couple of time, people changed and lately also the location did. But the spirit of the group remained intact: joyful and caring. Thank you for welcoming the first Italian and international PhD student as part of the group, and thank you for all your help when I did not understand one word of the UMC-U communications in Dutch. I would have probably not even signed my contract without you ;). In this group have also found two of my paranimfen and many very good friends:

**Annelisa**, thanks for being my PhD buddy from (almost) beginning till end. You were always there, having my back (sort of literally since you sat on the other side of the sunshine room ;) ). Thanks for all your scientific help that improved everything in this book. But especially thanks for the supportive talks and the warm hugs when I felt under the weather, and the crazy times at conferences and PhD retreats! **Esterina**, thanks for teaching me everything immune-related in the lab. You have been such a precious lab mentor and patient teacher that made every experiment a lot funnier and easier. Through time we have become not only collegues but good friends, getting to know that we were sharing so many passions outside the lab, like making home-made pizza,

drinking beers at carnival, wearing frogs suits and travelling. And not to forget that I have finally experienced horse-back riding thanks to you and the beautiful Lo!
**Bri**, I still remember that during the first lab meeting you started to talk about your latest trip somewhere on the other side of the world. I did not even know your name, but I knew we were going to be friends! Thanks for your enthusiasm and your determination, it always bring a sparkle of freshness and adventure. Thanks for listening to me over our billion coffee-breaks, the chair behind the door of the sunshine room will always have your name on it! **Coco and Celina**, thanks for being my wise big sisters in the group. When I started you were almost at the end of your PhD journey and always had important tips and tricks that helped me along the way. And after all, I am sure we wil be in touch, because a paranimf is forever ;). **Bas**, you are one of the most spontaneous persons I have met here, and I love it! With you there was always time for a random beer or a random lunch break! (Still sorry to have made you drunk with the jelly-bears! hahah). **Maud**, thanks for your supervision during the time I felt a bit lost. You are one of those people that always leaves a gentle and kind sign in your hearth. A big part of this thesis it also belongs to you and I am glad I got to know a bit of Maud-DCs world, thanks to all your help! I am sure in the future we will always find time to share a good IPA beer. **Shanice,** thanks for all the things I have learned about neuroscience, for your smile and precious care for others, looking forward for our lunch breaks in Amsterdam! **Suze**, we have got to know each other a lot more in the last few years when we started to collaborate. Thanks for our brainstorm sessions (often in front of a good beer) and thanks also for sharing with me the pressure of "the last year", I felt it was easier to carry when divided by two. **Jurgen, Denise, Konradin, Linde, Maike and Joyce** thanks for being (have been) part of this wonderful group, I am sure the legacy of the BoNi will be maintained in the Maxima!

**Doris, Kari and Yvonne**, my beloved students! Thanks for teaching me way more than what I tried to teach you. You were so different (for example Doris is in-famous for the "pasta pollo" and Kari is one of the best home-cook I have ever met! Hahah) the and yet so similar. With you I learned that there is never a fixed way to teach and relate with your students, you just have to listen and understand what they need.

The BoNi were part of an even bigger family called the **Center for Translational Immunology**. Thanks to all of you for making our environment a lot better than only a place to work, thanks for the borrels and the coffee break, the retreats and lab-outjes. Thanks to the ones that shared, many times, a hood with me (special thanks to **Petra and Anna** for making Fridays afternoon and late hours a bit sweeter), thanks to the ones that landed me a chair (**Tineke**, I know you miss finding me on your chair ;)), thanks to the ones that thought me how to play SET (**Thomas and Konradin**, I am much better now), thanks to the ones that have helped me with experiments (**Angelo, Inez and Sabine** I owe you a lot of chocolate!!), thanks to everyone that have tried to speak a bit of Italian (**Niels, Geert, Marjolein and Ruud** siete molto bravi!).

Thanks to the ones that suffered with me during a kickboxing lesson after work (**Kim and Anna**).

And as the tip of the boot (as from where we are from!)…an extra special thanks to **Domenico**, my Calabrian twin! Grazie a te e a **Vale** per averci accolto nella vostra famiglia come se ci conoscessimo da sempre! Grazie per i "maccaruni ra duminica", le discussioni sul sindaco e sui nuovi locali di Reggio. Ma soprattutto grazie per l'amore e la felicitá che ci regalano i nostril amati nipotini Martina e Lorenzo

Thanks also to all the people outside the department that I have met during those years. Just because I was Italian someone introduced me to another Italian in the corridor, **Virginia**! Grazie polla, for all the post-it left on my desk that made the start of my day a lot nicer, the coffees and the dinners outside work and especially thanks for introducing me to the "**Late-lunch group**". **Anil, Dan, Linglei, Aida, Dan, Mariona and Omnia** thanks for sharing with me most of my lunch breaks. In Italy we say that the food is important because it brings people together, and I am glad it brought you into my life. Unfortunately the pandemic kind of destroyed our tradition but I hope to share a nice lunch soon!

Thanks to the **MMB** and all the people at the fourth floor that always had a cold beer and some chips to share. I have met most of you during our wonderful PhD retreats **(Elena, Patrick, Henridk, Lisanne, Dennis, Gosha, Leire)** when we sang, danced, drunk and experienced the laughing Yoga. Those funny memories will always make me smile!

Among them one person deserves a bit more words to explain how important he was during those years. **Hendrik,** we were flatmates for a couple of weeks in the Biltstraat and it took us only a few days to pretend to be brother and sister (because you know, it makes sense when you are one Italian and a German and you totally do not look alike). But somehow while pretending to be siblings, we sort of became real ones. Thanks for passing by my office everyday for a coffee-break (and for stealing food!! ;p). Thanks for always being there for a concert, a drink, a carbonara, a walk in the nature and my many movings. Can't wait to shoot many more ugly pictures with you.

Last but not least my beloved **SUNSHINE ROOM**. The room itself deserves some credit, for being sunny and for welcoming all the food and coffee-breaks we had in it. But the people deserves a bigger thank you. **Kaylee**, we were the first "tenants" of the room and I couldn't ask for a better roommate. It was an amazing first year, when I learned a lot about Canada and climbing. I am so happy that we kept our coffee-tradition, and I can't wait to visit Canada with you!! **Eveline, Annelisa and Cristine**, for a long time we shared our week routine, our problems and our successes. We have made our small 'wonderwall' to collect memories of our lives. Thanks for sharing the daily life! **Bas, Mara and Geert** I have left the room in your hands, thanks for taking care of me (and of the room, replenishing food and coffe) in the last stressed "last months" of the PhD!

**A NEW HOME**

As all of you know life as a PhD student doesn't not finish in the lab (maybe some days it does, but not always). The first people I have met outside of the UMC-U were my lovely flatmates at the student house – the famous "**BILTSTRAAT HOUSE**".

Thanks to all the people that I have met, thanks to whom showed me the Gemente bike parking spot and the OV-chipkaart (Andreia), the ones that brought me to Roberto Gelato (Bruno). Thanks for the parties, the snowballs wars and the adventures. Thanks to the people that have cooked and shared a meal in one of the kitchens. And when it comes to meal-shared two people win the prizes: **Valeria e Lucia**. Grazie mille amiche, per aver portato un po' di orgoglio italiano nelle nostre serate, per il salmone condiviso, le discussioni sui broccoli e cavolfiori, la nostra passione per Grey's e le tisane di fine giornata. Vi sono bastati pochi mesi per conquistare un posto cosí grande nel mio cuore.

Thanks for who stayed around and became my "**book-club". Martine, Cisco, Lieke, Martina, Bruno, Bea, Cinta, Lefkios and Pascal** thanks for all the books we have already read and for many to come. You inspire me to read more everyday!

**Lieke and Francisco**, "obrigada" for becaming our board-game geeks, our helper with the new house and our MONSTRINI for life.

A "musical" thanks to **Susi**, the person that has never said no to a concert, explored the city with me and shared all the good memory of discovering a new place together on a bike road-trip.

A "green" thanks to **Claudia**, my favourite epidemiologist and plant lover! Thanks for keeping me company when we watch the thousands of basketball matches with Nino and Andrea and for scouting always new and interesting things to do around the NL.

After about 6 months of student-house time, my time had come to find my stable place in Utrecht. And with a lot of luck, my new house become a home, and the people living there my family, my beloved **Van Swinsi.**

**Rohith, Toby and Lotte**, I can not imagine my life in Utrecht without you. This amazing mix of cultures, culinary background and funny game-nights made my stay in the NL much better. And now I am so happy we became aunt and uncles of the lovely *Jess*!

## I TERRONI

There is a very popular saying in Italian that states “I veri amici si vedono nel momento del bisogno” (a friend in need is a friend indeed – sorry but the English version is terrible!). It was exactly the 13/11/2017, Italy vs Sweden, last chance for Italy to qualify for the World Cup and “c’era tanto bisogno di amici”. We all know how it ended, and many of you reading this sentences will still rub away a tear from their faces (if that is the case: remember that we are EUROPEAN CHAMPIONS at the moment ;)). But something good happened that night in front of the TVs at the Mick O’ Connells, a few strangers Italians offered their shoulders to cry our misery.

Somehow that bond was stronger than expected and something magical called “Terroni chi piú chi meno” was just about to begin.

**Massi e Gabri**, grazie per aver offerto quelle larghe spalle su cui piangere, quel paio di birrette per dimenticare e per aver fatto le facce confuse dopo aver ammesso di tifare Reggina. Sono passati quasi 5 anni da quel momento, nel frattempo l’Italia ha vinto un Europeo (che abbiamo rigorosamente seguito insieme) e ora rischia (aridaje) di non qualificarsi ai Mondiali; ma noi siamo sempre qui, sempre di piú, sempre piú belli, sempre piú dottori. Ognuno di voi é qualcosa di speciale nel mio cuore e per ringraziarvi descrivo un po’ come vi vedo e tutto il bello che mi avete donato:

**Massi**, l’amante degli anni ‘80 e il mio fedele compagno di tre sette in Calabria; **Gabri**, il politico e la persona che da piú soddisfazioni del mondo quando apre un regalo; **Serena**, la chimica idealista che combatterá a suon di insulti la sua innocenza a “Secret Hitler” ma che ti dimostrerá sempre un bene infinito; **Elena**, la PhD itinerante e la musicista rock del gruppo; **Emanuele**, quello che sembra il bullo ma che sotto sotto é il piú gentile del gruppo; **Silvia,** la piccoletta con piú forza e muscoli di tutti noi messi insieme; **Gabriele**, il designer chic che nasconde un cuore rivoluzionario e alternativo; **Irene**, la donna dale mille passioni, sarta provetta e produttrice di lievito madre tra l una talea e l’altra; **Raffi**, il bassista e fisico teorico che ha sempre la battuta giusta; **Eleonora**, gentilezza e eleganza; **dott. Porpiglia**, dottore da quando é nato, un uomo tutto d’un pezzo (anche quando si veste da samurai per carnevale), **Francesco**, il nostro spacciatore di caffé per anni; **Giovanni Torta**, un nome una garanzia: la tenerezza fatta persona; e i nuovi arrivati: **Greta**, la protettrice dei diritti umani e la mamma adottiva di tutti i gatti di Utrecht; **Enrico**, programmatore pazzo e campione indiscusso di Ping-Pong; **Giuliana**, la siciliana del nostro cuore, riserva indiscussa di cibo e medicine; **Gerardo**, il messicano italianizzato che riconosce piú attori italiani di me; **Fabrizio**, tutto Prosecco e ospitalitá; and the two people that have to bear us speaking Italian all the time: **Laura**, the sweetest and calmest of all that turns in a crazy dancer and party person when needed; and **Susi,** lovely and caring person with a special gift for teaching traditional mexican food.

GRAZIE MILLE per questi splendidi anni e per avermi insegnato la magia del **DAJE**!!!

**LE C.O.I ONE - The Italian Soccer Team**

Un nome una garanzia, per chi parla Italiano hahaha. Un nome che rappresenta un gruppo vero, fatto di mille sfaccettature ed una sola passione: il calcio…ok forse due passioni: il calcio e la voglia di fare festa insieme. Perché in fin dei conti a giocare a calcio facciamo proprio pena, ma a far festa siamo sempre campionesse. Grazie per essere state la luce della domenica mattina (nel senso vero del termine, perché non fosse stato per gli allenamenti non mi sarei mai alzata dal letto cosí presto), grazie per essere state il momento di sfogo della settimana sia sul campo che fuori, perché ognuna di voi era pronta ad ascoltarmi e aiutarmi qualsiasi cosa succedesse. Da portiere e capitano di questa squadra ho spesso preso tanti goal, ma non saranno mai abbastanza a farmi perdere la voglia di proteggere la vostra porta (e voi) con tutta la mia volontá!

Grazie alla **zia Ale** e al **cigno Lucia** per aver avuto la splendida idea di creare questa squadra con lo spirito giocoso e sorridente che le contraddistingue. E per ogni squadra che si rispetti vi ringrazieró a seconda del vostro ruolo in campo; mostrandovi in un paio di righe cosa siete per me.

First of all: **I nostril allenatori**, grazie **Mario e Giuseppe** per essere martiri di questa impresa e per farci ridere e imparare nello stesso tempo. E il nostro amato **presidente**: grazie **Roberto** di essere sponsor indiscusso (paga da bere, presidente paga da beeere!!!) di questo gruppo da anni!

**I miei fedeli difensori: Claudia**, grazie per aver accettato la mia proposta di matrimonio dopo i vari salvataggi della mia pellaccia; **Laura**, il muro, che tra un “che devo fare?!?” e una passeggiatina in campo é diventata invalicabile; **Alessia**, la mia sicurezza (mi raccomando lasciatela a digiuno prima delle partite!); **Chiara**, che sperava nessuno si accorgesse di lei in panchina, facendomi ridere a crepapelle ogni partita; **Lucia**, il cigno e mio leoncino preferito che si preoccupa sempre per me.

**Il centrocampo: Marghe**, colosso centrale e irremovibile; **Rosa**, il mio orgoglio, ha fatto vedere a tutti cosa significa voler diventare una vera giocatrice, **Monica e Sonia**, jolly di posizioni ma sempre pronte a sacrificarsi per la squadra, **Stefania**, vera giocatrice con tecnica e cuore; **Erika e Giuliana**, corridori indiscusse delle nostre fasce; **Michela**, si finge pigra come me; ma non salta un allenamento.

**L’attacco:** la prima vera bomber di questa squadra **Arianna**, correva come una gazzella e aspettava (illudendosi) che qualche pallone arrivasse da lei. Graize per averci fatto fare qualche goal e soprattutto per averci insegnato come salvare il pianeta dalla plastica. **Giulia**, un tornado di energia e forza fisica, spaventa le avversarie con un solo sguardo e punta la porta; **Irene**, sfiora la palla come fa con i suoi dischi, quando parte la sua muscia non c’é scampo; la zia Ale, la nostra gazzella Zen, che ti riporta sempre sulla retta via E poi le nostre special guests preferite: **Sonia**, pugliese e casinista (come piace a noi) ci aiuta sempre nei momenti di difficoltá con un passaggio perfetto in campo o

con l'intonazione di "Le bionde trecce", rigorosamente sulle note della nostra **Mylene**, paziente compagna di avventure.

E poi c'é il capitano. La persona che ha diviso con me questo ruolo per quattro anni. E quando dico diviso intendo davvero che é diventata la mia metá del cuore.

**Bea**, mio capitano, grazie di tutto. Ci sono poche parole per esprimere la tua vicinanza e il bene che riesci a trasmettere alle persone. Grazie per la tua gentilezza, il tuo sorriso e il tuo ottimismo e il tinto de verano che mi ha sempre fatto vedere l'arcobaleno anche nei momenti piú bui (anche nei giovedi degli ultimi mesi!!). Grazie per esserci sempre stata, alla fine del corridoio del secondo piano della UMC, sul campo, ad Utrecht, sei stata presenza costante di questo viaggio. Grazie per essere al mio fianco anche in questo giorno speciale in cui difendo il dottorato come mia paraninfa, sei speciale.

### THE REST OF THE NL

Utrecht has clearly become my home in the last years, but a few people around the NL have always let me feel at home in other cities too!

Thanks to the **Wageningen Squad**: thanks to Nino I have got to know a lot of lovely and interesting people that became part of our life. Grazie **Agata e Antonio**, per sopportare giornalmente Nino al mio posto :P. Grazie per la vostra follia e terronicitá! Grazie **Righe, Enrica, Bram e Sara** for the basketball matches watched together and the amazing shared food, siete i miei STRONZI preferiti! Grazie **Johanna**, for being my "art and craft" sister, for the girls afternoons and the sushi nights, but also thanks to **Jan**, for all the knowledge on food and whiskey and the little *Manuel* for filling with smiles our days.

Grazie **Ros, Rudy (and Alexis**) for our American reunions and your enthusiasm in following my career and life steps.

### GLI AMICI DI SEMPRE

Surely my life in the Netherlands has been full of new people and new friends, but there are some that have been there before (as I say from the title, "have always been there). Grazie agli amici di sempre, che cito per ultimi non perché sono meno importanti, ma perché hanno un posto nella parte piú sicura del mio cuore, quella che nessuno puó piú toccare.

Grazie alle **Trullo amiche**. Non avrei mai pensato entrando in quella classe al San Raffaele, piú di 10 anni fa, che avrei trovato sette ragazze che sarebbero diventate tra le mie piú care amiche. Nessuno avrebbe mai detto che la nostra amizia sarebbe durata "troppo diverse, troppo sparse per il mondo" eppure niente di tutto questo ha mai scalfito quello che abbiamo solidamente creato. Grazie per le chiacchierate costanti,

per i commenti alle presentazioni e ai papers, per le reunion in giro per il mondo. Grazie ad ognuna di voi in modo diverso. Grazie **Silvia**, per aver ascoltato ogni mio dubbio e per avermi aiutato a trovare risposte con chiacchierate lunghe ore; grazie **Ceci**, per essermi sempre stata accanto e aver sempre capito tutto ciò che avevo dentro con un solo sguardo; grazie **Elena**, per tutte le volte che mi hai fatto conoscere un canale nuovo di cucina o di comunicazione scientifica, per I tuoi "peaky comments" che mi hanno reso una persona, cuoca e scienziata migliore; grazie **Valto** pertutti i tuoi consigli sull'armocromia e la fisioterapia e grazie a te e *Nick* per il nostro primo fantastico nipotino *Edo*! Grazie **Luci**, per la tua modestia e immensa intelligenza che ci fa credere che possiamo essere tutte brave come te (anche se palesemente non é vero!! Ahah) e grazie per i messaggi vocali con *Beppone* su questioni di estrema importnza sociale/ scientifica ("ma chi gioca a basket diventa piú alto?!?!"); grazie **Marghe**, per la tua grinta e spensieratezza nell'affrontare la vita, ci hai sempre insegnato tanto!

Grazie a quelli dello **SBARRIO**, quelli che sono cresciuti piú o meno nel giro di 300 metri, che hanno visto trasformazioni inaspettate in ognuno di noi, che mi hanno visto andare da Milano, a Philadelphia, all' Olanda. Grazie a **Pietro, Andrea, Francesco, Umbe, Mario, Luana, Coco, Noemi e Teresa.** Voi ci siete e ci sarete sempre... perché possiamo andare a vivere in qualsiasi posto del mondo ma nel cuore rimaniamo sempre sbarroti. Grazie speciale alle mie **Pucci Purpette**, quelle amiche che non vanno molto d'accordo con il cellulare ma quando si vedono sono come sorelle; **Luana**, la mia designer e amatissima rana; **Teresa**, cugine di sangue ma amiche per scelta, razionale e ascoltatrice; **Noemi**, sognatrice un po' Peter Pan che ci rievoca sempre il bimbo dentro di noi.

**Vale e Sam**, amici di sempre significa anche riuscire a diventralo in poco tempo. Grazie per il vostro essere drolli, la vostra ospitalità e la vostra simpatia. Purtroppo per voi, ormai ci sentiamo proprio a casa in Sardegna!

Quel che rimane della **IIIE**. **Vincenzo**, grazie per essere accanto a me da quando avevamo 4 anni, criticando sempre le mie scelte ma nello stesso tempo guardandomi con occhi orgogliosi. Viva il comunismo! **Gerardo**, grazie per essere il mio piú caro confidente e medico di fiducia. In entrambi I casi basta parlare con te per sentirmi al sicuro. **Gionni**, grazie per la tua indiscrete presenza, per non dimenticarti mai di un compleanno e per la gioia nel continuare a vedere a distanza di anni che quel rapporto da conquilini non é mai cambiato (come non é cambiata la tua voglia di mandarmi i video mentre canti in macchina). **Martina** (Stitch), grazie per avere sempre una parola di incoraggiamento e un abbraccio caldo per me.

**LA FAMIGLIA**

La famiglia per me rimane qualcosa di cosí bello e complesso da spiegare. Vivere lontani dalla propria famiglia non é mai semplice, ma é anche un modo per ritrovarsi, per sentirsi piú uniti che mai. Vivere lontani significa trovare tante famiglie adottive, acquisire indipendenza e nuove abitudine; ma significa anche sapere che come la tua famiglia non c'é nessun altro, che le tue radici sono piú salde di prima, che ci sará sempre un posto e delle persone dove tornare che saranno la tua vera casa. Penso sempre alla mia famiglia come tanti cerchi, chi da più lontano e chi da più vicino mi abbraccia e mi accompagna sempre.

Grazie a chi famiglia é diventato da ormai parecchi anni. Grazie a tutti i **Crucitti/Miriello** che mi considerano ormai parte integrante del loro cerchio, in cui mi sento sempre a casa. Un grazie speciale ad **Emy, Angelo e Cristina** che mi fanno sentire l'amore di una seconda famiglia, l'interesse di chi ti guarda con occhi orgogliosi e ti sosterrà sempre. E un grazie speciale per tutto il cibo che ci fate arrivare per consolarci nelle fredde giornate d'inverno!

E poi un grazie infinito alla famiglia **Lo Presti/ Mellina**. Grazie zii e cuginetti, per le vostre case aperte ogni volta che sono a casa, il vostro gentile ricordi di ogni ricorrenza e il senso di appartenenza che ci lega da sempre. Grazie speciale a **zia Sabrina**, che continua a coccolarci come quando eravamo piccoli.

Grazie ai miei cari nonni. A **nonna Lina e nonno Giorgio**, alla forza del loro ricordo indissolubile, sono sempre con me con le ricette di nonna e le frasi tipiche del nonno. Graize **nonno Bibi** per essere sempre stata la tua principessa. Ci hai lasciato con il sorriso sulle labra e la voce dolce e solare che ti ha sempre contraddistinto. Non é stato facile accettare di non vederti e sentirti piú, ma so di averti accanto sempre, specialmente ogni volta che faccio un cruciverba o un bagno al mare, e ogni volta che, come con questa tesi, raggiungo un obiettivo. So che siete fieri di me, ovunque voi siate. Grazie **nonna Gianna**, baluardo di questa famiglia e fonte di ricordi e memorie. Grazie per esserci sempre e, anche se fragile, per tenerci insieme con la tua forza. Grazie per le nostre chaicchiere della Domenica pomeriggio, che mi fanno sentire a casa.

Grazie fratellone, la parte migliore di me. **Bebbo**, il mio scudo, la mia forza, semplicemente la mia persona da quando sono nata. Niente e nessuno potrá mai essere quello che tu sei per me. Con il tuo affetto (a volte anche appiccicoso), il tuo essere cosí diverso da me (specialemente a livello di lentezza), le nostre telefonate e le tue vacanze qui in Olanda non mi sono mai sentita sola per un secondo. Le persone dicono spesso che il rapport tra fratelli é difficile, per me invece rimane la cosa piú spontanea, trasparente e bell ache ci sia. Spesso vorrei potermi guardare con I tuoi occhi, perché attraverso di te mi sento sempre una persona migliore e non potró mai ringraziarti abbastanza per questo.

**Mamma e papá**, un grazie infinito a voi. Siete da sempre dei genitori fantastici. E io da sempre approfitto dei ringraziamenti delle tesi per ricordarvelo. Anche questa volta, come per le alter tesi, scrivo questi ringraziamenti da molto lontano. Eppure voi questa lontananza non me l'avete mai fatta pesare, mai una battuta, mai una richiesta di tornare a casa. Questo per me rimane il piú grande gesto d'amore di tutti, mi avete sempre lasciato libera di percorrere la mia strada, di fare le mie scelte e di crescere, senza interferire, ma senza mai lasciarmi sola, con quell'amore incondizionato di chi ti guarda da lontano e non vede l'ora di riabbraciarti. La distanza pesa, ma quando c'é la vicinanza del cuore poco importano i km che dividono. Grazie per i messaggi di fine giornata, i "reminder" che sta iniziando la partita della Reggina, le imitazioni di papá che si finge mamma al telefono, le discussioni di politica e  i milioni di PACCHI DA GIÚ che ci sfamavano durante l'anno!

Ed eccoci arrivati al cerchio piú piccolo di questa famiglia.

**Nino**, il grazie piú grande va a te che riempi quell'ultimo cerchio di amore ogni giorno. Grazie per avermi spronato ad iniziare questo dottorato, grazie per avermi consolato quando all'inizio mi sembrava tutto diverso e difficile, grazie per avermi accompagnato tenendomi per mano in ogni passo di questo percorso e grazie per essere stato il porto sicuro nei momenti di sconforto finale. Sei la persona che ha ascoltato ogni mio successo e ogni mio fallimento, ha imparato cosa sono le cellule T e gioito per un "successful KO", sei la persona che mi ha tirato su il morale se un paper veniva rigettato o le cellule morivano durante l'esperimento. Grazie per esserti sempre preso cura di me. Grazie per aver capito che a volte dietro quel sorriso che facevo vedere al mondo c'erano insicurezze e sconforto. Con una battuta stupida, con una buona cena, con un abbraccio, con una passeggiata, con "cane pazzo", con un concerto, con un viaggio insieme hai sempre fatto il massimo per far riapparire il mio sorriso genuino e l'arcobaleno nei miei pensieri. Grazie per amarmi con la felicitá negli occhi, senza chiedere niente e senza voler cambiare nulla di me. La nostra vita insieme (da veri coinquilini!!) è appena iniziata, e nonostante la pandemia, sono sicuramente stati tra gli anni piú belli della mia vita. Non vedo l'ora di vedere cosa ci riserva il futuro, ma sono sicura che sará insieme…

*" 'cause even when there is no star in sight*
*You'll always be my only guiding light!"*

- Mumford and sons


Grazie a tutti!!