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Towards GMP production of γδTCR engineered T cells

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Towards GMP production of γδTCR engineered T cells

DEVELOPING A UNIVERSAL CELL THERAPY TOOL

MASTER THESIS PROJECT

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Resumo

A leucemia mieloide aguda (AML) é uma das mais frequentes doenças malignas hematológicas descritas, e é caracterizada como uma anomalia heterogénea clonal de células progenitoras do sistema hemapoiético. Assim, células progenitoras hematopoiéticas (também designadas por "blasts") perdem descontroladamente a habilidade de se diferenciarem normalmente, incapacitando-as de responder a reguladores de proliferação. Consequentemente, depois do diagnóstico inicial, hemorragias, infecções, ou infiltrações em órgãos podem ser fatais na ausência de tratamento.

Ao longo dos anos, enquanto o uso de quimioterapia falhava em demonstrar eficácia em doentes que sofrem de AML, a imunoterapia surgiu como uma das estratégias mais promissoras para tratamento destes pacientes. Usando diversas estratégias, o transplante alogénico de células estaminais surgiu como tratamento de primeira linha. No entanto, a rejeição do transplante pelo recipiente (GVHD) ou a recaída de AML acarretam riscos fatais em pacientes que foram submetidos a este transplante. Com o intuito de fornecer eficácia anti tumoral e uma proteção duradoura, várias abordagens foram empregues, como a vacinação de células dendríticas ou infusão linfocítica (DLI). Porém, esta última não permite um controlo tumoral, impulsionando esforços na procura de soluções mais eficientes.

Atualmente, avanços na engenharia genética estabeleceram uma plataforma que permite a transferência genética para células T de recetores de células T (TCRs) com alta reatividade tumoral. O uso de receptores $\alpha\beta$ de células T ($\alpha\beta$TCRs) e recetores de antigénios quiméricos (CARS) demonstram limitações devido a restrição HLA, toxicidade "off-target" e imunogenicidade. Consequentemente, uma maior atenção tem-se dado a certos tipos de células T $\gamma\delta$ e aos seus recetores ($\gamma\delta$TCRs), devido ao reconhecimento de células em stress (células tumorais) não restrito pelo sistema antígeno leucocitário humano (HLA). Num contexto de terapia celular adotiva (ACT), este recetor de imunidade inata/adaptiva pode ser usado em células T $\alpha\beta$ para matar células tumorais sem as limitações dos TCRs e dos CARs, algo demonstrado pelo nosso grupo.

Nesta tese, centrámos os nossos objetivos no sentido de desenvolver um protocolo de boas práticas de fabricação (GMP) para a produção de células T geneticamente modificadas com o um recetor de células T $\gamma\delta$ altamente reativas a células de leucemia mieloide aguda. Para este fim, iremos otimizar diversos passos de um protocolo de investigação de transdução retroviral. Neste projeto, inserimos um Vγ9Vδ2 TCR clone 5 em células T $\alpha\beta$ estimuladas com anti-CD3/CD28 beads. Depois da expansão com IL-7 e IL-15, as células T geneticamente modificadas foram separadas de células T pouco ou não modificadas, usando um novo passo de depleção de células que expressam $\alpha\beta$TCRs. Como resultado fomos capazes de gerar uma grande população de células T geneticamente modificadas com $\gamma\delta$TCR, memoria efetoras e efetoras como estado de diferenciação fenotípica. Sobretudo, quando este produto celular foi testado contra amostras tumorais primarias de AML, estas células T geneticamente modificadas com $\gamma\delta$TCR libertaram INF-γ. O sucesso no escalonamento da produção destas células e o novo passo de purificação

destas, garantem uma fácil transição para a clinica, permitindo o seu uso em ensaios clinicos de fase I em pacientes de AML de baixo risco que sofreram um transplante alogénico de células estaminais (allo-SCT).

Palavras-chave: transplante alogénico de células estaminais (allo-SCT), terapia celular adoptiva (ACT), recetor γδ de células T (γδTCR), boas práticas de fabricação (GMP), células T geneticamente modificadas com γδTCR

Abstract

Acute Myeloid Leukemia (AML) is one of the most frequent hematological malignancy within those described to date. It is characterized as a heterogeneous clonal aberrance of progenitor cells of the human hematopoietic system. Hereby, these hematopoietic progenitor cells ("blasts") disorderly lose the ability to undergo a normal differentiation process consequently exerting a poor response to regular proliferative regulators. Therefore, after an initial diagnosis, fatal infections, organ infiltration and bleeding are consequences of this loss in the absence of treatment.

Throughout the years, whereas chemotherapy regimens fail to prove efficacy to accelerate and lengthen the remission state in patients suffering from AML, immunotherapy surfaced as one of the most promising AML therapeutic approaches. As several allogeneic-stem cell transplantation (allo-SCT) strategies remain first line treatments available for AML patients, graft-versus-host-disease (GVHD) or the relapse of the AML patient become major life-threatening complications post-allo-SCT. To deliver anti-tumor efficacy and long-term protection multiple approaches have been employed, such as dendritic cell (DC) vaccination or donor lymphocyte infusions (DLI). Nonetheless, this latter does not provide proper tumor control, driving efforts to seek more efficient solutions.

Currently, genetic engineering has evolved to provide a platform to transfer highly tumor reactive T cell receptors (TCRs) into T cells. The use of tumor specific $\alpha\beta$ T cell receptors ($\alpha\beta$TCRs) and chimeric antigen receptors (CARs) show their limitations due to HLA-restriction anti-tumor activity, off-target reactivity and immunogenicity. As a consequence, certain subsets of $\gamma\delta$ T cells and their receptors ($\gamma\delta$TCRs), have arisen attention due to their non-HLA restricted recognition of stressed cells (tumor cells). In the context of adoptive T cell therapy (ACT), this innate-like receptor can be used in $\alpha\beta$ T cells to deliver anti-tumor activity in tumor cells without the limitations of $\alpha\beta$TCRs or CARs, as demonstrated by our group.

In this thesis, we set our aims towards developing a good manufacturing production (GMP) protocol for the production of AML-reactive $\gamma\delta$TCR engineered T cells by optimizing several steps of a research-grade retroviral transduction protocol. We inserted a highly tumor reactive Vγ9Vδ2 TCR derived from clone 5 in anti-CD3/CD28 bead stimulated $\alpha\beta$ T cells. Following expansion with IL-7 and IL-15, engineered T cells were purified from poorly/non-engineered T cells using a novel $\alpha\beta$TCR depletion step. As a result, we were able to generate a major population of highly purified $\gamma\delta$TCR engineered T cells in an effector memory and effector differentiation phenotype. More importantly, when challenged against AML primary tumor samples, this engineered T cell product is able release INF-γ. The successful upscaling of the manufacturing process of this genetically modified T cell product using a novel purifying method, grants an easy translation for a clinical-grade setting enabling its use in a phase I clinical trial on poor/low risk AML patients that have undergone allogeneic – stem cell transplantation (allo-SCT).

Keywords: allo-stem cell transplantation (allo-SCT), Adoptive T cell transfer (ACT), γδT cell receptor (γδTCRs), Good Manufacturing Practice (GMP), γδTCR engineered T cells

Abbreviations

^{51}Cr	^{51}Cromium
AICD	Antibody Induced Cell Death
All-SCT	Allogeneic Stem Cell Transplantation
AML	Acute Myeloid Leukemia
BTN3A1	BuTyrophiliN subfamily 3 member A1
CAR	Chimeric Antigen Receptor
CD	Complement Determinant
CLL	Chronic Lymphocytic Leukemia
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
DLI	Donor Lymphocyte Infusion
DMEM	Dulbecco's Modified Eagle Medium
ELISpot	Enzime Linked Immunosorbent Spot Assay
EMEA	European Medicines Agency
FACs	Flow-Assisted Cell Separation
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
GMP	Good Manufacturing Practice
GVHD	Graft-versus-Host-Disease
GVL	Graft-versus-Leukemia
HLA	Human Leukocyte Antigen
Ig	Immunoglobulin
IL-(x)	Interleukin
INF-(x)	Interferon
iPSC	induced-Pluripotent Stem Cell
KIR	Killer Cell Immunoglobulin-like Receptor
LTR	Long Tandem Repeat
MACs	Magnetic Cell Separation
MART -1	Melanoma Antigen Recognized by T cell -1
MCB	Master Cell Bank
MHC I	Major Histocompatibility Complex Class I
MHC II	Major Histocompatibility Complex Class II
MPL	Myeloid Progenitor cell
NGFR	Nerve Growth Factor Receptor
NK	Natural Killer cell
NKG2D	Natural Killer cell Group 2D
PAM	Pamidronate

PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PD1-L	Programmed Cell Death 1 – Ligand
T_{CM}	T Central Memory phenotype
TCR	T Cell Receptor
T_{EFF}	T Effector phenotype
T_{EM}	T Effector Memory phenotype
Th1	T helper cell type 1
Th2	T helper cell type 2
TIL	Tumor Infiltrating Lymphocyte
T_N	T Naïve phenotype
Vγ9Vδ2	Variable gamma chain 9 Variable delta chain 2
αβ T cell	alpha beta T cell
γδ T cell	gamma delta T cell
αβ TCR	alpha beta T cell receptor
γδ TCR	gamma delta T cell receptor

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1. Introduction

Cancer has been extensively studied during the last century, being characterized by the transformation of normal cells into tumor cells, in a process called tumorigenesis. The dynamics underlying this process are related to a complex heterotypic set of interactions, between an aberrant proliferative cell mass and the tumor microenvironment. The immune evasion remains one of the most intriguing hallmarks of cancer, in which tumor cells limit the immune surveillance by avoiding detection or by hampering the cytotoxic killing of the immune system [4]. Indeed, this fact renders the opportunity to exploit the immune system as a therapeutic tool to treat cancer, especially in hematological malignancies that very often evade the immune responses [5, 6].

1.1. Acute Myeloid Leukemia

Acute Myeloid Leukemia (AML) is one of the most frequent type of disorders within the leukemias described. It is characterized as a heterogeneous clonal aberrance of progenitor cells of the human myeloid lineage of blood cells. The onset of the disease happens when myeloid progenitor cells (MPLs) disorderly lose the ability to undergo a normal differentiation process. These are often designated by AML blasts. The fact that AML blasts lack sensitivity to a wide spectrum of chemokines, responsible for controlling growth and proliferation, causes an uncontrolled growth of MPLs [7]. Therefore, after an initial diagnosis, fatal infections, organ infiltration and bleeding are consequences of this loss, in the absence of treatment [8].

1.1.1. Chemotherapy

Traditional treatment for AML included the use of chemical formulations - chemotherapy - to kill AML cells. Upon now, strategies using solely antibiotics [9], alkylating agents [10, 11] or, more recently, the use of targeting drugs [12], remained inefficient against this hematological malignancy [13]. The acquisition of resistance poses a major threat to AML patients, due to the vast heterogeneity of the malignant cell population. In fact, chemotherapy acts on active proliferating transformed cells, consequently some cellular populations, particularly low cycling stem-like cells remain unaffected while others, mostly the differentiated cells, respond better. Furthermore, it also appears that within a tumor, resistance cellular phenotypes may develop followed prolonged treatments increasing the risk of relapse after treatment [2].

1.1.2. Immunotherapy

1.1.2.1. Allogeneic Stem cell transplantation (Allo-SCT)

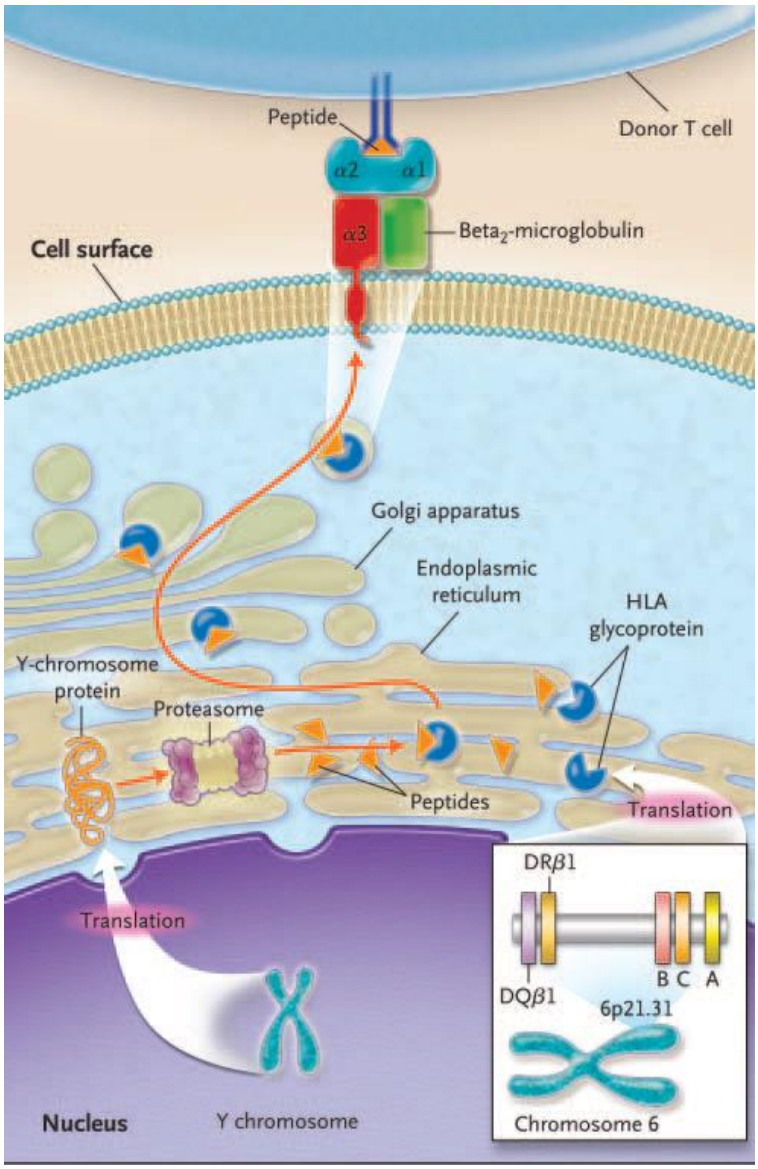

Figure 1 - Mechanism of Graft-versus-Leukemia mediated by Minor Histocompatibility Antigens. *The Y-chromosome gene encodes a protein of a male graft recipient that will subsequently be degraded by a proteasome. Therefore, this protein is transported through the endoplasmatic reticulum, where a HLA glycoprotein (encoded by HLA-complex of genes in chromosome 6) binds to it. Subsequently, the peptide bound to the HLA glycoprotein (class I) will travel together along the Golgi apparatus reaching the cell surface, where it will be recognized by female donor T cells as foreign. The class I gene encodes 4 subsets: (1) the peptide-binding domains (α1 and α2), (2) the immunoglobulin-like domain (α3), (3) the transmembrane domain and, (4) the cytoplasmatic domain. Beta2-microglobulin is encoded by chromosome 15 (not shown). Adapted from Copelan E. A. (2006)[2]*

Another therapeutic approach is the use of the immune system to kill AML blasts. The allogeneic stem cell transplantation (allo-SCT) – the infusion of stem-cells with immune cells from a healthy donor [14] into a patient - is a procedure being used for more than 50 years, and can be used either to consolidate the chemotherapy treatment, or as primary AML treatment. Yet, this strategy still lacks refinement as the success and major issues after the transplantation rely on an array of reciprocal immune responses between the donor cells and the recipient cells. The key events of these allogeneic grafts-resulting immune-reactions are associated with histocompatibility, particularly human leukocyte antigen (HLA) molecules of both class I and II. Hence, major histocompatibility (MHC) molecules derived from the HLA system have the role to alert the immune system by binding peptide derivatives marking cells for the T cell recognition. The fact that the HLA system is polymorphic, render cells from different individuals to be incompatible in the presence of a dissimilar original immune system. In the context of an allo-SCT, T cells from the recipient ought to detect and recognize donor cell antigens possibly rejecting grafts [2]. In contrast, donor T cells detect and recognize malignant cells (such as AML blasts) causing a graft-versus-leukemia effect (GVL), in which donor cells attack the tumor leading to its eradication [15], or a graft-versus-host-disease (GVHD), a misdirected immune response in other cell tissues other than the tumor

cells. Therefore, the vast majority of strong transplant reactions correlate with the incompatibility of major histocompatibility antigens (HLA) between the donor and the recipient. The generation of a range of degraded peptides from donor HLA molecules is directly proportional to the increase in the acuteness of the reaction. Other than major histocompatibility antigens, small peptides derived from a range of polymorphic proteins, namely minor histocompatibility antigens, can be presented by a minority of HLA molecules and are bound to initiate weak reactions, in contrast to major antigens. Different responses in the incidence of GVHD and relapse rate of AML have been observed, depending on the gender accounting the fact that some of the minor histocompatibility antigens are chromosome Y encoded. Generally, T cells derived from the donor are minor histocompatibility antigen reactive and act on proliferating cells leading to AML colonies inhibition, hereby called the so-called GVL effect previously mentioned [2] (Fig. 1) .

Overall, despite the fact that allo-SCT increases the survivability of high risk of hematological malignancies, the outcome of this strategy is still poor due to the high risk in developing life-threatening GVHD after this intervention, culminating in the patient relapse [14].

1.1.2.2. Dendritic Cell Vaccination

The GVL effect can also be stimulated by vaccination containing antigen presenting cells - dendritic cells (DCs) [16]. Thus for example, when loaded with tumor antigens [17], they stimulate antigen-specific cytotoxic T lymphocytes (CTLs) to target tumors expressing those antigens, towards a T helper cell type 1 (Th1) immune response (responsible for the inflammatory immunity against tumor cells) [18]. Besides the different and strategies to generate DCs for vaccination, this modality of treatment always rely on other cell subsets of the immune system. Consequently, insufficient stimulation of antigen specific-CTLs [19, 20] and natural killer cells (NKs)[16], a shift from Th1 to T helper cell type 2 (Th2) immune response (anti-inflammatory immunity against tumor cells) and the unwanted stimulation of T regulatory cells [21], are all part of the DC vaccination flaws to treat AML [16].

1.1.2.3. Adoptive T cell therapy (ACT)

Targeting malignant cells by expanding and infusing donor T lymphocytes into AML patients has become one of the most promising treatment approaches to complement allo-SCT. Adoptive T cell therapy comprises a T cell source that can be either autologous (from the patient), autologous (from a healthy donor) and are derived from antigen-specific T cells, peripheral blood mononuclear cells (PBMCs), tumor infiltrating lymphocytes (TILs) or, more recently, from induced pluripotent stem cells (iPSCs) [22](Fig. 2).

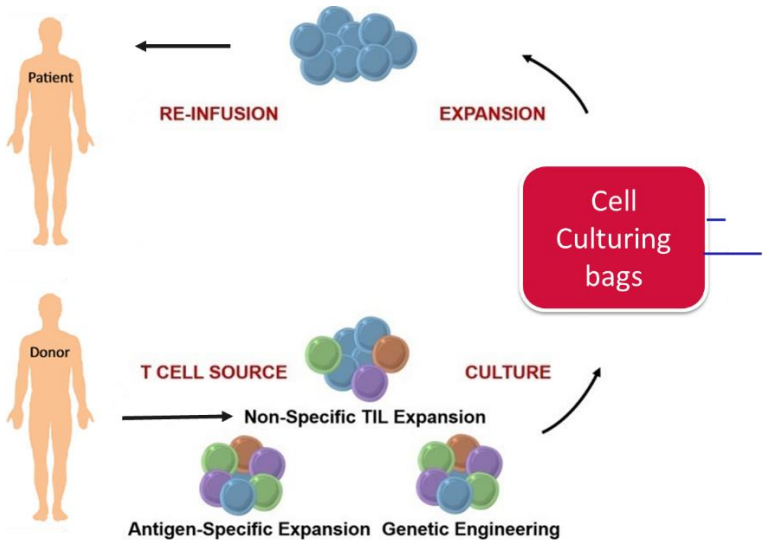

Figure 2 – Schematic synopsis of adoptive T cell immunotherapy. *Antigen specific T cells, Tumor infiltrating lymphocytes (TILs) or normal T cells (for genetic modification) are acquired from an healthy donor (allogeneic) or the patient for expansion in cell culturing bags. After this latter, re-infusion into the patient is done. Adapted from Perica et al. (2015) [23]*

This intervention, accounts for the need of less toxic stem cell transplantation regimens that create space for early immune interventions and, that do not need long term immune suppression. This can be achieved by the complete or, partial removal of immune cells from the allogeneic stem cells before transplantation (Fig. 3). Therefore, a T cell reconstitution via donor lymphocyte infusion (DLI), either using endogenous T cells or exogenous T cells, will result in lower transplantation-related mortality comparing to non-depleted T cell reconstitution after allo-SCT [24]. Selectively depleting subsets of CD3+ T cells [25] or enriching for CD34+ cells while completely deplete CD3+ T cells [26] are alternatives that result in low long-term GVHD in AML patients [14]. Depletion of $\alpha\beta$ T cells (a subset of T cells containing $\alpha\beta$ T cell receptors) from the allo-graft, is an alternative that maintain innate immune cells such as $\gamma\delta$ T cells and natural killer cells (NK cells), capable of tumor and infection control, preserving healthy tissue [27]. Nonetheless, the beneficial effect of this early innate immune reconstitution remains a concern due to the uncertainty of a required repertoire capable of controlling tumors and infections [28].

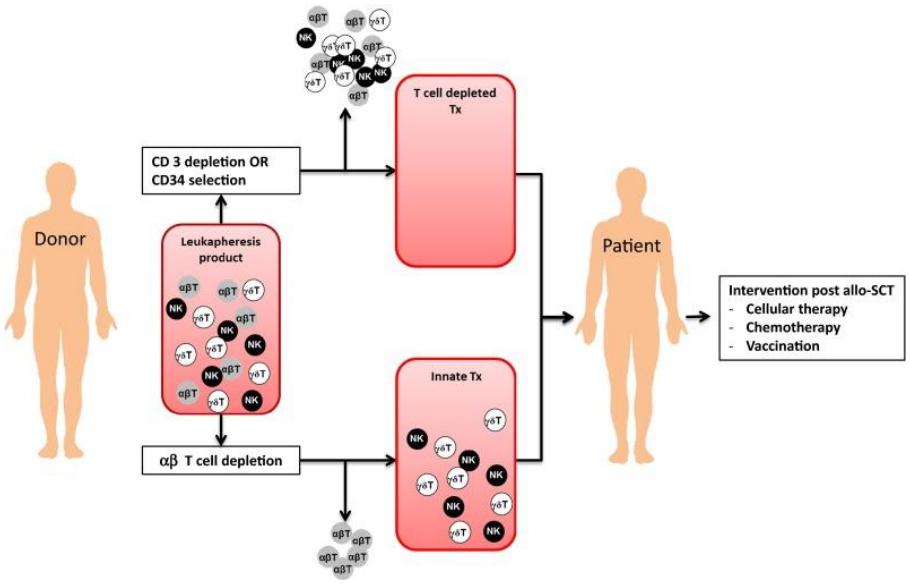

FIGURE 3 – Platforms for Low-GVHD allo-SCT. *T cells can be removed from the graft by CD34+ selection or CD3 depletion (upper part); Innate-allo-SCT can be generated by depleting $\alpha\beta$ T cells, allowing NK and $\gamma\delta$ T cell in the graft; Other interventions can be done post allo-SCT; Adapted from de Wite et al. (2015)[14]*

As DLI is the most potent immune intervention post-allo-SCT, it has been used for years in to treat relapsed patients in hematological malignancies, such as AML [29, 30]. However, the use of DLI can cause GVHD and does not necessarily provides tumor control [14]. The unpredictability of the repertoire presented in the total T cell pool is a major threat, as the frequency of T cells capable of causing GVL effect accounts for a small percentage [28].

1.1.2.3.1. Genetic engineering of T cells

Due to the need to improve the clinical outcome of the strategy mentioned above, the concept of engineering T cells with tumor specific receptors became a safe and effective approach [31]. Genetic modification of T cells can be done at several levels but a great majority of them are focused on the specificity of the T cell receptors (TCRs) and chimeric antigen receptors (CARs). For this purpose, several gene delivery methods were made available, comprising viral or non-viral based strategies. Due to great clinical successes and low insertional mutagenesis (in the gene intended to deliver), the majority of TCR gene transfers use retroviral vectors in a retroviral transduction protocol [32]. This strategy consists in a RNA sequence encoding a TCR gene that is released in the cytoplasm of the T cell and reversed transcribed to a DNA sequence in order to enter the nuclear envelope of the cell. Following mitosis in activated T cells, the desired DNA sequence is integrated into the T cells genome. Integration in the T cell genome is promoted by the long terminal repeats (LTRs) and a viral enzyme integrase. Indeed, to avoid pathogenicity due to replication competent retroviruses, mammalian packaging cell lines were created to overcome safety issues. These express *gag* (core proteins), *pol* (reverse transcriptase integrase) and *env* (envelope

proteins) proteins without generating infectious particles since the encoding mRNAs do not harbor the Ψ packaging signal. As result, virus particles do not encode integrase or reverse transcriptase proteins rendering the viruses the inability to replicate (Fig. 4) [1].

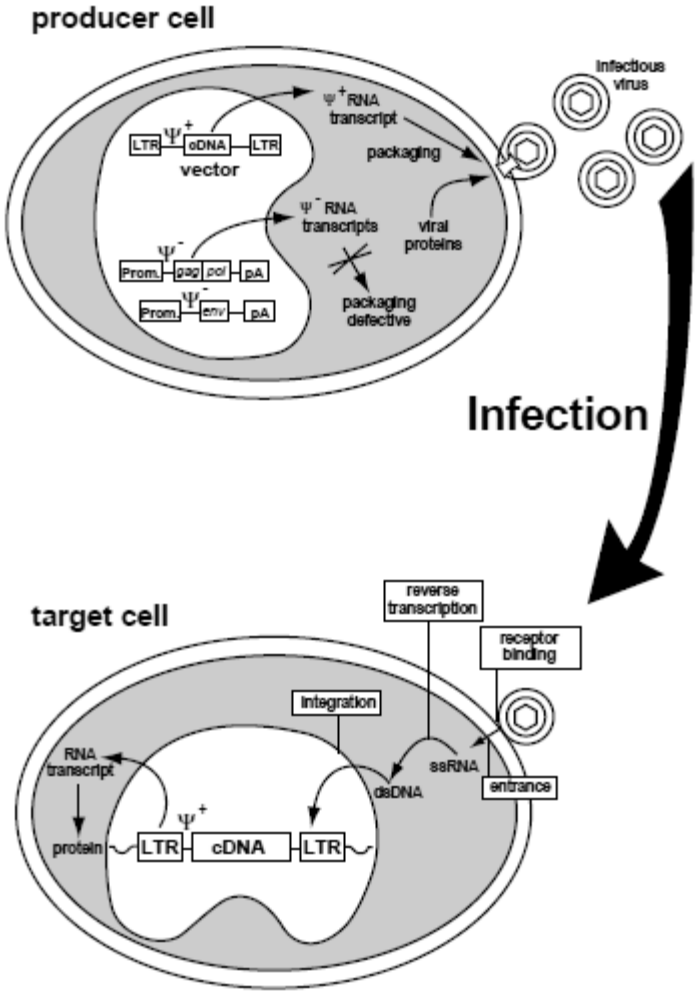

Figure 4 – Overview of retroviral transduction – *Packaging cell lines produces replication-defective infectious particles containing an intended packaged Ψ+ RNA sequence without gag, pol and env proteins; Target cells are infected with these virus particles and, by the usage of a host reverse transcriptase and integration via LTRs the gene is inserted in cells without viral replication safety issues. Adapted from Havenga et al. (1997) [1]*

1.1.2.3.2. The use of chimeric antigen receptors (CARs)

Modifying T cell specificity can comprise the introduction of a chimeric antigen receptor (CAR). These are composed of an extracellular antigen-specific single-chain variable fragment (scFv) linked to an intracellular signaling domain, and are derived from antibodies, thus endowed of non-MHC-restricted molecule recognition [33](Fig. 5). These have been successfully used in target tumor antigens. Recently, chronic lymphocytic leukemia (CLL) has been successfully targeted and treated using an anti-CD19 CAR [34]. Despite the promising results, potential immunogenicity might result in toxicity for the patient [35].

1.1.2.3.3. The use of αβ T cell receptors (αβTCRs)

Effector T cells have been extensively studied and two major subsets according to their T cell receptors (TCRs) – αβ and γδ T cells have been identified. αβ T cells (expressing αβTCRs) were the first T cell subtype to be genetically modified to express anti-tumor TCRs (Fig. 5). Currently, MART-1 (melanoma antigen recognized by T cells 1) αβTCR engineered T cells, have been used to successfully treat melanoma [36, 37]. On the other hand, the fact that αβTCRs recognize HLA-derived peptides presented by the MHC complex prevent these cells from causing an anti-tumor effect in other recipient other than the patient [22]. In addition, endogenous natural αβTCRs can still be expressed upon modification with a specific anti-tumor αβTCR, causing allo-reactivity when these tumor-specific αβTCR engineered αβ T cells are transferred in allogeneic setting [38].

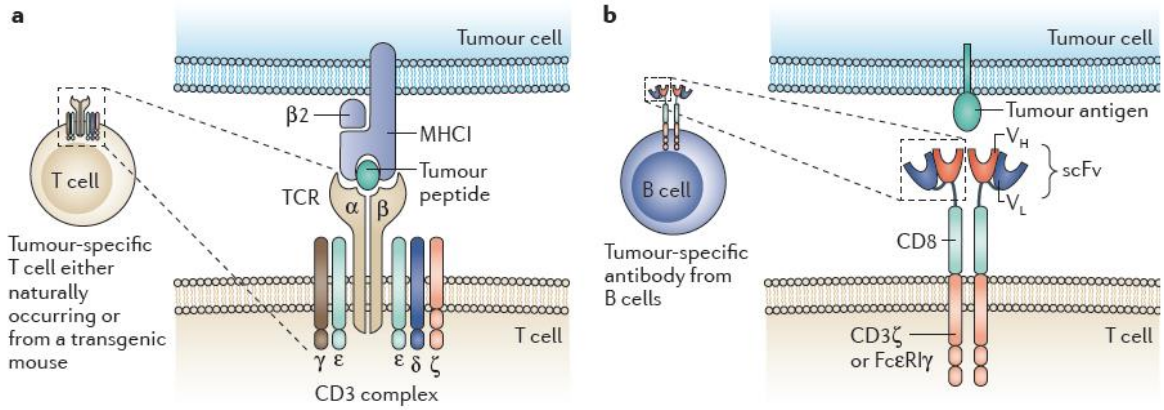

Figure 5 - Differences between TCRs and CARs. *(a) T Cell Receptor (TCRs) genes, composed of α- and β-chains, can be generated from different T cells, which can possibly occur naturally in the human body, or by humanization of human-leukocyte antigen (HLA)-transgenic mice. In contrast, they can also be generated from bacteriophages libraries of antibodies. Then the α- β- chains can associate with the chains from CD3 complex (γ- δ- ζ- and ε-chains. Finally, when the TCR recognizes a processed antigen displayed by the MHC complex of the tumor cell, activates the T-cell through a series of signaling events leading to a final secretion of cytokines and cytotoxic compounds. (b) Chimeric antigen receptors (CARs) have a single-chain antibody variable fragment (scFv) extracellular domain linked through a hinge and transmembrane domains to a cytoplasmatic signaling region. The genes that encode scFv domain derive from activated B cells. CARs recognize tumor antigens with no requirement for MHC presentation. Adapted from Kershaw et al. (2013)[3]*

1.1.2.3.4. γδT cells

Raising attention has been given to γδ T cells (expressing γδTCRs). The functions performed by γδ T cells are interestingly wide such as, cytolysis, Immunoglobulin-E (IgE) induction, antigen presentation, production of growth factors, among many others [39] (Fig. 6). Indeed, the possibility that γδ T cells have to differentiate into regulatory mediators promoting control over adaptive anti-tumor immune responses,

renders them a combinatorial array of roles, often associated to one specific type of immunity (adaptive or innate) [40].

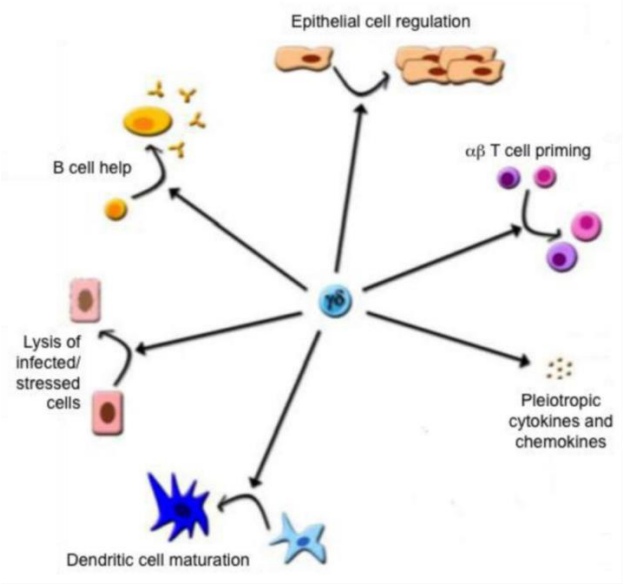

Figure 6 - Some of the best functions mediated by γδ T-cells. *A diversity of roles of γδ T-cells are performed to defend the human body from different infections and stress. Hereby, is shown that γδ T-cells are capable of : promoting dendritic-cell maturation, lysing infected cells/stressed cells, mediating B cell help, regulating epithelial cell, αβ T cell priming and, lastly, secreting cytokines and chemokines. Adapted from Vantourout & Hayday (2014)[39].*

Additionally, γδ T cell subsets recognize molecules in a non-HLA/MHC complex restrictive way. In particular, Vγ9Vδ2+ expressing γδ T-cells (a subset of γδ T cells), can sense stressed or virally infected cells that accumulate non-peptidic pyrophosphate molecules (phophoantigens), intermediates of a dysregulated malavonate pathway of the isoprenoid synthesis, via the expression of a changed conformation of BTN3A1 (Butyrophilin subfamily 3 member A1). Consequently, a high cytotoxicity against a wide range of tumors is displayed [33, 41, 42]. So far, mostly autologous Vγ9Vδ2+ expressing γδ T cells have been used in clinical trials. Although, data from these latter in a transplantation setting, is still scarce the results are rather promising [27]. However, there are limitations in using γδ T cells in adoptive T cell immunotherapy: (1) the aptitude to expand, *in vivo* and *ex vivo*, into acceptable numbers in a great number of patients [43]; (2) the inefficiency to home to tumor sites [44] and, lastly, (3) the inability to display high level of cytotoxity against tumors [45].

1.1.2.3.5. The use of γδ T cell receptors

Due to the functional advantages of the Vγ9Vδ2 TCR engineered T cell receptors and the proliferative advantage of αβ T cells, our group demonstrated that it is possible to redirect αβ T cells against tumors [41]. In addition, our group was able to demonstrate that γδTCR engineered T cells successfully recognized and killed AML blasts [33, 38, 41]. This genetic modification was achieved without the formation of mixed dimers with α and β TCR chains, often responsible for GVHD in patients infused

of tumor-specific αβTCR engineered αβ T cells [38, 41]. In addition, theoretically, the lack of HLA-restriction overcomes of γδTCR engineered T cells renders them the ability of being used in any ACT modality (autologous or allogeneic). Hence, our group created room for a promising immunotherapy approach using γδTCR engineered T cells in which this thesis focuses on.

1.1.2.3.6. Selecting γδTCR engineered αβ T cells

In an autologous ACT, unwanted transfer of regulatory T cells and the competition for homeostatic cytokines hamper an optimal anti-tumor activity [34, 46]. To avoid the unwanted transfer of cells (expanded during the culturing procedure), our group took advantage of an anti-αβTCR antibody and magnetic beads to negatively select γδTCR engineered αβ T cells after its flow through a magnetic column [38]. In contrast with a positive selection, in which engineered T cells are selected with antibodies ("touched" cells), we avoid problems such as suboptimal expression of the desired gene or the addition of immunogenic components for the positive selection [38]. Given the fact that, the γδTCR is a strong competitor for CD3 complex (harboring TCRs in the T cell surface), the endogenous αβTCRs are downregulated allowing efficiently engineered αβ T cells to escape the anti-αβ TCR antibody [38]. Hence, due to the lack of αβTCRs, these "untouched" γδTCR engineered αβ T cells are not allo-reactive when they encounter HLA-mismatched targets [27, 42]. Overall, this sheds new light on the treatments for the use γδTCR engineered T cells in an allogeneic ACT in poor/very low risk AML patients that have previously undergone an allo-SCT (Fig. 7).

1.1.2.3.7. Manufacturing γδTCR engineered αβ T cells

To reach a clinical-grade T cell product for adoptive T cell therapy, the manufacture of these must be done according to good manufacturing practice (GMP) procedures. GMP comprises a traceable final product accurately produced under defined standard operating procedures (SOPs) [47, 48]. Therefore, when building an adoptive T cell therapy, the source of T cells, the production process, the purity of the T cell product and its safety must be ensured to successfully reach a GMP-grade T cell product [47]. Until now, the GMP-grade translation of ACT remains a true challenge, despite the many advances in scientific knowledge as extensively review in Schumacher et al. (2015)[49]. By the use of this novel purifying αβ T cell depletion described above, a GMP-grade γδTCR engineered T cell product can be manufactured.

Taking advantage of a highly tumor reactive Vγ9Vδ2 TCR derived from clone 5 [33] and a novel αβTCR purifying method [38], we set our goals in optimizing a retroviral transduction protocol of AML-reactive γδTCR engineered αβ T cells towards a GMP-compliant manufacturing process. During this project, we optimize: (1) the infection enhancer; (2) the T cell activation; (3) the virus titers; (4) the T cell density; (5) the transduction spin and (6) the purifying method. Furthermore, we characterize and analyze the functional properties of our engineered T cell product. Ultimately, we upscale the manufacturing process of γδTCR engineered T cells to a larger cell culturing platform and αβTCR depletion method.

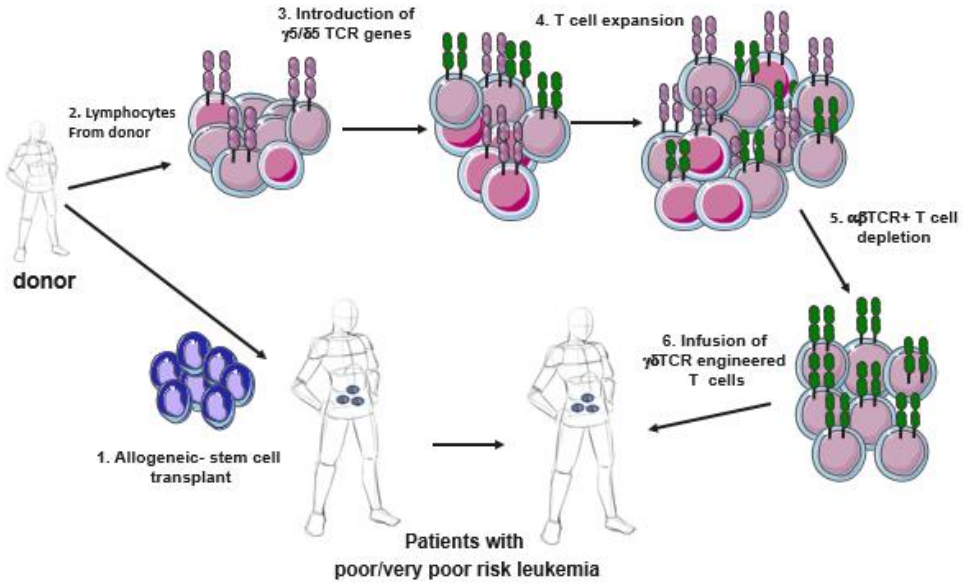

Figure 7 –Overview of γδTCR engineered T cell therapy in an allogeneic setting. *AML patients will undergo allo-SCT (1); The therapy will be performed after allo-SCT; Donor lymphocytes will be transduced with Vγ9Vδ2 clone 5 transgene (2&3), followed by a transduced T expansion (4), αβTCR+ T cell depletion (5) and infusion in poor/very poor risk leukemia (6).*

2. Materials and Methods

2.1. Cells and Cell lines

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy-coats obtained from Sanquin Blood Bank (Amsterdam, The Netherlands) frozen and stored in the liquid Nitrogen until further usage. PBMCs for the rapid T cell expansion protocol were obtained from the Institute for Transfusion Medicine and Immunohematology, Frankfurt, Germany. PBMC samples from AML for functional assays were obtained from the University Medical Center Utrecht Biobank and were collected according to GCP and Helsinki regulations. Daudi (Burkit's lymphoma;CCL-213) cell line was obtained from the American Type Culture Collection at the initiation of the study in 2010 [authentication by short tandem repeat (STR) profiling/karyotyping/isoenzyme analysis]. The RD114 (293VEC_RD114) and the master cell bank (MCB) #73 RD114 packaging cell lines were provided by EUFETs. These packaging cell lines were cultured every 3 days with DMEM (Dulbecco's Modified Eagle Medium; Invitrogen®) supplemented with 1% Pen/Strep (Invitrogen®) and 10% Fetal Calf Serum (FCS; Bodinco®) during a maximum of 2 months. On a routine basis, the cell lines were verified for growth rate, morphology, and/or flow cytometry and tested negative for mycoplasma using MycoAlert Mycoplasma Kit (Invitrogen®).

2.2. γδTCR gene

The transgene cassette we introduced in αβ T cells contains, a highly tumor reactive Vγ9Vδ2 TCR obtained from clone 5 [33] and a T2A peptide linker sequence, derived from the *Thosea asigna* virus [50] (Fig. 8). This sequence was cloned into an optimized retroviral vector pMP71 (provided by Miriam Heemskerk, LUMC, Leiden) to express simultaneously both TCR chains (pMP71:γ5-T2A-δ5). As a control in our retroviral transduction experiments, we used pMP71 expressions system with a truncated nerve growth factor receptor (NGFR; provided by Miriam Heemskerk).

2.3. Production of virus supernatant

2.3.1. Home produced supernatant

The 293VEC_RD114 packaging cell line (RD114) was seeded using DMEM with 10% FCS and 1% Pen/Strep (Invitrogen®) on a cell density of $2x10^6$/mL on day -1 (before activation). A transfection mix was prepared with pMP71:γ5-T2A-δ5 or pMP71:NGFR DNA, Fugene HD (Promega®) and room temperature DMEM (20 ug, 30 ul and 900 ul, respectively), during 20 minutes at room temperature, and added to the medium of the packaging cells on the day after seeding. One day after transfection, medium refreshment using in X-Vivo-15 mixed with gentamicin and phenol red (Lonza®) and supplemented with 10% AB+ Human Serum (HS; Heat inactivated) from Sanquin Blood Bank (Amsterdam, The Netherlands) and 1%

Pen/Strep. 2 days after the transfection the virus supernatant was harvested, filtered and used on the experiment or, snap-freezed and stored in -180ºC Nitrogen Tank.

2.3.2. Master cell clone derived supernatant

EUFETs GmbH (Germany) provided GMP-like virus supernatant from a packaging cell line producing constitutively retrovirus containing the pMP71:γ5-T2A-δ5 transgene. This cell line was called Master Cell Bank (MCB) VEC293_RD114 #73 (MCB #73 RD114). In addition we received this cell clone itself. This packaging cell line was seeded using DMEM with 10% FCS and 1% Pen/Strep on a cell density of $1x10^6$/mL. 5 days later, the cells were checked for confluence and medium refreshment was performed using in X-Vivo-15 mixed with gentamicin and phenol red and supplemented with 10% AB+ HS and 1% Pen/Strep. The next day the virus supernatant was harvested, filtered and used during the experiment or, snap-frozen and stored in -180ºC Nitrogen Tank until further usage. Virus supernatant was used undiluted or diluted as indicated for each experiment.

2.4. Retroviral Transduction and T Cell culturing

2.4.1. Small –Scale retroviral transduction protocol

The culture medium was X-Vivo-15 mixed with gentamicin and phenol red and, exogenously, supplemented with 10% AB+ HS and 1% Pen/Strep. PBMCs were gently thawed, centrifuged and resuspended in culture medium mentioned above containing premium grade IL-7 (500 IU/ml) and IL-15 (70 IU/ml) (Miltenyi Biotec®). Hereafter, T cells were incubated with Dynabeads® Human T-activator CD3/CD28 (Invitrogen®) on a ratio of 1:5 Bead:T cell ratio during 48h at 37ºC with 5% CO_2. The T cell activation was performed in an open system with 6-well plates (Thermo scientific®), using $4x10^6$ T cells in 2 mL per well. CD3+ T cell percentages were determined by flow cytometry on the same day. Prior to the retroviral transduction day (day 1), non-tissue culture treated Falcon 24-well plates (Beckton Dickison®) were coated with RetroNectin® (Takara®) and incubated overnight (O/N) at 4ºC. On the day of retroviral transduction, recovered T cells were harvested and resuspended at a T cell density of $0.5x10^6$/ mL in culture medium with cytokines. RetroNectin®-coated plates were blocked for unspecific binding with PBS with 2% FCS or PBS with 2% HS for 30 minutes at 37ºC with 5% CO_2. Following the blocking, a subsequent 90 minute pre-spin at 1000 g was performed using 2 mL of GMP-like virus supernatant or by home produced virus supernatant. After removing the virus supernatant, 1 mL of activated T cells per well was applied on the plate and incubated 24h at 37ºC with 5% CO_2. The next day, transduced T cells were resuspended in culture medium with IL-7 (500 IU/ml) and IL-15 (70 IU/mL) and transferred to a 24-well plate (Thermo scientific®).

Transduced T cells were counted and the culture medium with IL-7 (500 IU/ml) and IL-15 (70 IU/mL) was refreshed to a cell density of $5x10^5$/ mL on the 7^{th}, 10^{th} and 14^{th} of the protocol. Transduced T cells were transferred to 24-well plates, T_{25} Flasks or T 75 Flasks (<4 ml, 4-8 ml or >8 ml respectively).

To compare with our old research transduction protocol, we followed the same timeline and used: αCD3 (30 ng/mL; Ortho-190 clone OKT3; Janssen-Cilag®) for the activation of T cells, IL-2 (50 IU/mL; Proleukin, Novartis®) to supplement our culture medium, polybrene (6 mg/mL; Sigma-Aldrich) as an infection enhancer added on the T cell activation day and, performed a 30 min pre-spin at 1000g (with virus supernatant) followed by a 60 min spin at 1000g (with the T cells resuspended on the virus supernatant) on the day of the retroviral transduction. By using this protocol we did not use Dynabeads® Human T-activator CD3/CD28, RetroNectin, and IL-7 (500 IU/ml) and IL-15 (70 IU/mL) (Fig. 7).

2.4.2. Large–Scale retroviral transduction protocol

The culture medium was X-Vivo-15 mixed with gentamicin and phenol red and supplemented with 10% AB+ HS and 1% Pen/Strep. PBMCs were gently thawed, centrifuged and resuspended in culture medium containing premium grade IL-7 (500 IU/ml) and IL-15 (70 IU/ml). Hereafter, T cells were incubated with Dynabeads® Human T-activator CD3/CD28 on a ratio of 1:5 Bead: T cell ratio during 48h at 37ºC with 5% CO_2. The T cell activation was performed in an open system 500 mL Differentiation Bags, using $250x10^6$ CD3+T cells in 500 mL. CD3+ T cell percentages were determined by flow cytometry on the same day. Prior to the retroviral transduction day (day 1), non-tissue culture treated Falcon 24-well plates were coated with Retronectin® and incubated O/N at 4ºC. On the day of retroviral transduction, recovered T cells were harvested and resuspended at a T cell density of $0.5x10^6$/ mL in culture medium with cytokines. Retronectin®-coated plates were blocked for unspecific binding with PBS with 2% FCS or PBS with 2% HS for 30 minutes at 37ºC with 5% CO_2. Following the blocking, a subsequent 90 minute pre-spin at 1000 g was performed using 2 mL of home produced virus supernatant. After removing the virus supernatant, 1 mL of activated T cells per well was applied on the plate and incubated 24h at 37ºC with 5% CO_2. The next day, transduced T cells were resuspended in culture medium with IL-7 (500 IU/ml) and IL-15 (70 IU/mL) and transferred into a Miltenyi Biotec® differentiation bag T cell density of $2.5x10^5$/mL (respectively)..
Transduced T cells were counted the culture medium with IL-7 (500 IU/ml) and IL-15 (70 IU/mL) was refreshed to a cell density of $2.5x10^5$/ mL on the 7^{th} and to a cell density of $5x10^5$/ mL on the 10^{th} and 14^{th} of the protocol. In this protocol cells, were always cultured and expanded in 0.5-1L Miltenyi Biotec® differentiation Bags following the retroviral transduction step.

Here, we also tested on the retroviral transduction day, the use of a no pre-spin in which T cells were only incubated the virus supernatant O/N.

2.5. Depletion of non-engineered T cells

On day 14 after activation, transduced T cells were incubated with a biotin-labeled anti-αβTCR antibody (clone BW242/412; Miltenyi Biotec®) during 10 minutes at 4ºC, followed by an incubation of with anti-biotin antibodies cross-linked to magnetic beads (anti-biotin MicroBeads; Miltenyi Biotec®) during 15 min at 4ºC according to the manufacturer. After this procedure, the labeled cell suspensions were run into LD columns (Miltenyi Biotec®) and αβTCR-positive (αβTCR+) T cells were depleted by MACs

cell separation (Miltenyi Biotec®) according to the provided manufacturer protocol (Fig. 8). αβTCR+ depleted transduced T cells to use for functional assays, were expanded using Rapid T cell expansion protocol based on a previously described method by our group [41].

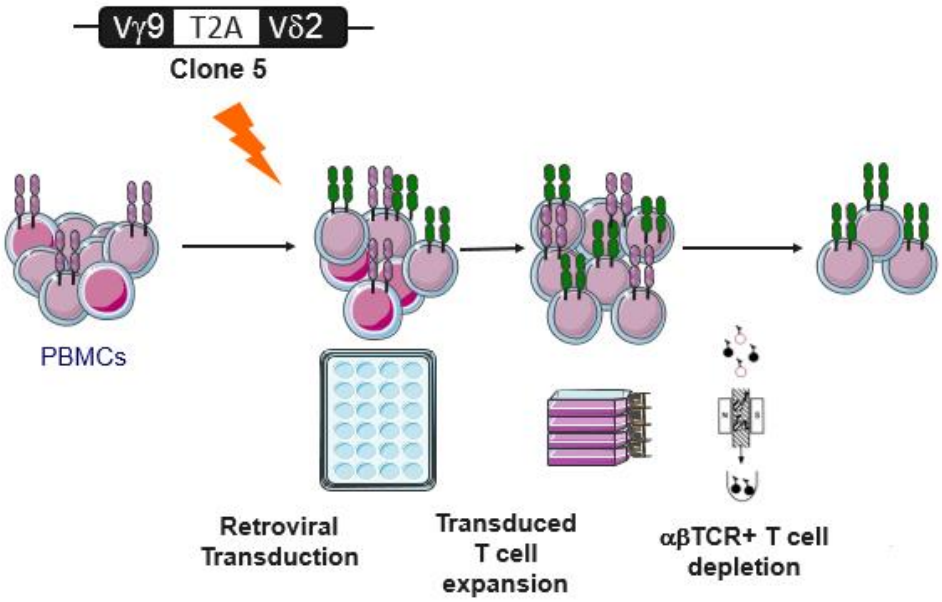

Figure 8 – Schematic for enriching γδTCR engineered T cells. *PBMCs are retrovirally transduced with Vγ9Vδ2 clone 5 transgene cassette; Transduced T cells are then expanded using cytokines and, finally, depleted from αβTCR expressing cells.*

2.6. Flow cytometry (FACs)

The number of CD3+ T cells was determined using a CD3 eFluor® 450 (clone OKT3; eBiosciences®). After performing the retroviral transduction protocol, transduced T cells were incubated with 3 main different antibody mixes: (1) (γδTCR expression) pan-γδTCR-PE (clone IMMU510; Beckman Coulter®), αβTCR-APC (clone IP26; Biolegend®), CD8-PerCP-Cy5.5 (clone RPA-T8; Biolegend®) and CD4-FITC (clone RPA-T4; BD Biosciences®), (2) (Phenotype markers expression) pan-γδTCR-PE (clone IMMU510; Beckman Coulter®), CD4-APC (clone SK3, Biolegend®), CD8-PerCP-Cy5.5 (clone RPA-T8; Biolegend®), CD45RA-Pacific Blue (clone HI100, Biolegend®), CD45RO PE-cy7 (clone UCHL1, BD Pharmigen®), CD62L FITC (clone Dreg-56, Invitrogen®),CD27 APC-eFluor780 (clone O323, eBiosciences®) and (3) (NK markers expression) CD3 eFluor450 (clone OKT3, eBiosciences®), CD56 APC (clone NCAM 16, BD Biosciences®), CD8-PerCP-Cy5.5 (clone RPA-T8; Biolegend®) and CD16 PE (clone eBioCB16, eBiosciences®).

In all flow cytometric assays, samples were washed with FACs Buffer containing 10% Bovine Serum Albumine (BSA; GE Healthcare Life Sciences®) and 1% Sodium Azide (Severn Biotech®), incubated with the antibody mixes for 20 minutes at room temperature and analysed on the 7th, 10th and 14th (both before

and after depletion of non-engineered T cells) days, on the BD LSRII using FACSDiva Software (BD Biosciences®).

2.7. IFNγ ELISpot assay

Target cells (Daudi and primary AML samples) and effector cells (γδTCR engineered T cells after 2 weeks of rapid T cell expansion protocol) (E:T 1:3) were incubated for 24 hours in the presence of pamidronate (PAM;10 mmol/L; Calbiochem®) where indicated.
Upon co-culture of γδTCR T cells with tumor cells, release of Interferon-γ (INF-γ) was measured by an ELISPOT using anti-human IFNγ mAb1-D1K (I) and a mAb7-B6-1 (II) (Mabtech®) following the manufacturer's recommended procedure.

3. Results

3.1. Establishing a small-scale GMP-ready protocol for the production of γδTCR engineered T cells

The small-scale manufacturing protocol for the production of γδTCR engineered T cells was based on the research protocol described on the materials and methods. We were able to build a timeline of 14 days (Fig. 9), based on the results shown in this section.

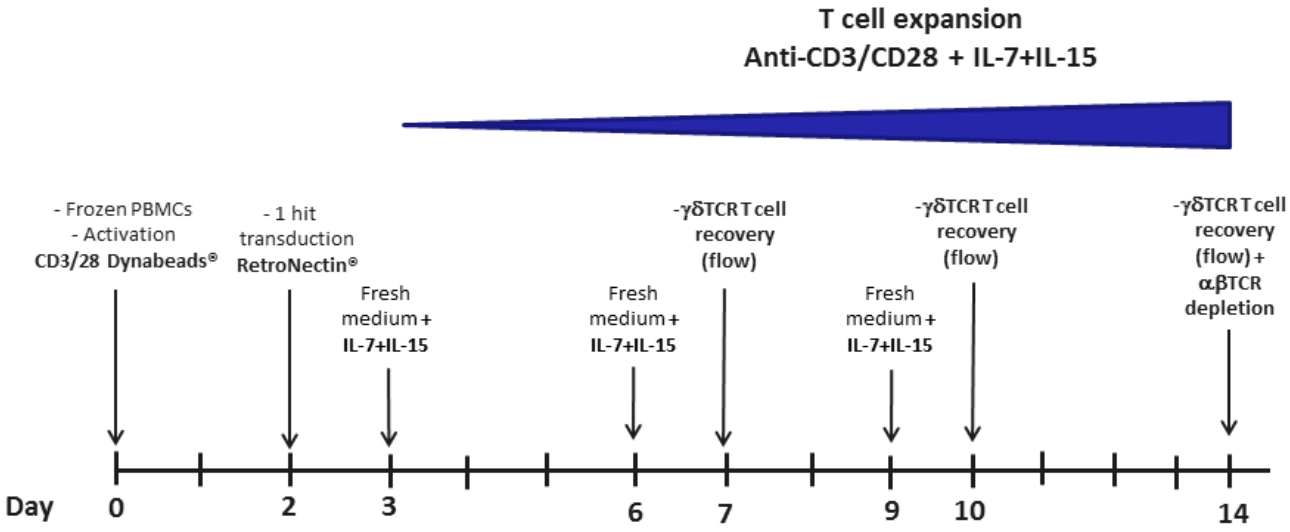

Figure 9 – Timeline for the large-scale manufacturing protocol of γδTCR engineered T cells. *Frozen PBMCs and the activation of T cells with CD3/CD28 Dynabeads® will be performed on day 0 in 6 well plates; One transduction step will be done in a 24-well plate with RetroNectin® and using a 90 minute pre-spin on day 2 after activation; Fresh medium with IL-7 and IL-15 will be renewed to the 24-well plates, T_{25} flasks or T_{75} flasks on day 3, 7, and 10 after activation; Cell culturing will be performed at a density of 0,5x10^6 T cells/mL after transduction; Flow cytometric assays (flow) will be performed to assess γδTCR T cell recovery percentage on day 7, 10 and 14 after activation (This latter before and after αβTCR depletion); The αβTCR depletion step will be performed on day 14 after activation.*

3.1.1. Infection Enhancer and T Cell activation

In a retroviral transduction, one of the most important steps, is the facilitation of the infection by the retrovirus. Our old protocol for this procedure already included a polycation (polybrene) that facilitates the virus adsorption due to electrostatic interactions (nullifying charge repulsion between virus and T cells) [51]. However, we needed to change this component of the protocol due to the lack of its availability in a GMP-compliant version. In alternative, we used a chimeric peptide, named RetroNectin®, containing an integrin domain connecting to T cells and a heparin domain connecting to virus particles.

Hence, this molecule is able to facilitate infection when virus and T cells, are spinned together in a RetroNectin® pre-coated plate [52]. Therefore, our first question aimed to test a GMP compliant infection enhancer (GMP- RetroNectin®) with, at least, the ability to match the transduction efficiency of a protocol using polybrene. In fact, our data indicated a difference of more than 10% in γδTCR+ T cells using the same activation method and GMP-RetroNectin® compared to Polybrene (Fig. 10). This indicates that GMP-RetroNectin® can be used as an infection enhancer, to provide an optimal transduction efficiency in a small-scale retroviral transduction protocol.

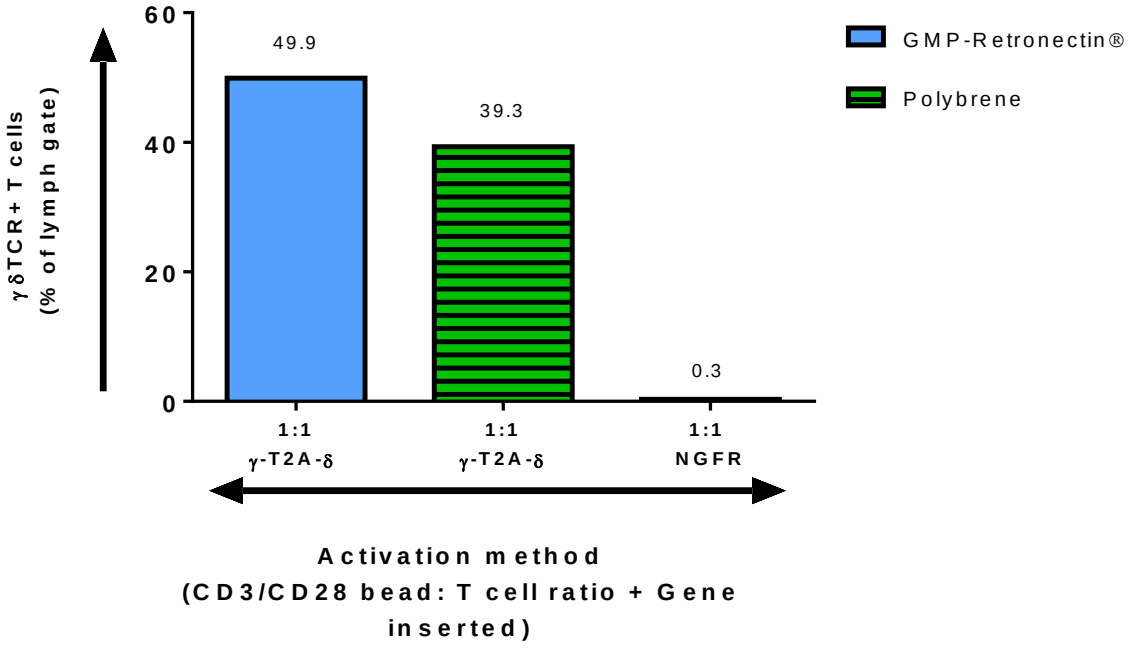

Figure 10 - GMP-Retronectin® matches Polybrene in transgene expression. *Different gdTCR+ T cell percentages resulting from transducing PBMCs with Polybrene or GMP-Retronectin®. OKT3+IL-2 activation in combination with polybrene is used as a standard positive control for the research-grade transduction protocol; Nerve growth factor receptor (NGFR) is used as a negative control for γδTCR transgene expression in the cells; The results shown are based on a n=1*

3.1.2. T cell activation

As reported by others [1, 53, 54], T cell stimulation influences the transduction efficiencies, becoming a crucial step to determine the expression of the transgene. Time prevented us from using the GMP compliant version of an anti-αCD3 monoclonal antibody (OKT3) for T cell activation, due to the lack of its availability in a GMP-compliant form. Therefore, we seeked a different T cell activation stimulus yet with the same or higher performance compared to the research grade protocol. When using GMP-RetroNectin®, we could achieve a comparable γδTCR+ T cell percentage (Fig. 11 - A) and number of γδTCR expressed per cell (Mean Fluorescence Intensity; MFI; Fig. 11 - B) when comparing magnetic beads coated

with anti-CD3 and anti-CD28 antibodies with IL-7&IL-15 with OKT3 and IL-2 stimulation from the research grade protocol (Fig. 11 - A). However, the number of beads per T cell (bead: T cell ratio) result in different γδTCR+ T cell numbers. Data from the 10th day of the transduction protocol emphasized the need to use a 1:5 bead:T cell ratio to obtain a maximal number of γδTCR+ T cells (5,7x10^6 cells), when comparing with ratios using the same conditions and with the OKT3+IL-2 (Fig. 11 - C). In summary, this data was determinant to switch from OKT3+IL-2 to Dynabeads® Human T-activator CD3/CD28 activation with IL-7& IL-15.

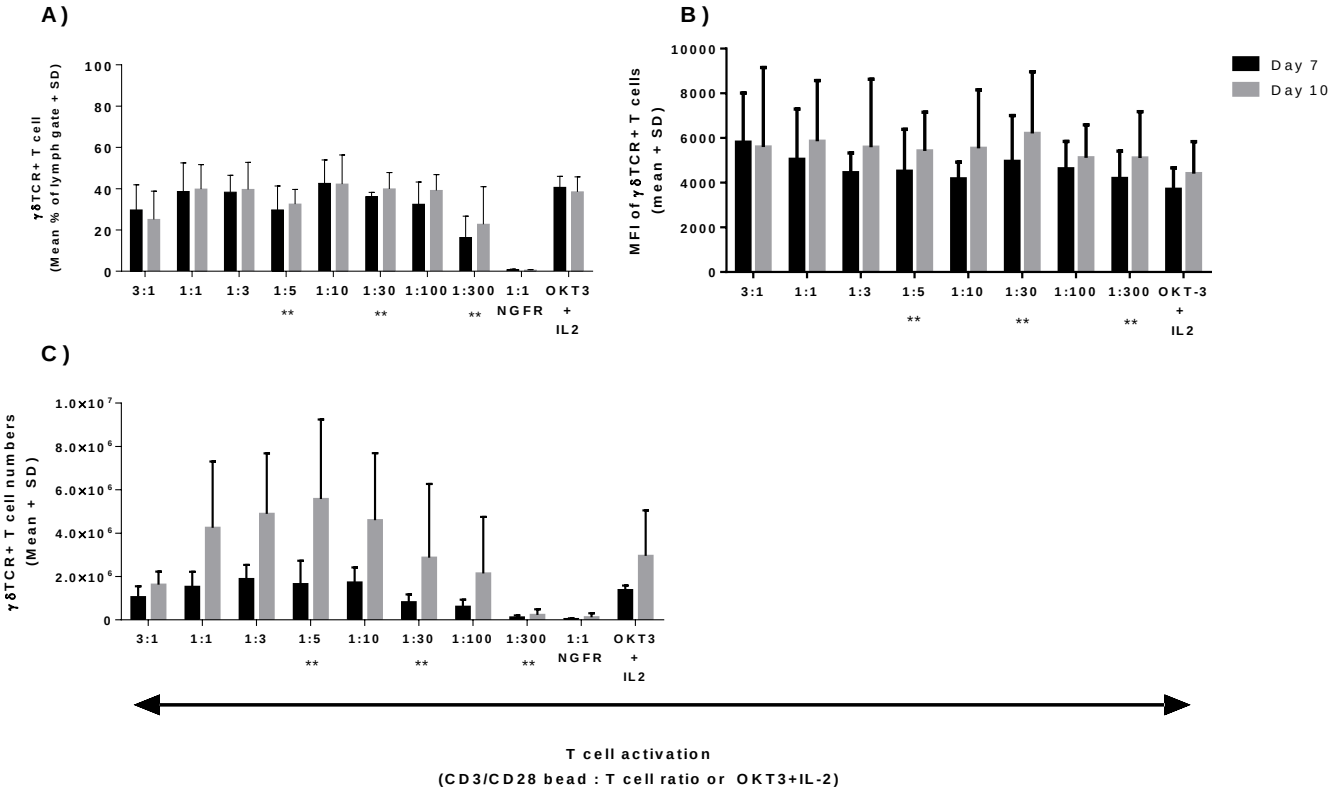

FIGURE 11 - 1:5 CD3/CD28 Bead: T cell Ratio results in a better number of γδTCR+ T cells. *(A) Different CD3/CD28 beads: T cell ratios result in gdTCR+ T cell % on day 7 and day 10; (B) Mean fluorescence intensity (MFI) was determined by flow cytometry for the different activation methods on day 7 and day 10; (C) γδTCR+ T cell numbers were obtained by applying the γδTCR+ T cell % on the cell numbers of the different activation methods, on day 7 and 10; OKT3+IL-2 activation is used as a positive control for the research transduction protocol; Nerve growth factor receptor (NGFR) is used as a negative control to confirm γ5-T2A-δ5 receptor transgene expression in the cells; CD3/CD28 beads: T cell ratio mean ± 1 SD, n=4 (**n=3)*

3.1.3. Virus Titration

Established the activation method and the infection enhancer, we focused our efforts in optimizing the number of virus particles needed for an optimal transduction efficiency. Hence with this number, we could establish a basal range of titers that could retain reproducibility in an upscaled GMP production setting and predict donor heterogeneity. To assess this question, several GMP-like virus titers with different infectious particles per mL and produced by the MCB clone #73 packaging cell line, were analyze for the ability to insert the transgene into αβ T cells. Our data shows that, there is a raising percentage of γδTCR+ T cells (transduction efficiency) along with γδTCR+ αβTCR- T cells in a virus particle dependent fashion from Day 7 to Day 10 (Fig. 12 - A). We were able to observe a stabilization of transduction efficiency at 2,73x10^5 ips/mL (infectious particles/mL) corresponding to 72,5% of the total γδTCR+ T cells in which 50,6% are γδTCR+ αβTCR- T cells.

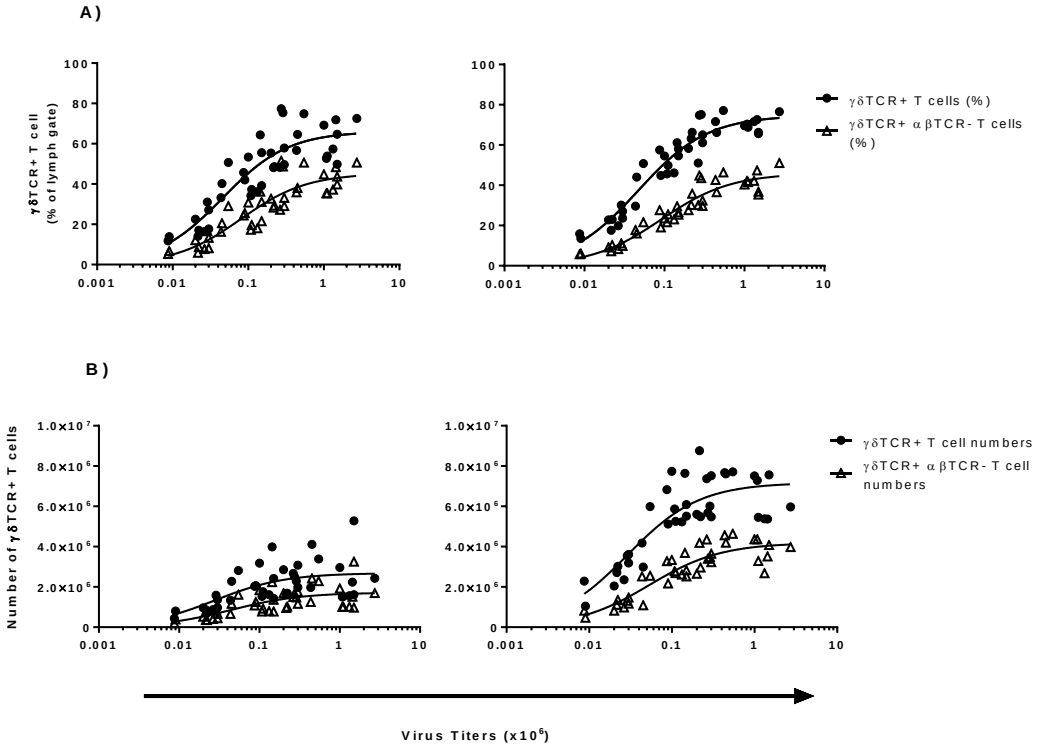

Figure 12 - *1x10^5 ips/mL and 1x10^6 ips/mL sets a virus titer window titer for a stable transdution efficiency*. *(A) (Left) γδTCR+ αβTCR+ and γδTCR+ αβTCR- T cell percentages and the virus titers were compared in which each dot represent a different virus titer tested and the percentage of cells obtained. These percentages were followed until Day 10; (B) (Left) γδTCR+ αβTCR+ and γδTCR+ αβTCR- T cell numbers and the virus titers were compared in which each dot represent a different virus titer tested and the number of cells obtained. These numbers were followed until Day 10 (Right). Virus titers were previously determined and provided by the manufacturer and the comparison was made using a non-linear regression; Virus titers are in a Log scale; Results show a n=40 for each curve*

Based on these percentages, we calculated the numbers of T cells that were γδTCR+ and γδTCR+ αβTCR-. Indeed, there is an increasing number of γδTCR+ and γδTCR+αβTCR- expressing T cells along with the increase of virus titers, from day 7 to day 10 (Fig. 12 - B). Thus we were able to obtain a large number of total γδTCR+ T cells ($5.97x10^7$) and γδTCR+ αβTCR+ T cells ($3.98x10^6$) on the 10^{th} day of the protocol using the previously referred virus titer. Given this data, we were able to determine a stable plateau of transduction efficiency between $1x10^5$ ips/mL and $1x10^6$ ips/mL.

3.1.4. T cell density

In order to maximize the percentage of the transduced T cells with the γδTCR after transduction step, we tested whether we could increase the number of activated T cells per well for the same amount virus particles per mL. Despite the different T cell densities, side by side comparison reveals that the percentages of γδTCR+ T cells are very similar on both days of the protocol, after flow cytometry analysis (Fig. 13). Nonetheless, on the two days of screenings, we were still able see differences between the percentages of γδTCR+ T cells by using lower T cell densities on lower virus titers. Although, the $0,1x10^6$/mL T cell density has a better percentage of γδTCR+ T cells than the currently used $0,5x10^6$/mL T cell density (29,1% and 25,9%, respectively; Fig. 13 - A), we maintained the currently used T cell density (because: (1) the difference in γδTCR+ T cell percentages do not justify the further dilution of and (2) it is not economically profitable to further dilute the T cells.

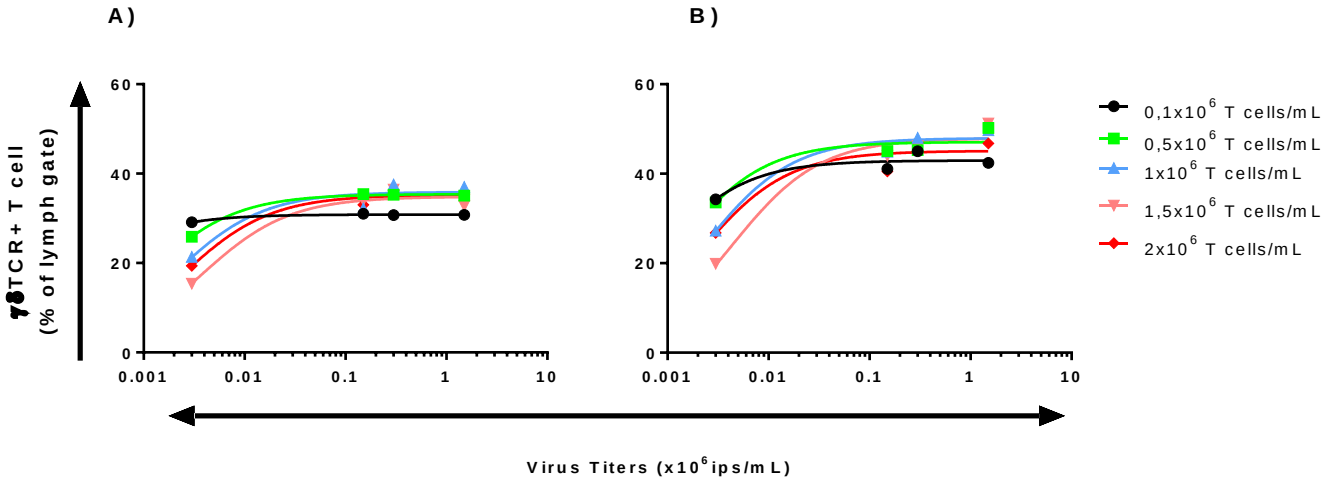

Figure 13 - $0,5x10^6$ T cells/mL is the optimal T cell density. *A) γδTCR+ T cell % is assessed by relating the T cell density with different virus titers on day 7; B) γδTCR+ T cell % is assessed by relating the T cell density with different virus titers on day 10; Virus titers are in a Log scale; Results show a n=1 for each curve*

3.1.5. Transduction Spin

The transduction step in our protocol consisted in, pre-spinning the virus supernatant on the pre-coated Retronectin® wells during 30 minutes, followed by a 60 minutes spin with activated T cells resuspended in virus supernatant. As mentioned previously, the pre-spin is used to bring the virus particles onto the RetroNectin® molecules already pre-coating the plates, and the spin is used to bring together the activated T cells and the virus particles by binding the RetroNectin® molecules. Other retroviral transduction protocols [36, 47], T cell transduction is achieved with one pre-spin with the virus supernatant either with two hits of retroviral transduction, or with selection and expansion after transduction. Due to economic reasons, it would be more cost-effective to achieve a maximal transduction efficiency using one transduction hit and one pre-spin. Therefore, we tested whether the use of only one transduction pre-spin would result in maximal transduction efficiency. We did this by comparing transduction efficiency from T cells transduced only with one 90 minute pre-spin or by a 30 minute pre-spin and a 60 spin. Data from day 7 indicates that the percentage of γδTCR+ T cells is very similar using the two options mentioned above with different virus dilutions (78% and 68.3%, respectively; Fig. 14 - A). Supporting our idea of using only one 90 minutes pre-spin, the number of γδTCR+ T cells is higher using this modality comparing to the 2 spin research protocol used before, in a virus supernatant dilution of 5x ($3{,}2x10^6$ and $2{,}2x10^6$ respectively; Fig. 14 - B). Moreover, the use of a 90 minute pre spin allows this γδTCR+ T cell number advantage until a dilution of 10x, which can reduce costs of production. It is clear by these results, that the use of one 90 minute transduction spin would be the most cost-effective due to: (1) higher transduction efficiency and transduced T cell numbers and (2) reduction in the overall costs of production.

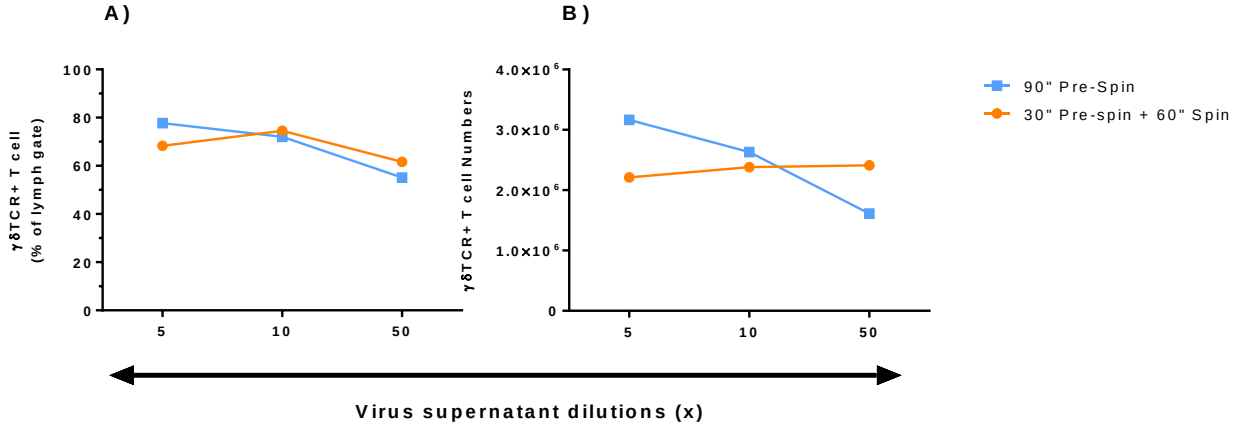

Figure 14 – 90 minute pre-spin favors the number of γδTCR engineered T cells - *(A) γδTCR+ T cell percentage (γδTCR+ αβTCR+ and γδTCR+ αβTCR- T cells) was determined by flow cytometry on Day 7 according their respective virus supernatant dilutions; (B) γδTCR+ T cell numbers (γδTCR+ αβTCR+ and γδTCR+ αβTCR- T cells) for each spin timeframe was calculated according to the percentage of cells in the lymph gate on day 7; Dilutions were made on a fresh produced virus supernatant from a clone of a RD114 cell line producing virus containing Vγ9Vδ2 clone 5; Results shown are from a n=1*

3.1.6. Purifying γδTCR engineered cells

Upon engineering αβ T cells with the Vγ9Vδ2 clone 5 transgene cassette it is needed to purify the non-engineered T cell fraction. For this purification method, the use of a biotin labeled anti-αβTCR GMP-grade antibody followed by a run in a MACs® cell separation column, has demonstrated to be a crucial step concordant with previous work from our group [38]. Data provided by flow cytometry analysis demonstrated that, this procedure was able to completely deplete all present αβTCR+ T cells (55,4% to 0,023%; Fig. 15 - A). Taking this into account, we can calculate the percentage of recovery of γδTCR+ T cells obtained after depletion if we compare to: (1) the total cell numbers before depletion, (2) the γδTCR+ T cells before depletion or (3) the number of γδTCR+ αβTCR- T cells before depletion (Fig. 15 - B). Among all the three approaches given, we noted that purifying γδTCR engineered T cells, with minimal loss of these, remains a true challenge. The resulting fraction of γδTCR engineered T cells after αβTCR depletion is only 10% of the γδTCR+ T cells we had before αβTCR depletion (Fig. 15 - B; central column). According

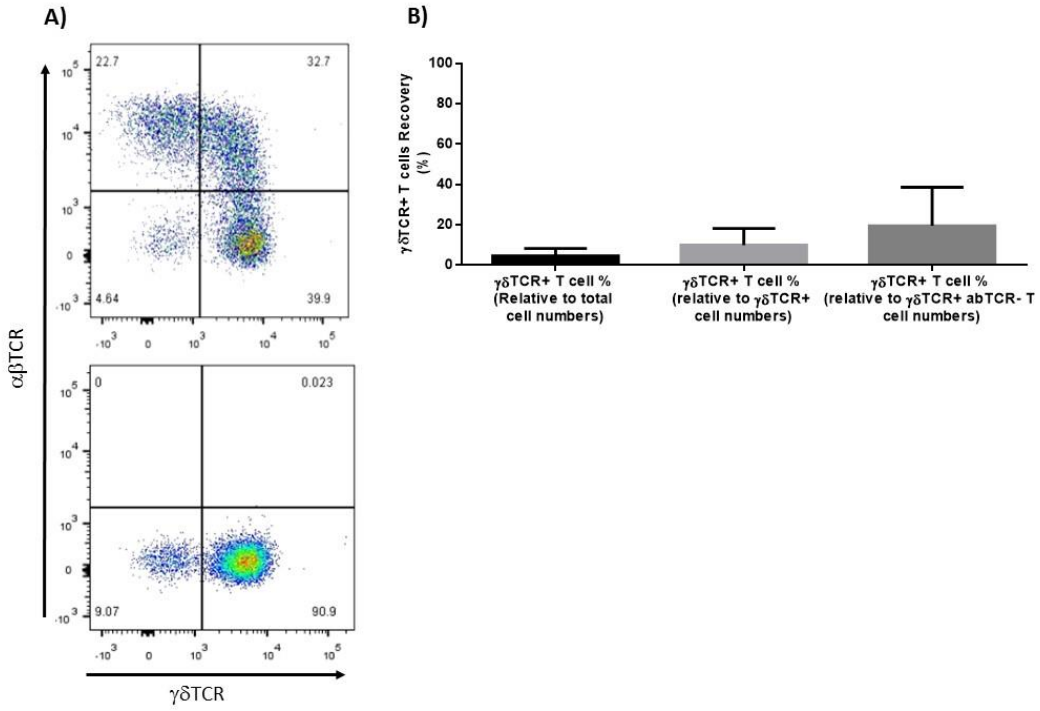

FIGURE 15 – A pure population of γδTCR engineered T cells was obtained upon αβTCR depletion. *A) Flow cytometry plots from before (upper part) and after (lower part) αβTCR depletion. B) Overview of the different γδTCR+ T cell recovery % comparing to the total cell numbers before depletion (left column) the γδTCR+ T cells before depletion (central column) and the number of γδTCR+ αβTCR- T cells before depletion (right column), on day 14; Results show mean ± 1 SD, n=4 (containing 4 individual experiments)*

to this data, we set our next goal in finding the most favorable day for depletion of the non-engineered T cells according to two main criteria: a) expansion profile of γδTCR+ T cell and γδTCR+ T cell recovery after depletion. We observed that, transduced T cells activated with 1:5 CD3/CD28 bead: T cell ratio expand in large numbers between the 10th and 14th day of the protocol (Fig. 16; upper part), suggesting a higher

γδTCR+ T cell recovery % and after depletion on this latter day. Additionally, if we apply the recovery percentage of γδTCR+ T cells mentioned before (10%) we can predict the number of γδTCR+ T cells we would obtain if we αβTCR-depleted the γδTCR engineered T cells in the different days of the retroviral transduction protocol. Despite a low cell number of γδTCR engineered T cells obtained after αβTCR depletion in the different days, we can still see that we acquire more engineered T cells in the 14th day of the protocol (Fig. 16; lower part). Also, the higher the output of engineered T cells we deplete the higher the number of purified γδTCR engineered T cells we can obtain after αβTCR depletion.

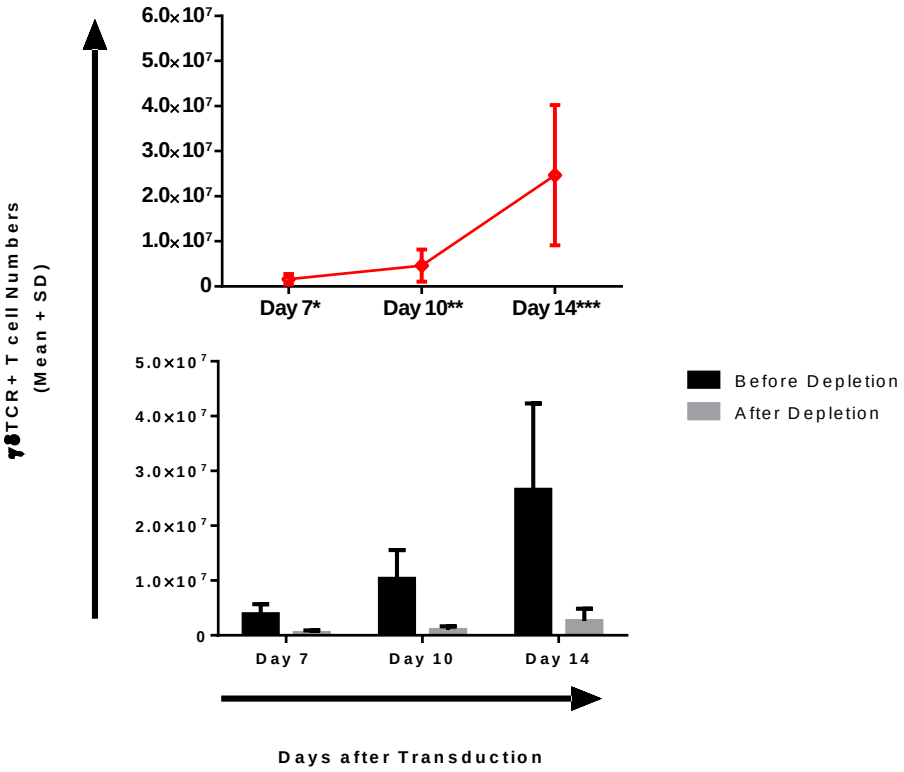

Figure 16 - ***Day 14 of the small-scale production protocol is the best day to deplete αβTCR+ T cells.*** *(Upper part) γδTCR+ T cell proliferation numbers on day 7, 10 and 14, activated and transduced with 1:5 CD3/CD28 Bead:cell ratio; (lower part) γδTCR+ T cell numbers (γδTCR+ αβTCR+ and γδTCR+ αβTCR- T cells) obtained on the different important timepoints after transduction of the protocol either before depletion (representing solely the proliferation) and after the depletion of the untouched population of cells (representing the proliferation and recovery percentage applied on them); Results shown mean ± SD, n=4 (contained 4 individual experiments) (upper part; *n=93, **n=75 and ***n=17)*

Overall, it can be concluded that an activation of T cells with 1:5 CD3/CD28 bead: T cell ratio in combination with an αβ-depletion step on day 14 make an ideal combination to obtain a cell product with, the highest recovery of γδTCR T cells.

3.1.7. Cell Product Characterization

Until now, we gathered information that allowed us to successfully establish a small-scale production protocol of γδTCR engineered T cells. However, for clinical purposes, we need to describe complete production process [47]. To this end, we characterized the cellular end product. After depleting our population of γδTCR engineered T cells from αβTCR+ T cells, flow cytometry data allowed us to conclude that the purified resulting cell fraction is composed in its great majority by cells expressing Vγ9Vδ2 clone 5 transgene cassette (Fig. 17 - A). On the other hand, it is still interesting to observe that a mean percentage of residual CD3- cells comprises more than 10% after depletion (3,7% to 18,2%). In addition, we tried to uncover the subset phenotype of this CD3- cell fraction by screening surface markers that are known to be expressed on NK cells, such CD56 and CD16 [55, 56]. CD56 is lost upon maturation into high cytotoxic NK cells and CD16 (FcγRIIIa) expressed in maturated NK cells [55, 56]. Upon screening, we were able to identify at least one NK cell subset: $CD56^{bright}$ CD16- (34,2%). Nevertheless, we still obtained a $CD56^{dim}$ CD16- (40,8%) and $CD56^{bright}$ CD16+ (20,3%) cell population.

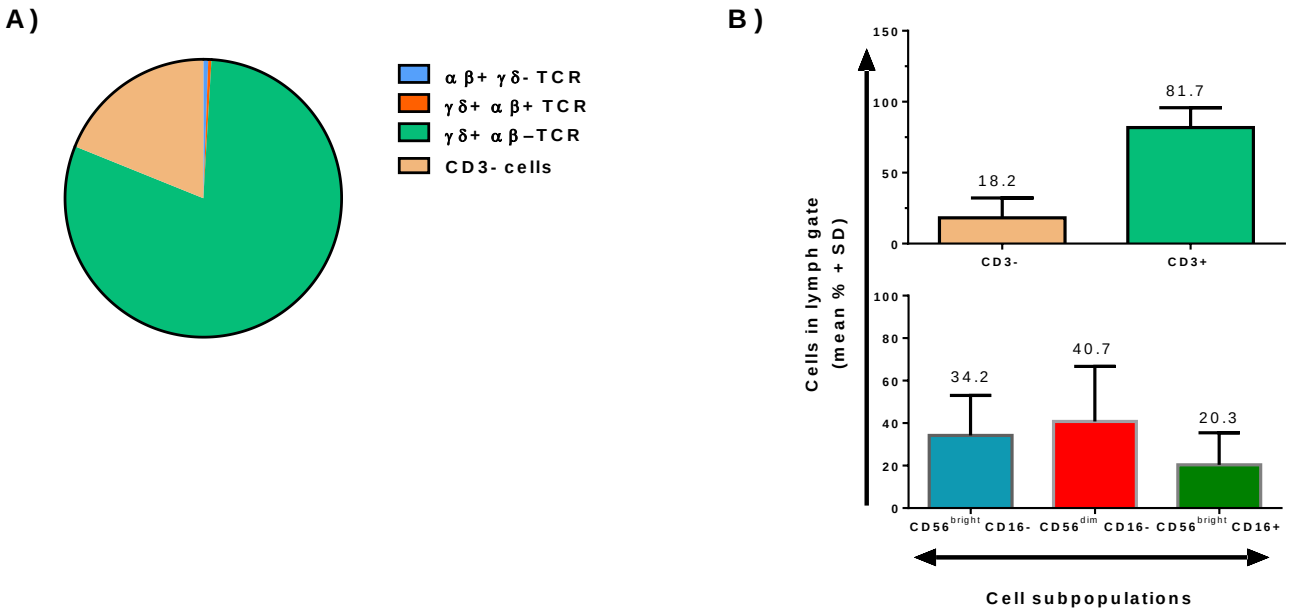

Figure 17 - γδTCR engineered T cells compose the majority of the final cell product. *A) Within the CD3+ T cell fraction different subpopulations of interest were determined by flow cytometry on day 14 after depletion of non-engineered cells; Flow cytometry data indicates that a CD3- fraction is still present in our end-product; B) flow cytometry data from CD3- (left columns) and CD3+ (right column) on day 14 after αβTCR depletion (upper part); $CD56^{bright}$ CD16- (left column) $CD56^{dim}$ CD16- (central column) $CD56^{bright}$ CD16+ (right column) subpopulations were assessed by gating on CD3- cells after αβTCR depletion (lower part); Results show mean ± 1 SD, n=4 (contained 4 individual experiments)*

To characterize the differentiation phenotype of the γδTCR engineered T cell product obtained after purification, we chose two cell surface markers - CD62L and CD27 [57]. The expression of CD62L, a cell surface adhesion molecule required for the migration of T-cells into peripheral lymph nodes, by antigen experienced T cells and the progressive downregulation of CD27, a co-stimulatory molecule that enhances T cell receptor induced-activation, proliferation and memory-formation, can be used to build up a differentiation profile to describe our cell product [57]. Using the T cell differentiation definition indicated by Klebanoff and his colleagues (2006)[57] (Fig. 18 - A) and by staining our engineered T cells with antibodies against these two molecules, flow cytometry data indicates a great presence of effector memory (T_{EM}) and effector (T_{EFF}) CD4+ and CD8+ γδTCR+ T cells. These percentages increase from the 7th to the 10th day of our small-scale production protocol (Fig. 18 - B). These results indicate that we predominantly manufacture T_{EM} and T_{EFF} γδTCR engineered T cells, with low impurity, characterized by NKs.

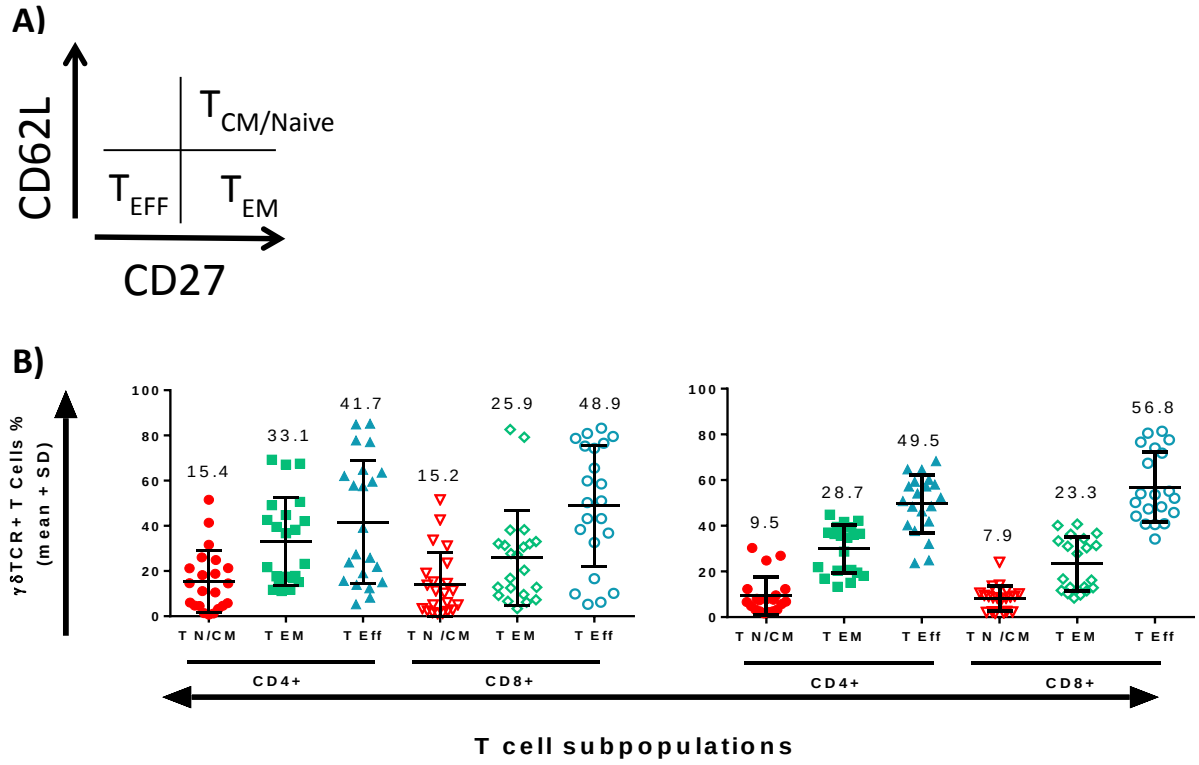

Figure 18 - Effector memory and Effector T cells compose the majority of our end product. *(A) overview of the differentiation status according to the markers used: CD62L- CD27- (T effector; T_{EFF}), CD62L- CD27+ (T effector memory; T_{EM}) CD62L+ CD27+ (T central memory/naïve; $T_{CM/N}$); (B) CD4+ and CD8+ γδTCR+ T cells were investigated by flow cytometry for their differentiation status on day 7 (left) and day 10 (right); Results show mean ± 1 SD, n=22 (for each differentiation status; day 7), n=20 (for each differentiation status; Day 10)*

3.1.8. Functional Properties

As previously referred in this thesis, the Vγ9Vδ2 clone 5 has already been tested for its tumor specific lysis by our group, demonstrating a high release of INF-γ [33, 38]. Despite the fact, that these results are promising using a research grade protocol, it is crucial to confirm these results in our newly established small-scale γδTCR engineered T cell production protocol. To address whether the γδTCR engineered T cells were able to successfully recognize a variety of primary tumor samples we set up an INF-γ ELISpot assay. INF-γ is an essential cytokine produced upon an anti-tumor γδT cell immune response [58]. Upon blocking the malavonate pathway of the isoprenoid synthesis with aminobisphosphonates (such as pamidronate; Pam), we observed that 6 out of 8 primary tumor samples acquired from different patients, were recognized by the γδTCR engineered T cells using the small-scale production protocol (Fig. 19). It is still interesting to notice that, 2 out of the 6 primary tumor samples were from B-ALL patients (Fig. 19). To summarize, not only the γδTCR engineered T cells, produced by the small scale production protocol, can induce a functional immune response against AML blasts but also, it might be able to recognize other subsets of tumor cells.

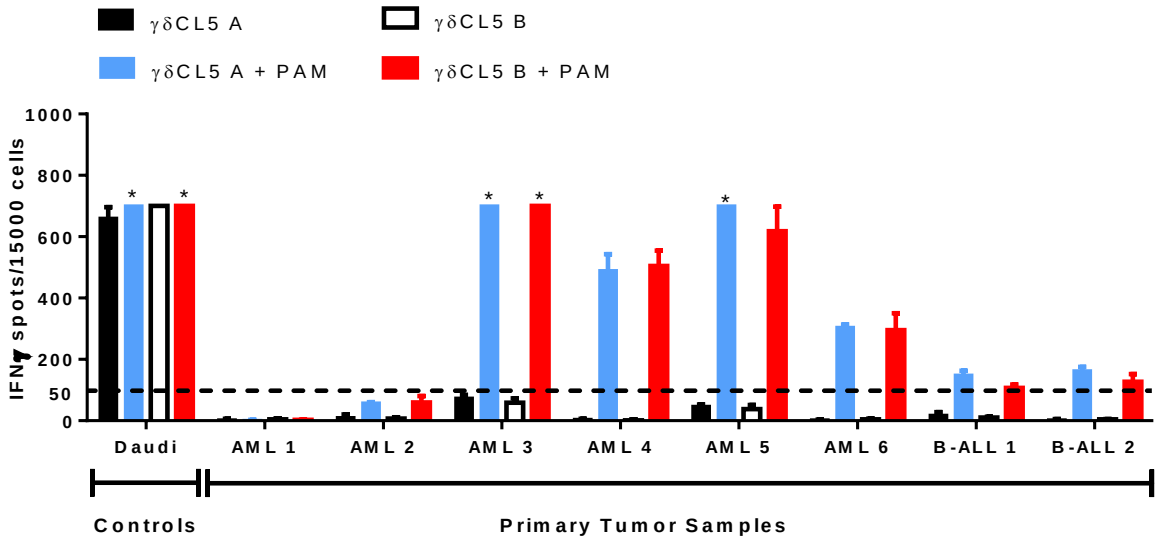

Figure 19 – 6 out of 9 primary tumor samples are recognized by γδTCR engineered T cells. *Primary tumor samples (AML 1-6; B-ALL 1-2) were incubated with γδTCR-transduced T cells that were αβTCR depleted (>90% γδTCR+) with or without 10 mmol/L pamidronate (PAM) and IFN-γ secretion was measured by ELISPOT. Daudi cell lines were used as a positive control; IFNg spots per 15,000 T cells are shown as mean of triplicates (±SD); 50 spots/15,000 cells were considered as a positive antitumor response and indicated by the black horizontal dashed line (*>700 spots/ 15,000 cells);*

3.2. Towards a large-scale production of γδTCR engineered T cells

As previously mentioned in this thesis, the γδTCR engineered T cell product is intended to be infused in an AML patient. Depending on the timeline of the clinical study, we aim to infuse the engineered T cells in three doses: (1) $1x10^6$ engineered T cells/Kg, (2) $1x10^7$ engineered T cells/Kg and (3) $1x10^8$ engineered T cells/Kg. Consequently, we needed to upscale the manufacturing process in order to obtain a higher output of γδTCR engineered T cells to meet AML patients requirements.

As previously described in the methodological section, to build a large-scale manufacturing process we had to modify: (1) the cell culturing platform, and (2) the cell separation system. To this end, we used cell culturing bags and a GMP-compliant cell separation system (CliniMACs) that allowed us to handle a higher number of T cells (Fig. 20).

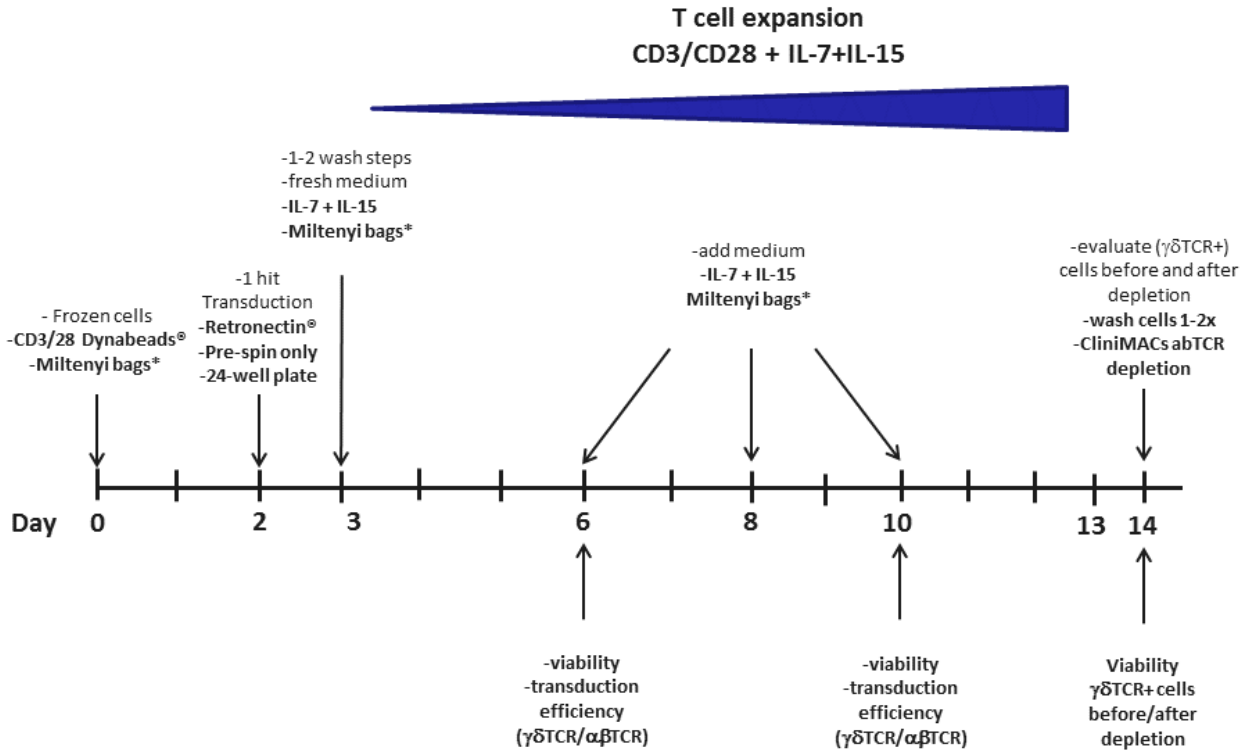

FIGURE 20 - Timeline for the large-scale manufacturing protocol of γδTCR engineered T cells. *Frozen PBMCs and the activation of T cells with CD3/CD28 Dynabeads® will be performed on day 0 in Miltenyi® cell culturing bags; One transduction step will be done in a 24-well plate with Retronectin® and using a 90 minute pre-spin on day 2 after activation ; Fresh medium with IL-7 and IL-15 will be added to the cell culturing bags on day 3, 6, 8 and 10 after activation; Cell culturing will be performed at a density of $0,5x10^6$ T cells/mL (day 3) and $0,25x10^6$ T cells/mL (day 6, 8 and 10) after transduction; Flow cytometric assays (flow) will be performed to assess γδTCR T cell recovery percentage on day 6, 10 and 14 after activation (This latter before and after αβTCR depletion); The αβTCR depletion step will be performed on day 14 after activation*

3.2.1. Transduction efficiency

Our first question resided in whether, the transduction efficiency would be the same or better than the small-scale production protocol. To be able to compare this, we performed the transduction into 24-well plates as described in the methodological section. Flow cytometry data from the 10th day of both production protocols indicate that there is a difference in transduction efficiency of less than 5% between the large-scale protocol and the small-scale protocol (49,1% and 53,7% respectively; Fig. 21). In addition, when we compared the use of the 90 minute pre-spin, with the absence of a pre-spin on the transduction step of the large-scale production of γδTCR engineered T cells, we had a loss of less than 4% in transduction efficiency (49,1% and 45,6% respectively; Fig. 21 –A). Ultimately, we confirmed that we could upscale the small-scale to a large-scale manufacturing of γδTCR engineered T cells with the use of a 90 minute pre-spin transduction step and with minimal loss of transduction efficiency.

3.2.2. γδTCR engineered T cell separation

We also compared the research-grade MACs used in our small-scale production protocol, with the GMP-CliniMACs cell separation system to be used in the large-scale production protocol, to assess whether we could obtain a similar γδTCR+ T cell recovery by upscaling the manufacturing of γδTCR engineered T cells. In the large-scale production setting, we were able to recover from the CliniMACs system 23% of γδTCR+ T cells in comparison with 12% γδTCR+ T cells recovered from research grade MACs system (both percentages relative to the number of γδTCR+ T cells acquired before the depletion; Fig. 21 -A). In addition, this γδTCR+ T cell recovery differed in 5% between the large scale and small-scale production protocol (23% against 18%, respectively; Fig. 21). Summarizing, data shows that we can upscale the manufacturing of γδTCR engineered T cells achieving a higher γδTCR+ T cell recovery.

3.2.3. *γδTCR engineered T cell* expansion profile

Finally, we analyzed how the γδTCR engineered T cells would expand in cell culturing bags comparing with the use of 24-well plates, T_{25} flasks and T_{75} flasks. Side by side comparison showed that, we could recover on day 2, 88,4% of the activated T cells compared to 38% of T cells activated in a 6-well (large-scale vs small-scale; Tab. 1). When comparing the cell transduction recovery (cells recovered after the retroviral transduction step; day 3) we observed a difference in 11% between the small-scale and the large scale protocol (130% and 119%, respectively; Tab. 1). If we compare the γδTCR+ T cell recovery (reflecting the total GMP process including T cell expansion) we can observe that a high percentage of γδTCR+ T cells can be generated from the starting T cell numbers in this large-scale production protocol comparing with the small scale protocol (1565% and 118%, respectively; Tab. 1). At the same time, we followed the expansion profile of the transduced T cells generated on the large-scale production protocol during 14 days to address if, we could obtain more γδTCR engineered T cells comparing with the small-scale production protocol. By counting T cells on keypoint days of the protocol, we were able to expand

116.5x more transduced T cell in cell culturing bags (large-Scale protocol) than in T_{75} flasks (small-scale protocol) (167,1x and 50,6x respectively; Tab. 2). Ultimately, these findings suggest that the use of cell culturing bags will allow us to get a higher number of T cells until the day of the depletion of non-engineered T cells.

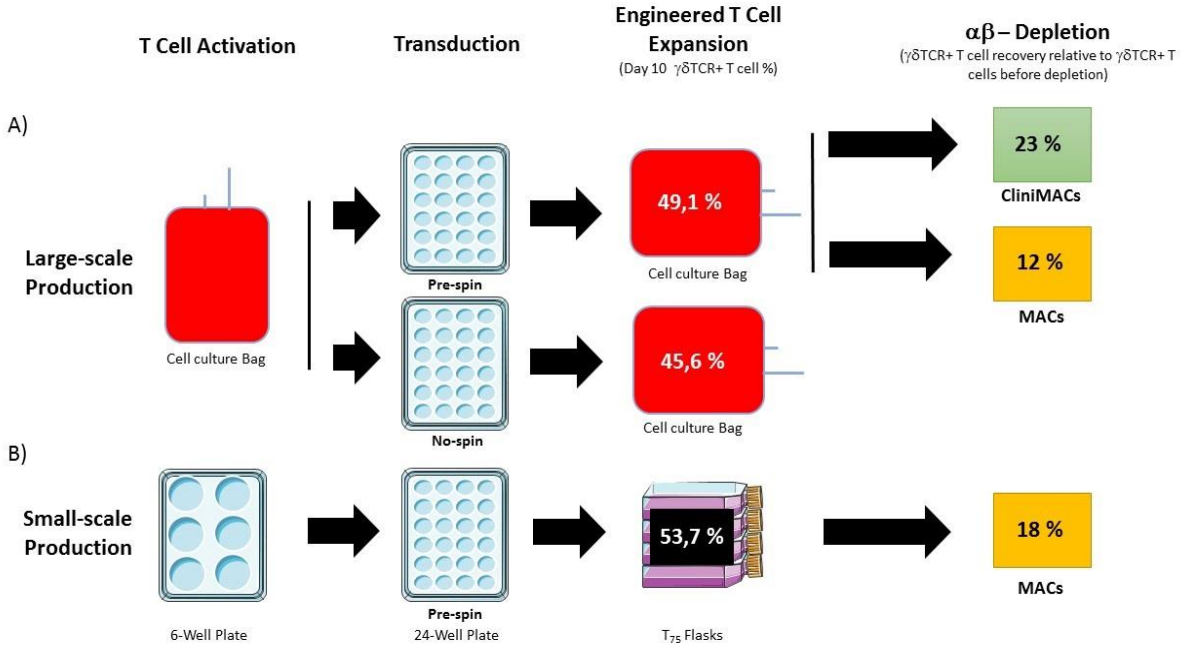

Figure 21 – Upscaling experiment from a small-scale to a large-scale production protocol for γδTCR engineered T cells. *(A) Large-scale production experiment setup; cells activated in a cell culturing bag were transduced using a 90 minute pre-spin or no pre-spin; After expansion, the γδTCR+ T cell % was evaluated on day 7 and 10 (only shown γδTCR+ T cell % from day 10); The αβTCR depletion was performed on transduced T cells generated from a 90 minute pre-spin on a GMP-compliant (CliniMACs) or using a research grade (MACs) cell separation system; (B) Small-scale production experiment setup; In parallel cells activated in a 6-well plate were transduced using a 90 minute pre-spin; After expansion, the γδTCR+ T cell % was evaluated on day 7 and 10 (only shown γδTCR+ T cell % from day 10); The αβTCR depletion was performed on transduced T cells generated from a 90 minute pre-spin using a research grade (MACs) cell separation system. γδTCR+ T cell recovery % presented in the image are relative to the γδTCR+ T cell numbers before αβTCR depletion. Results shown are based on a n=1*

Table 1 –Comparison between the overall small-scale and large-scale production of γδTCR engineered T cells. *Results shown below are based on different ns: *n=77, **n=10, ***n=1*

	Start CD3+ cells	Activation CD3+ cells	Cell recovery activation (relative to start CD3+ cells)	Cell recovery transduction	Total γδTCR+ recovery (γδTCR+ relative to start PBMCs number)
Small-scale*	$4x10^6$	$2x10^6$/ml	38%	130%	**118%****
Large-scale***	$250x10^6$	$0,5x10^6$/ml	88%	119%	**1565%**

Table 2- Overview of the expansion profile of the small-scale and large scale protocol for the production of γδTCR engineered T cells. *Results shown below are based on different ns: *n=56, **n=42, ***n=20, ****n=1; *****=cell culturing density of 0,25x10^6 T cell/ mL*

Expansion	Cell Density Expansion	Fold Expansion				Total Fold Expansion
Small-scale	0,5x10^6/ml	day 3-7*: **3.3x**	day 7-10**: **2.6x**	n.d.	day 10-14***: **5,9x**	day 3-14***: **50,6x**
Large-scale****	0.5x10^6/ml	day 3-6: **7,8x**	day 6-8*****: **3,5x**	day 8-10*****: **1,8x**	day 10-14*****: **3,4x**	day 3-14: **167,1x**

4. Discussion

Until now, autologous engineered T lymphocytes as adoptive immunotherapy post allo-SCT was extensively explored using either CARs or αβ T cell as mediators of specific antitumor immunity. To our knowledge, we describe here for the first time a translation for a clinical grade tumor-specific γδTCR engineered T cells to be used in an allogeneic setting for AML patients that have undergone an allo-SCT intervention.

To achieve an engineered T cell product, upgrading our previous research transduction protocol was our first task. To reach this goal, we seeked to improve several crucial steps: (1) the activation of αβ T cells and expansion of transduced T cells, (2) the efficient insertion of the Vγ9Vδ2 clone 5 T cell receptor transgene during the transduction step, (3) the separation of our target engineered T cells from non-engineered T cells and (4) preserving an active phenotype and functionality. Secondly, we upscaled the manufacturing of γδTCR engineered T cells for a large cell culturing platform together with a separation system capable of

Likewise other methods of producing engineered T cells [36, 59], we searched for the most suitable T cell activation. Regarding this parameter, it is known that TCR engagement, cytokine signaling and a co-stimulatory signal (such as CD28) is required, leading us to switch from OKT3 (clone from anti-αCD3 antibody) to CD3/CD28 Dynabeads® to better mimic an *in vivo* activation. Gomez-Eerland R. and her colleagues (2014)[36] used 3:1 CD3/CD28 bead: T cell. However, the use of this ratio in our small-scale production protocol resulted in high death cell numbers (unpublished data). This cell death may be a result of overstimulation [60], given the fact that by using a lower CD3/CD28 bead: T cell ratio (1:5) we could obtain a lower number of dead cells.

To expand the cells, we previously used IL-2 on the research protocol. This cytokine is widely known for being a potent growth and proliferation factor for T-cells [61]. From a functional point of view, we wanted to preserve the CD8+ T cell subset in order to obtain a maximum delivery of anti-tumor activity [62]. Despite the successes of using IL-2 to expand T cells in an *ex vivo* setting, it is known that this cytokine is reported to cause activation-induced cell death (AICD) and inhibit cell proliferation and survivability of CD8+ T cells after antigen-priming [61, 63]. To this end, the alternative was to use IL-15 and IL-7. The combinatorial effect of enhanced anti-tumor activity (IL-15), and promotion of survivability and differentiation of CD8+ T cells (IL-7) is reported to result into a high active anti-tumor T cell numbers in other protocols [36, 59, 61, 63, 64]. Although CD4+ T cells were transduced in higher numbers (due to donor PBMC heterogeneity), these participate in promoting a robust immune response [65] and mature DCs [41], discarding a possibility of an hampered anti-tumor effect by these cells.

In addition, we inserted the Vγ9Vδ2 clone 5 transgene into αβ T lymphocytes using retroviral transduction, a delivery system known to be very efficient upon human T cell TCR gene transfer [66]. We discarded the use of other gene delivery systems such as, the Sleeping Beauty and the PiggyBac that are

reported to give high TCR gene transfer but poor clinical translation [67-69]. In alternative, the use of electroporation/nucleofection is a limited option due to short TCR RNA half-life in T cells[70]. Moreover, we used an optimized pMP71 retroviral vector [38], containing murine myeloproliferative sarcoma virus (MPSV) enhancer promoter sequences, that are known to efficiently express genes in T lymphocytes [66]. This retroviral vector expressed an optimized transgene cassette containing a T2A ribosomal skipping sequence [38] with a γ5-T2A-δ5 specific orientation, known to affect transcription of this transgene in T cells [71]. The use of a packaging cell line constitutively producing RD114-derived retroviral particles with Vγ9Vδ2 clone 5 transgene, enabled us to home produce a high virus titer as reported by other authors [72]. From a clinical point of view, this emphasize that the obtention of these high virus titers, allow a production of a large-scale virus batch needed for clinical grade-setting. Reflecting the optimized gene delivery system, transgene cassette and home produced virus titers, we were able to obtain a maximal transduction efficiency of 77% with a 90 minute pre-spin (Fig. 14 – A). These results are very good, putting aside the need to use another transduction hit in the retroviral-transduction protocol. Although, we saw that donor heterogeneity might compromise transduction efficiency reinforcing the need, to assess the number of infectious particles per mL this particular virus batch and, to discover a set of optimal virus titers to be used among different donor PBMCs. Hence, in a clinical-grade setting it is possible to predict an outcome of engineered T cells independently of the donor.

Among the T cell densities we tested in the transduction step, we saw that low T cell densities ($0{,}1x10^6$ and $0{,}5x10^6$ T cells/mL) result into higher transduction efficiencies at a low virus titer ($3x10^4$ ips/mL), possibly due to a higher ratio of virus particles [73]. On the other hand, the use of higher virus titers did not influence transduction efficiency upon the testing of different T cell densities. Therefore, this reinforces the need of a retroviral transduction protocol using of a virus supernatant with a high number of viruses in order to obtain always a maximal transduction efficiency.

From a purity point of view, our method for the negative selection of produced γδTCR engineered T cells allows us to achieve almost 100% of engineered T cells. Whereas this purity is achieved, there is an additional loss of γδTCR engineered T cells. To evaluate the number of γδTCR engineered T cells that end up in the αβ-TCR deleted fraction, we chose a percentage that was the fairest by comparing the cells expressing γδTCRs before and after the αβTCR depletion. Given the fact that, this percentage does not exceed 10%, we speculate that likewise non-engineered T cells, γδTCR engineered T cells get stuck in the column while our bulk transduced T cells (transduced T cells before αβ-TCR depletion) flows through the column. This suggests that a careful revision to the manufacturer's protocol can be done to increase the recovery numbers of acquired engineered T cells, therefore increasing the efficiency of this purification method. From a clinical point of view, the obtention of untouched engineered T cells will avoid AICD upon infusion in patients [74, 75], drawing a major advantage of this exclusive purification method compared to other engineered cell selection methods.

Upon enriching for γδTCR engineered T cells after αβTCR depletion, we also noticed that a fraction of CD3 negative cells was present in our end cell product. Therefore suspicions arose for the presence of NK cells, since IL-15 (we used to expand T cells) promotes NK cell development and proliferation [76].

According to this knowledge, we screened our bulk transduced T cells (before αβTCR depletion) and the αβTCR depleted fraction for markers known for their association with the NK cell presence. We detected a subset of NK cells (CD3- $CD56^{bright}$ CD16-) that is described to be present on lymphoid organs [56]. This might be due to the presence of IL-15 in our cytokine mix, which is known to be needed for the expansion of NK cell subsets [76].

In a therapeutic setting, the administration of engineered T cells in patients relies on anti-tumor efficacy and persistence. Regarding the anti-tumor activity, other authors shown that the naïve T cell (T_N) differentiation phenotype has a therapeutic advantage when compared with central memory (T_{CM})[77-79] and T_{EM} [63, 80]. Furthermore, persistence of T_{CM} cells *in vivo* is obtained by the reexpression of CD62L [81]. Similarly to other protocols [36, 82], and because T_N cells are potent mediators of GVHD [83, 84], we aimed to produce T_{CM} engineered cells. However, the majority of our end cell product was composed of T_{EM} and T_{EFF} engineered cells probably due to the cytokine mix used in this small-scale protocol. An alternative to achieve a T_{CM} differentiation phenotype would be to optimize the cytokine concentrations of IL-7 and IL-15 or even the usage of IL-21 [85, 86], since the data we generated supports the idea that the retroviral transduction protocol we use should be different than 14 days.

Our end cell product, contained an almost pure population of γδTCR engineered T cells with a T_{EM} and T_E differentiation phenotype. Similarly to previous work from our group [33, 38], the αβ T cells transduced with the Vγ9Vδ2 clone 5 are able to recognize and release INF-γ on most of the primary tumor samples treated with PAM, including AML blasts and B-ALL blasts. This demonstrates the capability of this TCR to universally sense stressed cells that accumulate derivatives from a dysregulated malavonate pathway of the isoprenoid synthesis. More importantly, in an AML therapeutic point of view, our engineered T cell product can be used to treat AML patients. Nevertheless, to support the functional data generated in this thesis, a ^{51}Cr-release assay should not be discarded as it measures direct lysis of tumor cells [33]. Despite the fact that a residual NK subset is present on the αβTCR depleted end fraction, NKs can equally target cells self-stress molecules present in hematological tumor cells using receptors such as the NKG2D [55, 87]. However, alloreactivity of these cells is prevented by killer cell immunoglobulin-like receptors (KIRs) which sense MHC class I molecules. These cells when administered in an allogeneic (HLA-mismatched) setting, will have the KIRs disengaged allowing the killing of allogeneic transformed cells, and in this case AML blasts [88]. More importantly, we discard the possibility that NK cells can hamper a high anti-tumor activity against AML blasts.

Similar to other authors [36], we upscaled the manufacturing of γδTCR engineered T cells by using cell culturing bags and a GMP-compliant MACs cell separation system. Both of these platforms were crucial to generate a higher number of engineered T cells and to purify them from non-engineered T cells. To this end, our data indicates we were successful in mimicking the same results of our small-scale production protocol in a large-scale setting. In addition, our engineered T cells were able to expand in high numbers due to the availability of more cytokines and a higher volume to expand. However, even with the use of supporting cytokines and medium refreshment every 3 days, we noticed a decrease in numbers of engineered T cells when the culture process surpassed 14 days (unpublished data). This might be due

to the secretion of ligands such as programmed cell death-1 (PD-1L) or glucose deprivation [89]. Therefore, the culturing process should not be extended over 14 days. Nevertheless these preliminary results, it is still needed to evaluate the reproducibility of this data and to perform the transduction step in bags, in alternative to 24-well plates, to avoid the contamination probability.

5. Conclusion & Future Remarks

Warranting the delivery of a safe GMP-compliant γδTCR engineered T cell product is crucial to make it available on the market. To this end, several design issues are imposed by the food and drug administration (FDA; United States of America) and the European medicines agency (EMEA; Europe), placing cell therapy in a new level of regulation [47].

As toxicity remains one of the major concerns in cell therapy field, risk assessment of TCR engineered T cells can regard two aspects: (1) off-target effects and (2) on-target effects. As mentioned previously in this thesis, the off-target effect regards allo-reactivity on healthy tissue upon antigens that are not targeted by the TCR engineered T cells due to mispairing of TCR chains [90]. On the other hand, the on-target effect regards cross-reactivity on healthy tissue upon target-antigens that are simultaneously expressed in tumor and healthy cells [91]. Severe inflammatory colitis [92], destruction of healthy tissue [93] and fatal cardiac toxicity [94] are reported to be on-target toxicity events in clinical trials. Despite the fact that our γδTCR engineered T cell product does not have off-target issues, the on-target effect can become an issue. Although our group already demonstrated low toxicity in a pre-clinical mouse model [38], an *ex vivo* healthy tissue screening is needed to fully assess the toxicity of these engineered T cells and avoid harmful consequences upon infusion into AML patients.

In addition, taking into account that our purified engineered T cell product also includes a residual subset of unwanted cells, it is important to include more markers to further characterize other cell subpopulations by flow cytometry. Therefore, we can guarantee a minimal purity of the manufactured γδTCR engineered T cells, meet the release criteria of regulatory agencies [47], and have a full description of a safe cell therapy product.

Furthermore, the γδTCR engineered T cells were manufactured under a semi-closed system which still complies open cell transfer steps under a flow cabinet. To complete the translation of this large-scale production protocol to GMP manufacturing protocol the system must be closed and the product must be done under a certified GMP clean room [47]. This comprise the use of

To finalize, we need to deliver this GMP-compliant engineered T cell product in a phase I clinical trial. Despite the preclinical data already shown by our group [38], safety and effectiveness must be evaluted *in vivo* (post-allo-SCT AML patients). Only then, γδTCR engineered T cells can prove to be a potent immunotherapy asset for the treatment of AML patients that have undergone an allo-SCT intervention. More importantly, the versatility and properties of γδTCRs, the manufacturing protocol described and the novel purifying method used in this thesis, create a universal toolbox for the use of γδTCR gene modified αβ T cells that can revolutionize adoptive T cell immunotherapy for other malignancies.

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