QATAR UNIVERSITY

COLLEGE OF HEALTH SCIENCES

GENERATION AND CHARACTERIZATION OF "OFF-THE-SHELF" CHIMERIC ANTIGEN RECEPTOR ENGINEERED T CELLS (CAR-T) TO TARGET CANCER PATIENTS WITH HEMATOLOGICAL MALIGNANCIES

BY

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ABSTRACT

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Title: Generation and Characterization of "off-the-shelf" Chimeric Antigen Receptor Engineered T Cells (CAR-T) to Target Cancer Patients with Hematological Malignancies

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Background: The usage of T cells engineered with a tumor-specific chimeric antigen receptor (CAR) for the therapeutic treatment of some types of hematological malignancies demonstrated great clinical success. However, the manufacturing of these medicinal products is commonly performed in an autologous setting, with a relatively lengthy and complex process. Therefore, not all the patients with the defined blood cancer can have access to this type of therapy. Allogenic CAR-T cells, due to their "off-the-shelf" availability can overcome some of the limitations associated with the use of the autologous approach. The principal aim of this study was to optimize the generation of "off-the-shelf" CAR-T cells utilizing the umbilical cord blood (UCB) as a source of T lymphocytes.

Methods: UCBs were collected at Sidra Medicine from newborn babies at the time of birth. T lymphocytes where isolated by magnetic selection and then activated *in vitro* using anti-CD3/CD28 agonistic monoclonal antibodies. The activated T cells were then transduced with lentiviral vectors encoding for two types of CARs, CD19-CD28ζ and CD19-4-1BBζ CARs. The characterization of the efficiency of transduction, the *in vitro* expansion, phenotype analyses and anti-tumor activity of these CD19-CAR-T cells was performed. PBMCs were utilized as source of CAR-T cells as reference of our experiments.

Results: UCB-derived CD19-CAR-T cells showed an enrichment of CAR-T cells at early stage of differentiation (T stem cell memory/central memory T cells) associated with different activation markers. These cells demonstrated high *in vitro* expansion and exerted an efficient anti-tumor activity against target cell lines.

Conclusions: Our results allowed to identify the method for the efficient isolation *ex vivo* of UCB-derived CD19-CAR-T cells. UCB can be considered an efficient source of T lymphocytes to generate "off the shelf" CAR-T cells endowed with anti-tumor activity and favorable phenotypic properties.

DEDICATION

This thesis is dedicated to my Family, for their continuous support and encouragement during the time of my studies. This work is also dedicated to my thesis supervisors for their guide and mentorship.

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CHAPTER 1: INTRODUCTION

The concept of tumor immunosurveillance was initially proposed by Paul Ehrlich back in 1909 and further developed in 1957 by Macfarlane Burnet and in 1959 by Lewis Thomas. Back in that time, the only known function of thymus-derived T lymphocytes was that to recognize and kill foreign pathogens. However, Burnet revealed that T lymphocytes in cell-mediated immunity serve the role of identifying neo-antigens expressed on tumor cells leading to immunological reaction and eliminating cancer cells at the tumor site [1]. Thomas' theory hypothesized that the mechanisms to protect against cancer cells was similar to those mediating homograft rejections [2]. Over the past several decades, scientists dedicated efforts to investigate the immune system's functions in cancer development and progression, leading to understanding its role in the control cancer growth.

The immunological system is not only focused on destroying cancer cells and suppressing their growth, but it can also facilitate the tumor progression by determining tumor cell variants that are most likely to escape the immune response or by establishing immunosuppressive conditions in the tumor microenvironment (TME) that can promote tumor outgrowth [3]. Based on these findings, the control of tumor growth by the immune system involves three phases that can occur in sequences, including tumor elimination, tumor equilibrium and tumor escape (Figure 1) [4]. This has been denominated the "Three E" theory, by Dunn and Schreiber [5, 6].

In this regard, cancer immunotherapy aims at restoring the active immunosurveillance to fight tumor cells and bring them back to the elimination phase. The rapid progress in cancer immunotherapy allowed the development of many therapeutic approaches [7].

Among these, the adoptive cell therapy (ACT), which involves the use of the patient's anti-tumor immune cells, their expansion to obtain large number of cells and the re-infusion into patients. One of the most promising approaches of ACT is represented by T lymphocytes engineered with either lentiviral or retroviral vectors encoding for chimeric antigen receptor (CAR). These cells recognize tumor associated-antigens (TAAs) expressed on the surface of the cancer cells and, upon engagement of the CAR, undergo cell activation and proliferation [8].

The usage of autologous CD19-CAR-T cells have shown clinical activity in patients with some types of B-cell hematologic malignancy, leading to the approval of three therapeutic drugs for relapsed or refractory B cell acute lymphoblastic leukemia (ALL) and lymphoma, including diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL) and indolent follicular lymphoma [9-11]. However, autologous CAR-T cells production is costly, relatively lengthy, and associated with several disease-related challenges such as T cell dysfunction in heavily pre-treated patients [12].

UCB represents a good source of allogenic T lymphocytes due to its enrichment of naïve immune cells and allowing broader mismatching in terms of major histocompatibility complex (MHC). Currently, few studies are available demonstrating the anti-tumor activity of cord blood-derived engineered T lymphocytes [13-15]. However, the use of this novel approach still requires further understanding in terms of clinical safety and efficacy.

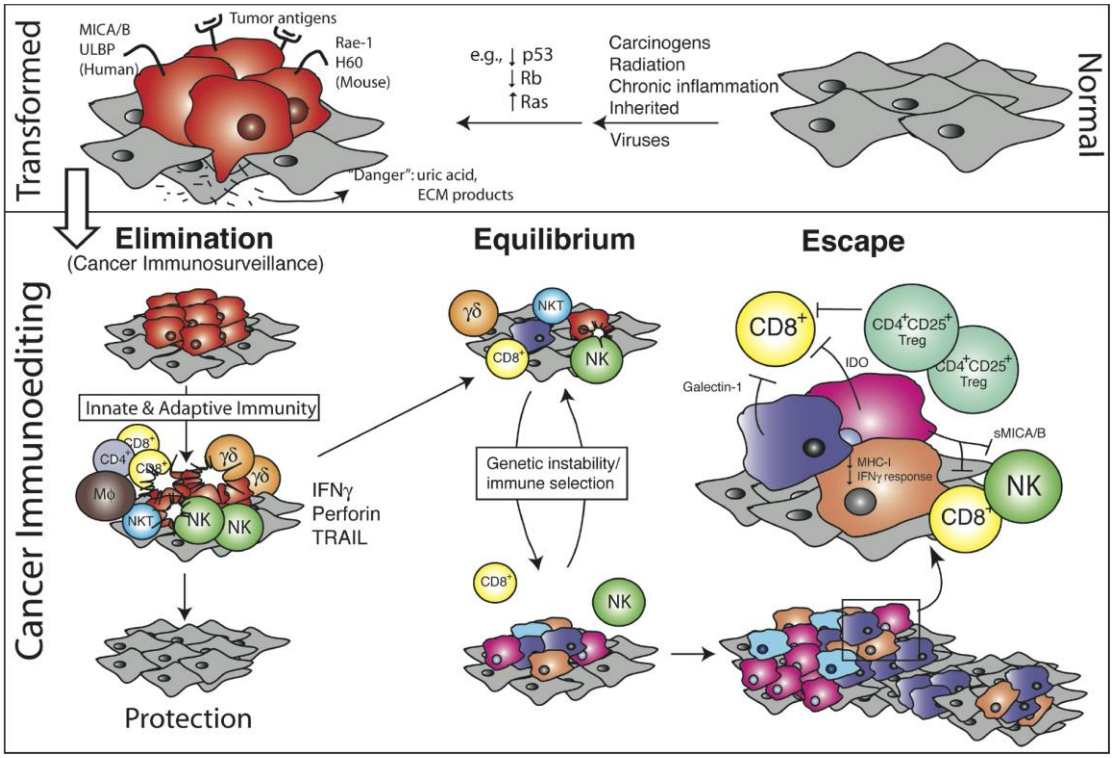

Figure 1: Three phases of cancer immunoediting process.
When transformed cells are actively recognized by the immune cells, they are eliminated (***elimination phase***) by the host's immunosurveillance. In this process, invasive growth of transformed cells triggers the release of inflammatory signals leading to the migration of innate and adaptive immune cells to the tumor site. Cancer cells variants that escaped eliminations enters the (***equilibrium phase)*** in which the Darwinian selection leads to the formation of new variants with enhanced resistance to the immune response. In addition, inflammatory and immunosuppressive signaling can occur in the TME. All these mechanisms are part of the cancer immunoediting (***escape phase***). During the phase of tumor escape, new tumor cell variants acquire a set of epigenetic and genetic changes rendering them resistant to the immune recognition leading to the tumor progression. ECM: extracellular matrix; IDO: Indoleamine-pyrrole 2,3-dioxygenase; IFN: interferon; MHC: Major Histocompatibility Complex; MICA/MICB: MHC class I polypeptide–related sequence A and B; NK: natural killer cells; gd: gd T cells; Rae: retinoic acid early inducible-1 gene; RB: retinoblastoma protein; Ras: oncogenic protein RAS; sMICA/MICB: soluble MICA and MICB; TRAIL: retinoic acid early inducible-1 gene; T reg: T regulatory cells; ULBP: UL16-binding protein. (Figure by Dunn, G.P., et al., 2004 [6]).

The principal aims of this study are represented by i. the optimization of the *in vitro* manufacturing of CAR-T cells utilizing UCB as source of immune cells and ii. to obtain a comprehensive characterization of these cells. These objectives have been achieved through:

1) CD19 target molecules, overexpressed on malignant B cells, have been selected to validate the generation of CAR-T cells from UCB.
2) Optimization of T cell isolation from UCB and their activation, transduction, and expansion *in vitro*.
3) The design and development of deep phenotype analysis and functional assays.
4) To compare the obtained results to obtain a comprehensive profiling of UCB-derived CAR-T cells.

The significance of this project is represented by the development of "off-the-shelf" CAR-T cells for cancer immunotherapy, knowing that only few clinical studies are ongoing with the use of allogenic CAR-T cells. The manufacturing *in vitro* of CAR-T cells still requires optimization. Therefore, this study was aimed at understanding the best method of activation of T cells and to compare two different lentiviral vectors.

The obtained results will allow the identify the procedures for the manufacturing *in vitro* of "off-the-shelf" CD19-CAR-T cells, starting from UCB that could be exploited for the future clinical grade development.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to cancer immunotherapy

2.1.1 Immune cell function in tumor immune response

The immune system can be divided into two different categories, innate and adaptive (Figure 2). The first line of defense against pathogens is primarily done by the innate arm of the immune system, and include the use of soluble factors and immune cells including granulocytes, mast cells, macrophages, and dendritic cells (DC) [16]. The adaptive immune system is constituted by T and antibodies-producing B lymphocytes [17]. At the junction point of immune system, between the innate and adaptive branches, are the γδ T cells and the natural killer T cells (NKT).

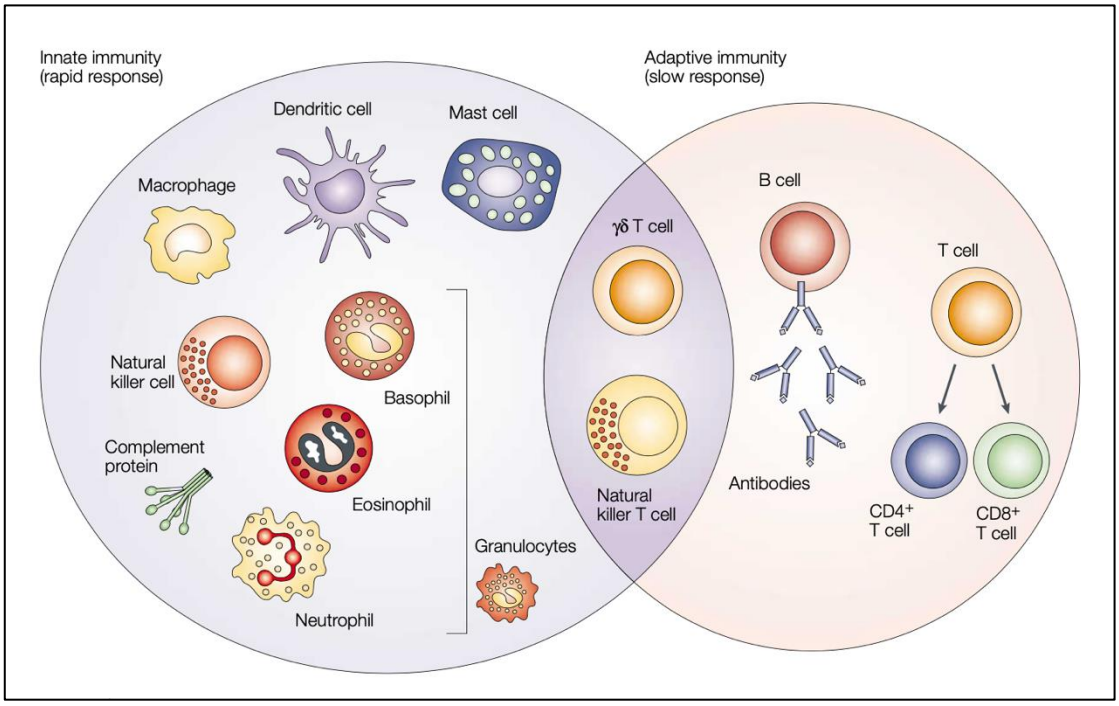

Figure 2: Cells from innate and adaptive immune systems.
The innate division serves as first defense line against infections and pathogens and includes macrophages, eosinophils, basophils, DC and mast cells, granulocytes, and natural killer cells (NK). The adaptive immune system includes both B and T lymphocytes. Natural killer-like and γδ Tcells represent the connection between the innate and the adaptive immunity. (Figure by Dranoff, G., 2004 [18])

Antigen presenting cells (APC), such as macrophages and DCs are professional cells deputed to antigen presentation to innate and adaptive immune cells. Macrophages and DC in turn can recognize and engulf apoptotic cancer cells using cell surface CD36 and αvβ5 integrin [19]. In addition to that, DC and macrophages uses CD91 molecules and other scavenger receptors to uptake heat-shock proteins and cancer-cell peptides released from necrotic cancer cells [20] (Figure 3, Panel A).

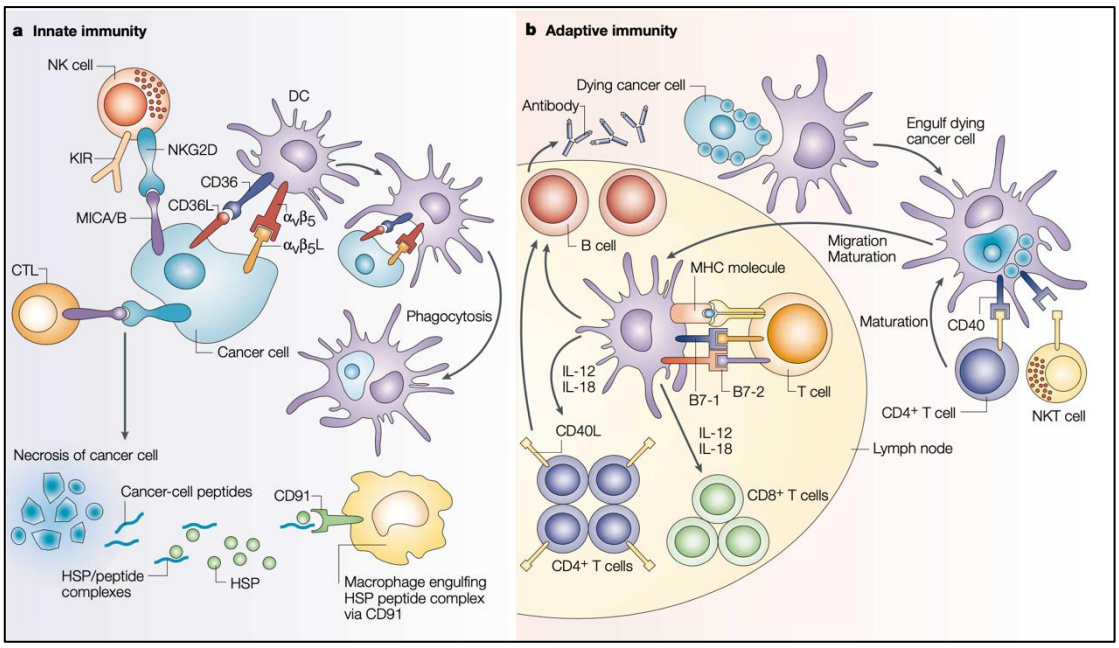

Figure 3: The complexity of the crosstalk between cells from the innate and adaptive immune systems. (Figure by Dranoff, G., 2004 [18])

Dendritic cells engulf dead tumor cells and travel via lymphatic system to lymph nodes where antigen presentation to the adaptive components of immune system takes place. NK cells can exert the anti-tumor activity through specific receptors which ligands that are expressed on the surface of APCs or of cancer cells (such as the stress-related molecules MICA and MICB that serve as ligand for the receptor NKG2D expressed on NK cells) [21] (Figure 3, Panel A).

T cells on the other hand recognize cancer cells through the interaction of T cell receptor (TCR) with TAAs presented by MHC molecules on APC or present on the surface of tumor cells [22]. However, the engagement of the TCR alone is not sufficient to sustain their proliferation, long term activation and anti-tumor activity. The activation involvement of the second signaling by co-stimulatory molecules is necessary. Molecules, such as B7-1 and B7-2 and CD40, that are efficiently expressed by mature APC can trigger the T cell-mediated immune responses [23, 24] (Figure 3, Panel B). Of note in some cases B7-1 and B7-2, although at lower levels as compared to APC can be detected on tumor cells. Upon antigen recognition, naïve T cells become activated and can proliferate and differentiate to different and temporarily related subsets. This process of T cell differentiation is associated with expression and co-expression of specific molecules (Figure 4) [25].

	Naive	SCM	CM	EM	TE
CD45RA	+	+	-	-	+
CD45RO	-	-	+	+	-
CCR7	+	+	+	-	-
CD127	+	+	+	+	+/-
HLA-DR	-	+	+	-	-
CD62L	+	+	+	-	-
CD28	+	+	+	+/-	-
CD27	+	+	+	+/-	-
CD25	+	+	+	+/-	-
CXCR3	-	+	+	-	-
CD58	-	+	+	+	+
CD57	-	-	-	+/-	+
CTLA-4	-	+	+	+	+
LAG3	-	-	+	+	+
TIM3	-	-	+	+	+
PD-1	-	-	-	+	+

Figure 4: The process of maturation of T cells from naïve to terminally differentiated effector memory T cells indicating the associated markers. Tn, naïve T cell; Tscm, stem cell memory T cell, Tcm, central memory T cell; Tem, effector memory T cell; Tte, terminal effector memory T cell.

T lymphocytes can be divided into different subsets based on their differentiation status and the expression of extracellular markers such as naïve (Tn), stem cell memory (Tscm), central memory (Tcm), effector memory (Tem), effector cell (Teff) T cells. The most common extracellular markers to distinguish Tn cells from Tem cells are CD45RA, CD25, CD62L, and CCR7. For instance, Tn $CD8^+$ cells expressing CD45RA will transform to $CD45RO^+$ Tcm cells along with their maturation [26].

The regulation of T cell activation is orchestrated by a set of costimulatory and coinhibitory signaling molecules [27]. These signaling molecules are capable of modulating the T cell immune response by either enhancing or inhibiting T cell function [28]. Several costimulatory and coinhibitory molecules have been recognized including the B7-CD28 family such as CD28, programmed death-1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), B and T lymphocyte attenuator (BTLA), and inducible costimulatory molecule (ICOS) [29]. The signaling lymphocyte activation molecule (SLAM) family including CD150, CD48, and 2B4 [30, 31]. The immunoglobulin (Ig) family such as lymphocyte-activation gene 3 (LAG-3) or T cell immunoglobulin mucin-3 (TIM-3) [32, 33]. The tumor necrosis factor receptor (TNF) receptor superfamily such as CD27 [34].

While some of these molecules are involved in the positive stimulation of T cell response such as CD28, CD27 and ICOS expressed on naïve T cells upon activation to trigger T cell expansion and survival [34-36]. Other such as CTLA-4, PD-1, BTLA, SLAM, LAG-3 and TIM-3 serves as negative regulators for the inhibition of the T function [37-41]. For example, Tumor infiltrating lymphocytes (TILs) of CD8 T cells expressing TIM-3 and PD-1, have been significantly detected in progressed melanoma patients [42]. The population CD8 cells expressing both inhibitory molecules demonstrated impaired T cell function. The targeting of such coinhibitory molecules

were shown to restore T cells antitumor function [41]. Overall, the expression of these co-stimulatory and co-inhibitory molecules in both CD4 and CD8 T cells in differential and associated with the differentiation status (Figure 4).

2.1.2 The emergence of cancer immunotherapy

Cancer is a heterogeneous disease caused by an evolutionary process of genetic and epigenetic modifications of somatic cells within the body tissues [43]. These phenomena favors adaptive environmental mechanisms such as abnormal cell division, de-regulation of cell-contact regulation, neo-angiogenesis, cell migration and invasive phenotypes [44]. For more than fifty years, cancer treatment has relied on three main strategies: surgery, radiation, and chemotherapy [45]. Immunotherapy is centered on triggering the host's immune system to recognize and destroy tumor cells.

The concept of using the immune system to treat cancer can be traced back in 1893, when William Coley from the New York Cancer Hospital was pioneering this approach. During that time, Coley has developed a preparation for the treatment of malignant tumors that consisted of cultures of deactivated bacteria named coley's toxins. This treatment was repeatedly injected into cancer patients and showed promising results. However, due to the high associated mortality, this approach was stopped in the 1930's [46].

Over the past few years, huge progress has been made in understanding cancer immunosurveillance, leading to the development of new treatment modalities for cancer. Immunotherapeutic approaches can be divided into passive and active. Passive therapy involves the administration of immune components such as antibodies, cytokines etc., to unleash or sustain the immune responses. On the other hand, active immunotherapy aims at eliciting patients' durable immune responses towards the tumor

cells (such as cancer vaccines) [47]. Overall, all major categories of cancer immunotherapy treatments, including cancer vaccines, cytokines, oncolytic viruses, immune checkpoint modulators and adoptive cell therapy, have shown promising results [7]. The proof of concept clinical trials corroborated these observations. One of the most notable achievement of immunotherapy was the improvement of the overall survival of metastatic melanoma patients using the antagonistic anti-CTLA-4 antibody ipilimumab, representing the first immunotherapy drug to be granted a Food and Drug Administration (FDA) approval and revolutionizing the paradigm of cancer treatment [48, 49].

2.1.3 Overview of immunotherapeutic approaches

Cytokine-based therapies are one of the initial non-specific cancer treatments. The release of cytokines from immune cells in response to a specific pathogen orchestrates cellular interactions and the immunosurveillance [50]. One example of a therapeutic cytokine is the interleukin-2 (IL-2), displaying the ability to induce T cell proliferation [51, 52]. Interferon-alpha (IFN-α) is another therapeutic cytokine and a major player in anti-tumor immunity [53]. Using IFN-α has been reflected in melanoma and chronic myeloid leukemia patients, where a high dosage of this agent resulted in delayed disease progression and prolonged survival. Regardless of this clinical success, the use of this therapeutic cytokine exhibited in some cases severe toxicity limiting its usage in specific indications [54, 55].

Cancer vaccines on the other hand trigger cell-mediated anti-tumor responses utilizing immunogenic proteins or peptides from TAAs [56] dissolved in adjuvants and either directly administered into patients or vectors encoding for TAAs. In addition,

mRNA encoding for TAAs, dissolved in adjuvant or encapsulated in nanoparticles, can be also utilized for cancer vaccines [57, 58].

The different types of TAAs used in the development of cancer vaccines: 1. differentiation antigens, which are overexpressed on tumor cells but shared also by normal cells. 2. cancer testis antigens, expressed in several cancer types and in normal germ cells and placenta. 3. neoantigens generated by non-synonymous mutations of cancer cells. Unlike other types of antigens, the neoantigens are rarely shared among cancer patients and each patient should be characterized for a tailored antigenic vaccine. In addition, clinical studies utilizing the use of such vaccines led to superior antigen-specific T cell responses. Thus, tumor neoantigens are characterized by higher immunogenic properties compared to other types of antigens [59]. Furthermore, neoantigens derived from non-synonymous somatic mutations are highly detectable in TILs within the lesions of some type of tumors. TILs play a key role in anti-tumor response upon *ex vivo* isolation, expansion and reinfusion into patients in the context of cell-based therapies.

The use of oncolytic viruses is another emerging class of immunotherapy. Oncolytic viruses are capable of the selective replication in tumor cells without harming normal cells. They are able to induce an anti-tumor response via a number of mechanisms like the direct killing of infected tumor cells or indirect killing caused by immune-mediated destruction [60]. Recent advances in genetic engineering provided the ability to design highly efficient and tumor selective viruses that gained remarkable clinical success. Rigvir, for example, has been approved for treatment of melanoma, Oncorine H101m for head and neck malignancies, and talimogene laherparepvec, for metastatic melanoma treatment [61].

Another group of immunotherapeutic strategy is represented by agents targeting the immune checkpoints. These molecules are modulators of immune cell functions. Moreover, their ligands can be overexpressed by tumor cells. [62]. Immune checkpoint inhibitors can be utilized to unleash the cell-mediated immune responses against malignant cells. Multiple antagonistic monoclonal antibodies (mAbs) have been developed for clinical application and, nowadays, represent standard of care for tumors with different histological origins [63]. Mostly, these agents are PD-1/ programmed cell death ligand 1 (PD-L1) signaling and CTLA-4 [63]. Notably, still a significant percentage of patients do not respond or develop resistance to these types of therapy. The search for biomarkers for the clinical response to the immune checkpoint blockade showed that the tumor mutational burden, the microsatellite instability, and the frequencies of cell blood populations or the presence in the plasma of soluble factors can be associated with the patients' clinical outcome [64, 65]

Adoptive cell transfer (ACT) is another type of personalized cancer immunotherapy involving the isolation of *ex vivo* tumor-specific lymphocytes and their expansion followed by re-infusion into patients to exert their anti-tumor effect [66]. ACT was initially investigated in the treatment of a mice model bearing local and disseminated tumor, where the systemic delivery of lymphoid cells expanded ex vivo in the presence of IL-2 led to tumor regression [67]. Eventually, several investigations aimed toward understanding the possible clinical applications for the use of T cells and other immune cells to treat cancer and other diseases [68]. Luckily, the development of *ex vivo* expansion and maintenance strategies allowed their sufficient expansion for the clinical application [69].

There are several forms of ACT such as the following: 1. the exploitation of NK cells owning anti-tumor capabilities against solid and hematological tumors; 2. the use

of TILs isolated directly from solid tumors; 3. the use of genetically engineered T or NK cells bearing either TCR or CAR with the capability to recognize and attack tumor cells [70, 71].

Rosenberg et al. demonstrated that the systemic administration of autologous lymphokine-activated killer (LAK) cells along with high doses of IL-2, enabled tumor regression in metastatic cancers [53]. The ability to isolate TILs from surgically resected tumor lesions and to expand them in the presence of recombinant IL-2 allowed their exploitation for ACT studies in melanoma patients, upon non-myeloablative chemotherapy and/or total body radiotherapy to overcome the immunosuppressive environment of cancer patients [72].

This therapeutic strategy showed clear improvement of patients' clinical outcome, even in a metastatic setting [73]. Notably, it has been shown that TILs exerting clinical activity in metastatic cancer patients with different histological origins, could recognize TAAs that have been molecularly identified, including neo-antigens [74, 75]. However, TILs cannot be commonly isolated form cancer patients and sufficient efficiency in different type of tumors. In addition, several tumor escape mechanisms, including MHC downregulation and antigen loss can lead to weak immunogenicity and failure of the treatment [76].

This limitation was overcome through the development of T cells genetically engineered to express antigen-specific TCR endowed with high avidity [77, 78]. This approach is dependent on the efficiency of antigen processing and presentation in association with MHC molecules by tumor cells.

2.2 Introduction to engineered immune cells with CARs

2.2.1 The development of CAR-T cells

In the late 1980's, scientists developed a method to design an artificial MHC-independent chimeric TCR composed of a single-chain variable fragment (ScFv) domain, conferring mAb specificity for the target molecules, fused to an antibody variable domain [79]. The structure of the CAR holds different functional properties compared to the TCR (Figure 5). The mechanisms underlying CARs specific antigens recognition is MHC-independent and regulated by the ScFv domain. Currently, CAR-T cell is one of the promising approaches in immunotherapy, by which T-cells are redirected to recognize TAAs. Nowadays, the usage of autologous CAR-T cells has shown clinical activity for some type of hematological malignancies. Hence, up to now three medicinal products have been approved by both the Food and Drug Administration (FDA) and the European Medicinal Agency (EMA) for clinical administration, tisagenlecleucel for B cell ALL, axicabtagene ciloleucel for the diffuse large B cell lymphoma (DLBCL) and brexucabtagene autoleucel for MCL [9, 10, 80].

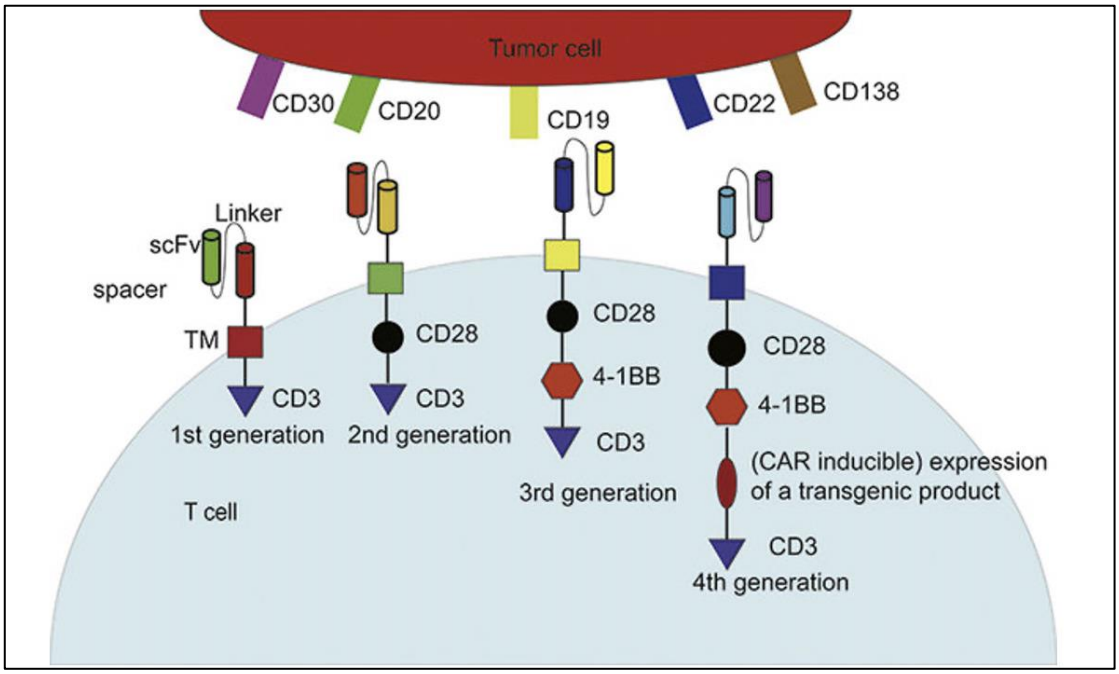

Figure 5: Basic structure of four CAR-T cells generations and common tumor cell target antigens. The structure of CAR includes three domains, extracellular (scFv), transmembrane (TM), and CD3ζ signaling intracellular domain. Different generation of CAR incorporates one or more co-stimulatory domains. (Figure by Zhao, Z., et al., 2018 [81])

2.2.2 Evolution of the CAR construct

2.2.2.1 Ectodomian

The ScFvs is a commonly used binding domain for CARs. Several studies demonstrated the correlation between ScFv affinity and CAR anti-tumor efficiency. For example, a study to evaluate the function of T cells expressing a CAR specific for the receptor tyrosine kinase-like orphan receptor 1 (ROR1) with different ScFv affinities, indicated that CARs with high ScFv affinity displayed enhanced tumor recognition and T cell effector function [82].

Other studies demonstrated that high-affinity immunoreceptors do not induce potent T cell activation as compared to low-affinity receptors. High-affinity receptors

have also the ability to distinguish cells that express high or low levels of the antigen [83].

CAR-T cells are designed to display through the high-affinity ScFvs specific binding to TAAs [84]. Another factor that can also impact CAR efficacy is the location of the target antigen epitope. For instance, CD22 CAR-T cells targeting different CD22 epitopes exhibited superior anti-leukemic activity towards membrane-proximal epitope compared to CAR-T cells recognizing membrane distal epitopes [85]. Currently, most of the ScFvs used in the clinic are derived from murine monoclonal antibodies with heavy and light chain antigen-binding domains. Though, murine ScFvs can be neutralized by human anti-mouse antibody that can be generated *in vivo*, resulting in the decrease of the activity of CARs and their poor persistence *in vivo*. Recently, efforts are ongoing to substitute murine ScFvs with single-domain antibodies such as camelid-derived nanobodies [86].

Other strategies to reduce CAR immunogenicity involve the use of functionally active CAR of human origin [87]. Unfortunately, this approach couldn't escape the chance of developing anti-idiotype antibodies that can exhibit a similar inhibitory function of the CAR. The CAR construct is not limited to the usage of ScFvs domains; other molecules recognized by target cells such as ligands and cytokines have been used for binding [88]. For example, CAR constructed with IL-13 showed anti-glioma activity **t**hrough its ability to recognize the interleukin-13Rα2 expressed specifically by the tumor cells [89].

2.2.2.2 Hinge region

The hinge region refers to the spacer sequence that connects the binding region (ectodomain) and the transmembrane domain together. Not only the ectodomain is

involved in the functionality of CAR, but also the hinge region can affect the stability and activity of the CAR. Up to date, most CAR-T cells use immunoglobulins-like domains (Ig) based hinge regions. Besides its function in separating the ectodomain from the transmembrane domain, it also affects the CARs flexibility in terms of binding to the specific antigen [90]. A study by Guest *et al.* investigated the effect of a hinge region derived from IgG1 molecule called CH2CH3 in the structure of the CARs recognizing the TAAs, carcinoembryonic antigen (CEA), neural cell adhesion molecule (NCAM), 5T4, and CD19. Even though all the CARs were able to target the antigens, the anti-5T4 and anti-NCAM CARs showed anti-tumor activity and strong cytokine release when bearing the CH2CH3 hinge. In contrast, CAR-T cell activity against CEA and CD19 was superior in the absence of spacer region. To further investigate this effect, the tracing of the ScFv epitopes has been performed and revealed that CEA specific CAR-T cells bind the target antigen in regions close to amino-terminal part that are greatly accessed by CARs lacking the hing region. On the other hand, CARs with a hinge domain seems to bind to epitopes near the cell membrane, indicating that a spacer region is required for the binding of CAR to membrane distal epitopes [91].

Several studies indicated that the length of the hinge region has to be adjusted based on the targeted epitope. Long spacer regions provide more flexibility to access membrane-proximal epitopes [92]. By contrast, short hinges are more functional in binding membrane distal epitopes [82]. Besides flexibility and stability, hinges can also affect the overall function of the CAR. IgG1 derived CH2 and CH3 hinge domains are commonly used as a spacer domain due to their exploitation to detect the expression of CARs on T cells, the low immunogenicity, and the efficient antigen recognition. However, some groups showed that CARs with Fc spacer domain can be recognized through the binding to the Fc gamma receptors (FcγRs) on the innate immune cells

through their CH2 domain. This binding results in an un-intended innate immune response, secretion of inflammatory cytokines and cytolysis of FcγRs^{+} innate immune cells [93]. This feature also leads to off-tumor localization and severe toxicity in xenograft animal models [94]. Replacement of IgG1 residues involved in FcγR binding with IgG2 amino acids lowered the chance of off-target activation and prevented CAR-T activation [93]. In addition, point mutations in Fc spacer reduced FcγRs binding, improved T cell persistence and function *in vivo* [95].

2.2.2.3 Transmembrane domain

The transmembrane domain is located between the hinge and endodomain and mediates the anchoring of CAR to T cell membrane. This domain can be represented by CD3-ζ, CD4, CD8, or CD28 structures with several studies suggesting their influence on CAR-T cell effector functions[96-99].

Similar to the TCR, it has been proposed that the activity of CARs can be associated with other molecules associated with signal initiation and activation. For example, it has been demonstrated that the first-generation of CARs harboring a CD3ζ transmembrane domain requires specific regions within the intact CD3 transmembrane for the dimerization and optimal T cell activation [96]. However, other investigations of first generation CD19 specific CAR including the transmembrane domain CD3ζ revealed lower stability when compared with the second generation that harbored CD3ζ and co-stimulatory domain CD28 or 4-1BB [100, 101].

Second and third generation CARs have been designed to integrate a variety of transmembrane domain derived from CD4, CD8α, or CD28. Recent studies showed the incorporation of ICOS instead of CD8α showed enhanced CAR-T cell resilience and resistance to tumor activity [102]. All of the previous observations suggest that the

transmembrane domain harboring specific co-stimulatory molecules are involved in overall CAR signaling and function.

2.2.2.4 Endodomain

The intracellular domain of the CAR is responsible for the activation and the engagement of the signaling of T cells upon the binding of the ectodomain to the TAA. The evolution of the construct of CARs involved the implementation of several changes in the intracellular domain by adding a variety of co-stimulatory molecules. The addition of co-stimulatory molecules defined each generation of CAR and enhanced the anti-tumor efficacy of each generation. For example, the first generation of CAR-T cells containing only CD3ζ in its intracellular domain exhibited limited anti-tumor response [103]. The main reason for this was the lack of co-stimulatory molecules and their activity in initiating T cell responses [104]. Later on, the co-stimulatory signaling molecules CD28, 4-1BB, CD27, CD278, and OX40 were integrated into the new generations of CARs and fortified the division and cytokine release by T cells [10, 100, 105-107].

The second generation of CAR-T cells was developed to carry CD3ζ along with one co-stimulatory domain and showed remarkable clinical success [108, 109]. The third generation of the CAR constructs included two different co-stimulatory molecules and revealed superior activation and proliferation compared to the second generation of CARs [106]. However, this approach was rapidly dismissed due to high grade of toxicities. Lastly, the fourth generation of CARs termed TRUCKs are modified to include an inducible transgenic expression cassette to enable cytokine secretion, such as IL-2, once the CAR-T cells reach the tumor site and upon the engagement of the

receptor. The secretion of IL-2 can enhance the T cell activation, modulate the TME and recruit additional immune cells to attack cancer cells [110].

Currently, CD28 and 4-1BB are the most commonly used co-stimulatory domains. Both co-stimulatory molecules showed a similar response in patients with hematological malignancies. Studies have shown that both co-stimulatory domains exert a different effect in terms of the *in vivo* persistence of T cells [111-113].

2.2.3 The manufacturing of CAR-T cells

The production of CAR-T cells for clinical application is a multi-step process that follows several quality-controlled steps, independently on the autologous or allogeneic source of T cells . The manufacturing process typically involves the following: isolation of T lymphocytes by leukapheresis, their activation and transduction with viral vector encoding for CARs, expansion, harvest, and cryopreservation. All of the previous steps require procedures according to the good manufacturing practice (GMP) [114].

Initially, blood leukapheresis is performed in order to harvest and concentrate in a small volume the mononuclear cells and return the plasma components back to circulation [115]. The resulting product is then further enriched for mononuclear cells, to eliminate the red cells through gradient centrifugation, where cells are separated based on size and density [116]. Afterward, either the magnetic positive enrichment of $CD4^+$ and $CD8^+$ T lymphocytes or the negative depletion of non-T cells can be further performed using magnetic beads conjugated with specific antibodies [117].

After the T cell enrichment, their activation *ex vivo* occurs using an artificial antigen-presenting cells system to mimic normal T cell activation by APC. This method was developed to allow T cell activation without the need to purify autologous APC from patient and involve the use of anti-CD3/anti-CD28 agonistic mAb coated magnetic

beads [97, 114]. Later, the beads are removed from the culture with magnetic separation [118].

T cells are then transduced with viral vectors encoding the CARs. Upon viral transduction, the vector integrates into the cellular DNA. To this scope lentiviral vectors (LVs) and gamma retroviral vectors are commonly used. However, LVs are associated with lower chances to induce mutational insertions and have superior efficiency of transduction [119, 120]. The usage of adeno-associated viral vectors has been also explored for the generation of CAR-T cells [121]. Other non-viral methods for the T cell transduction are the sleeping beauty system or the transfection of the cells with mRNA but these are at the pre-clinical phase of investigation [122, 123].

For large-scale CAR-T cell production in GMP conditions, regular and constant mixing of culture media with gas exchange are required. Therefore, for this purpose several models of bioreactors have been developed. One example is the WAVE bioreactor. Using this rocking platform, a continuous supply of fresh media is pumped into the system as equal volume of media is pumped out [124]. When starting from low cell density, Wilson Wolf manufacturing developed a new cell culture platform termed G-REX. The cell culture plates were developed to hold large media volumes with permeability of gases through a membrane located in the base of the cell vessel, allowing gas exchange and up to 100-fold expansion *in vitro* of the cell over ten days of culture [125].

Automated technologies such as the CliniMACS Prodigy device allow to perform the CAR-T cell manufacturing within a continuous flow and procedure [126]. Although this system can implement all of the steps at one run, a new high throughput system, the cocoon platform, allows the processing of multiple units all at once [127].

Safety of CAR-T cell product is monitored with the use of releasing tests for lack of contaminations such as mycoplasma, endotoxin level, replication-competent for retrovirus or LVs, and determining the insertional and copy numbers of the vectors, and the purity and potency of the final cellular product [128]. Finally, cells are either cryopreserved or transferred directly for patient infusion after the obtainment of adequate cell number.

2.2.4 Autologous vs. allogeneic CAR-T cell manufacturing

The production of CAR-T cells can be achieved through both autologous and allogeneic approaches. The autologous creation of CAR-T cells utilizes patients' circulating T lymphocytes that upon their engineering are expanded to reach large numbers ($5x10^8$-$1x10^9$ cells) for the administration into patients. This process is relatively complex, costly and can take up to three-four weeks [12]. In addition, it requires the availability of clinical grade facilities. The time required for the manufacturing of CAR-T cells can lead to possible significant delays in the treatment of the patients and the risk of progression of the disease. In some cases, autologous CAR-T cells display cellular dysfunction induced by the tumor and the immunosuppressive environment or by the heavily treatment of patients with standard therapies [129]. In other cases, the patient might not be able to receive treatment due to manufacturing failure. This occurred during the kymriah trial where 9% of the enrolled subjects didn't receive treatment due to failure in production [130]. Finally, the cost of CAR-T cell-based cancer therapy is very high for the health care system. For example the approved anti-CD19 therapies costs about 300,000-400,000 US dollars each dose [131].

Allogeneic CAR-T cells have several benefits compared to the autologous products, such as the decreased cost due to the scaled-up and "off-the-shelf" production, the availability of high number of cells from donors and the availability of cryopreserved CAR-T cells required for redosing or regular treatment. Allogeneic CAR-T cells are generated from healthy donor's peripheral blood that did not encounter the suppressive TME or chemotherapeutic agents [132]. Nevertheless, the Human Leucocyte Antigen (HLA) mismatch between the donors and recipients of the cellular product can induce allogeneic reactions. Therefore, several gene-editing techniques, such as zinc finger nucleases, CRISPR/Cas9, megaTAL nucleases, transcription activator-like effector nuclease (TALEN), and engineered I-CreI Homing endonuclease, have been developed to overcome this issue [133]. Several studies utilized these tools in the production of universal CAR-T cells (UCART) from allogenic donors to vanish either the TCR-mediated reactivity by CAR-T cells or the host's T cell -mediated reactivity. One example includes the use of zinc finger nucleases to knockout (KO) HLA-A locus [134]. Other researchers used CRISPR/Cas9 for the simultaneous KO of β2 microglobulin, TCRαβ, and PD-1 in a NALM6 NSG murine model, the results demonstrated an enhanced anti-tumor activity without inducing graft versus host disease (GVHD) [134, 135]. Other strategies targeting TCRαβ and CD52 involve the use TALEN showed that the developed gene edited CAR-T cells did not induce GVHD and were resistant to the lymphodepleting agent alemtuzumab [136]. Clinical proof of concept has been shown in a clinical study using TALEN-edited CD19 UCART for the treatment of infants with ALL; the study demonstrated great clinical success with complete remission and no sign of GVHD [137].

2.2.4.1 The use of UCB–derived T cells for CAR-T cell manufacturing

UCB represents another promising source of T cells. In terms of HLA matching, a study performed by Eapen et al. evaluated three sources of allogeneic hemopoietic stem cells including UCB, peripheral blood mononuclear cells (PBMC), and bone marrow (BM) for the treatment of acute leukemia [138]. The results showed that leukemia-free survival of 4-6/6 HLA-matched UCB was similar to 8/8 and 7/8 HLA matched PBMC and BM. Furthermore, the usage of UCB was associated with reduced severity of GVHD compared to the other two sources. Suggesting that the use of UCB allows broader HLA mismatching [138]. In addition to superior antigen naïve phenotype that might result in reduced alloreactivity and also improved homing properties [139]. Finally, UCB T cells have reduced expression of the nuclear factor of activated T cells 1 (NFAT1). Impaired NFAT signaling may reduce the response of UCB T cells towards the patient's alloantigen's, thus limiting the severity of GVHD following the transplantation [140].

2.2.4.2 Allogenic CAR-T cells: *ex-vivo* expansion and *in-vivo* persistence

There is a remarkable linkage between CAR-T cell clinical efficacy and expansion. Lymphodepleting therapies are needed for the expansion of infused T cells *in vivo*, where these cells undergo a process termed homeostatic expansion [141]. This homeostatic proliferation is driven by the post-lymphodepletion increased release of the homeostatic cytokines IL-7 and IL-15 [142, 143]. Earlier clinical trials using CAR-T cells did not include the lymphodepletion step resulting in lower persistence of the engineered T cells. Thus, lymphodepletion has been integrated into the therapeutic protocol to get rid of the immunosuppressive environment of the host [144].

The time of persistence of CAR-T cells to achieve favorable clinical outcome is unclear. The presence of these cells in circulation in association with the remission of the disease depends on the type of the disease, its biology and host's background. For example, it has been proposed that longer persistence is required for the control of some malignancies such as B-ALL [9]. In contrast, patient with DLBCL receiving axicabtagene ciloleucel can remain tumor-free after achieving CR even at six months post-infusion [10].

Another factor contributing significantly to CAR-T cells division and survival is the co-stimulatory domains. CAR-T cells bearing the CD28ζ exhibited a greater proliferative capacity and frequency of effector memory T cells while CAR with 4-1BBζ showed the superior persistence and central memory phenotype [145].

Compared to the autologous approach, the allogenic approach is associated with lower CAR-T cell persistence. Several methodologies have been proposed to prevent the rejection of allogeneic CAR-T cells and to improve both the expansion and the persistence. One strategy is the biobanking of T cells expressing a variety of combination of HLA alleles according to the population under consideration. The results from organ and UCB transplantation highlighted that the most alleles for matching are HLA-A, HLA-B, and HLA-DR [146-148]. A biobank of 150 HLA-typed homologous participant could correspond to 93% of United Kingdom population [149]. Supporting the idea that this number of donors that are homozygous for all three HLA alleles could be beneficial. However, based on the experience of ACT with third-party virus-specific T cells, it has been shown that the persistence of infused cells with partial HLA matching is lower compared to autologous T cells, where cells were able to persist for 14-90 days after infusion. It has been also shown that donor-derived Epstein Barr Virus (EBV)-specific T cells demonstrated persistence *in vivo* for over ten years [150].

Another possible approach to reinforce allogenic CAR-T cell persistence is to increase the lymphodepletion with the addition of alemtuzumab, a monoclonal antibody that binds to CD52, leaving CAR-T cells function unaffected. In fact, along with the development of gene editing technologies, it has been proved in an animal model of lymphoma that CAR-T with the knocked-down of CD52 and TCR could be successfully engrafted and were resistant to the elimination by alemtuzumab while maintaining their anti-tumor activity [136]. Similar results have been obtained using universal anti-CD19 CAR-T cells in ALL patients in two-phase I clinical trials [137, 151]. Similarly, the deletion of deoxycytidine kinase, which is responsible for purine nucleotide analogues toxicity can prevent CAR-T cell alloreactivity and enhance resistance against lymphodepleting regimens [152]. However, the use of lymphodepletion regimens can also increase the possibility of introducing opportunistic infections.

Another approach is represented by the generation of universal CAR-T cells lacking HLA class I expression through the deletion of β2-microglobulin [153]. In addition, $CD4^+$ T cells can also lead to allogenic CAR-T cell rejection. So, the deletion of HLA class II genes, e.g., the associated transcription factors such as *RFXANK* or *CIITA* could be a possible solution [154].

Finally, the selection of some immunomodulatory genes to disturb immune rejection has been also proposed. In terms of T cells, PD-L1 and FASL represent possible targets. In addition, the targeting of CD47 and CD200 could lead to the escape of macrophage-mediated rejection [155]. It is not clear yet if all of the modifications mentioned above will be required to avoid allo-rejection.

2.2.5 Selection and identification of the optimal T cell subsets for allogenic CAR-T manufacturing

T cells are composed of heterogeneous subpopulations characterized by the combination of different markers, cytokine profile, functions, and a role in disease pathology [156]. From a functional point of view, T cells can either display tumor-specific effector functions or immunoregulatory properties. Hence, the manufacturing of optimal and most efficient CAR-T cell product requires the selection of the T cell subsets with favorable functional compositions [157].

All the reported clinical trials for CAR-T cells showed heterogeneity in terms of the composition of T cells, including either $CD4^+$ and $CD8^+$ lymphocytes both with different phenotype subsets. A study by Sommermeyeret al. demonstrated that $CD4^+$ and $CD8^+$ T cells derived from different subsets (Tn, Tcm, Tem) exhibited variability in terms of proliferation and effector functions both *in vitro* and *in vivo* [158]. Compared to other differentiated subsets, memory cells can persist longer in the lack of antigen exposure and demonstrate increased activity upon re-exposure [159]. Despite that $CD8^+$ Tem cells can exhibit cytolytic activity, only $CD8^+$ T cell clones isolated from Tcm cells exhibited long-term persistence *in vivo* as shown in a non-human primate model [160]. In addition, less differentiated cells such as Tscm hold a greater therapeutic efficacy [160, 161].

In addition to T cell phenotype, the ratio of $CD4^+$ and $CD8^+$ subsets can also affect the anti-tumor capacity of CAR-T cell therapy. In a phase I/II clinical trials, ALL and non-Hodgkin lymphoma patients have been infused with a 1:1 ratio of $CD4^+$ and $CD8^+$ CAR-T cell product. The trial showed that the administration of a cellular product with defined T cell subsets is safe and effective at low doses [162, 163]. Therefore, the CAR-T cell manufacturing process is not only revolved around generating enough product

for therapy, but also to generate a unified product with a sufficient number and specified ratio of potent T cell subsets.

CAR-T cell persistence was shown to depend on the percentage of $CD4^+$ cells and Tcm cells in the infused product [164]. Indeed, it has been previously demonstrated that $CD4^+$ T cells can support the generation of $CD8^+$ memory T cells [165], highlighting the importance of both combinations in immunotherapy. Obtaining the desired populations of T cell subsets might require some adjustments to culture conditions, type of cell culture medium or the method of T cell transduction. For example, the use of IL-7 and IL-15 in culture conditions can increase the frequency of early differentiated $CD8^+CD45RA^+CCR7^+$ CAR-T cells, a phenotype that corresponds to cells at early stage of differentiation characterized by higher anti-tumor activity and *in vivo* persistence [166, 167]. In addition, the component of the cell culture medium can also affect the metabolic activity and state of T cell differentiation. For instance, the addition of exogenous L-arginine to the culture media has been shown to induce Tcm phenotype expressing CCR7 and CD62L [168]. Finally, the comparison between two different methods of transduction, piggyBac transposition and LVs, demonstrated that the piggyBac system contributed to an enhanced expression of early differentiation markers of Tscm phenotype [169].

2.3 The use of CAR-T cells for the treatment of hematological malignancies

2.3.1 Acute lymphoblastic leukemia (ALL)

CD19 has been chosen as a target antigen for CAR-T cells due to its high expression in B cell malignancies and lower levels on normal B cells [170]. One advantage of using CD19 as a marker for B cell malignancies is the limited side effects of off-target effects that can happen when CAR-T cells recognize the same antigen on other type of non-

malignant cells. The reason for that is that CD19 is only specific to B cells and not expressed on hematopoietic stem cells nor other normal cells [171]. It has been shown, that upon the treatment and the depletion of normal B cells, these cells can be regenerated by the body.

Several clinical investigational studies performed with anti-CD19 CAR-T cells in adults and children with ALL showed complete or partial remission rates [172, 173]. In one study, autologous anti-CD19 CAR-T cells with CD28ζ signaling domain was administered to sixteen ALL patients. All patients received cyclophosphamide conditioning before the infusion. The overall remission rate was 88% and the safety was documented [174]. Another study reported complete remission in 70% of young adults and children with ALL with detectable molecular remission in 60% of the patients. In addition, CAR-T cells had a short-term persistence of approximately two months. However, this short-term persistence was equalized by rapid tumor remission [172, 175].

A single center Phase I-II study evaluated the use of CD19-4-1BB-CAR-T cell therapy, tisagenlecleucel, also termed Kymriah, in 59 children with ALL. During this trial, high rate of complete remission (93%) has been achieved. However, cytokine release syndrome that is associated with CAR-T cell therapy occurred in 88% of patients. These toxicities have been effectively managed using the anti-cytokine (IL-6) therapy [176].

Based on the previous study, a phase 2 pivotal clinical study, ELIANA, involving 25 clinical centers tested the use of tisagenlecleucel in a total of 75 pediatric and young adult patients with ALL. Notably, 61% of the enrolled patients relapsed after the allogenic hematopoietic stem cell transplantation. Overall, the remission rate of 81% has been achieved within three months after the infusion of CAR-T cells, that had long-

term persistence (twenty months after the treatment). Moreover, relapse-free and overall survival of 73% and 90% and of 50% and 76% at six and twelve months after treatment, respectively were found [9].

A study evaluated CD19 CAR-T cells with isolated $CD4^+$ and $CD8^+$ Tn, Tcm and Tem T cell subsets. Both results *in vivo* and *in vitro* experiments demonstrated a difference in cell expansion and effector functions [158, 177]. Additionally, patient relapses in multiple clinical trials due to antigen escape raised the need to target also other TAAs. In this regard, CAR-T cells targeting CD20, another common cell marker expressed by B leukemic cells, have been developed to overcome the loss of CD19 expression by leukemic cells [178].

Overall, these studies have led to the approval for the first time of a cell-based immunotherapy approach for hematological malignancies.

2.3.2 Lymphoma

According to cancer statistics, lymphoma has been considered as the most common type of lymphoid malignancies and also ranked globally among the ten most common types of human cancers [179]. Heterogeneous cancer of the lymphatic system can be divided into Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL). HL is the least common type. The majority of lymphatic malignancies is NHL which includes B-cell, T-cell, and NK-cell NHLs. Chemotherapy is considered as the first-line of treatment for lymphoma followed by immunotherapeutic agents that significantly altered its therapeutic landscape [180, 181]. However, CAR-T cell therapy has emerged among the newly developed advanced immunotherapies for some subtypes of NHL [10, 182].

Several CARs have been developed to target a variety of lymphoma-associated surface antigens. A multicenter phase 1 clinical trial, ZUMA-1, evaluated the efficacy of using Yescarta, a CD19-CD28ζ-CAR-T cell, in patients with DLBCL. Four out of seven patients (57%) showed complete response (CR) while five patients (71%) achieved overall response (OR). CAR-T cells were detected in the circulation of patients with long-term CR for up to 12 months following infusion [183]. Subsequent second phase trial, ZUMA-1, comprised of 111 patients with refractory DLBCL, transformed follicular lymphoma, and primary mediastinal B-cell lymphoma. The trial achieved 82% of objective response rate and 54% of CR rate. Interestingly, 23 of the patients achieved CR at 15 months after infusion [10]. Moreover, a two year follow up study of this trial exhibited an objective response of 82% and 58 % of CR [184].

Besides the use of Yescarta, a phase II clinical trial, JULIET, in patients with DLBCL evaluated the use of Kymriah. The trial resulted in 52% of overall response rate and 40% of CR, with the remaining patients experiencing at least a partial response [185]. Later in May 2018, Kymriah received a second approval for treatment of adult patient with DLBCL [185].

Besides axicabtagene ciloleucel and tisagenlecleucel, a third anti-CD19-4-1BBζ CAR-T cell product named lisocabtagene maraleucel has been recently investigated in phase I clinical trial (NCT02631044) for patients with B-NHL. This product was manufactured with a defined ratio (1:1) of $CD4^+$ and $CD8^+$ T cells, showing durable clinical responses [169].

In July 2020, the FDA granted a new approval for the third CAR-T cell therapy, brexucabtagene autoleucel (Tecartus) to treat MCL. Tecartus was tested in phase II clinical trial, ZUMA-2, where 85% and 59% of patients experienced overall response rate or CR, respectively. Unlike other approved CAR-T cells, the production of

Tecartus involves a T cell selection step to exclude circulating tumor cells which upon transduction can result in tumor resistance [69].

Other studies targeting CD20, CD22, and CD30 with CAR-T cells have been performed. In phase II clinical trial (NCT01735604) the treatment of patients with $CD20^+$ NHL demonstrated an overall response rate of 81.8% [170]. A phase I clinical trial (NCT02315612) evaluated the use of anti-CD22 CAR-T cells in ALL patients resilient to anti-CD19 CAR-T cell treatment, showing the achievement of remissions in 73% of patients [171]. A phase I trial (NCT01306146) exploiting anti-CD30 CAR-T cells in HL and anaplastic large cell lymphoma (ALCL) patients showed clinical response in 28.6% and complete remission in 50% of HL and ALCL patients, respectively [172]. Instead, the development of CAR-T cells targeting T cell malignancies is more challenging due to the same type of cells in normal, tumor, and CAR-T cells leading to auto-killing of CAR-T cells [173].

2.3.3 Chronic lymphocytic leukemia (CLL)

CLL is a common chronic lymphoid hemopathy with a highly variable clinical course and prognosis. In some cases, CLL patients can survive for many years without any medical interventions, while other patients can display a more rapidly aggressive disease [186]. Allogenic stem cell transplantation represented the optimal treatment for treating high-risk patients. However, the development of novel therapeutic strategies such as B cell antigen receptor (BCR) pathway inhibitors, ibrutinib, venetoclax, and idelalisib demonstrated clinical success and introduced a challenge in assessing the most suitable treatment [187].

In the past, CLL was one of the first disease in which CAR-T cell therapy has been explored [188]. Although, the use of CAR-T cell therapy in CLL is less extensive

compared to other hematological malignancies. The infusion of allogenic CD19 CAR-T cells in relapsed patients post hematopoietic stem cell transplant demonstrated clinical safety, efficacy in the absence of GVHD [189, 190]. However, the effect of prior therapy or the immunosuppression caused by CLL pathogenesis represents one of the main obstacles in CAR-T cell therapy in CLL patients. Indeed, the investigation of T cell phenotype and function in CLL patients demonstrated that $CD4^+$ and $CD8^+$ T cells exhibited an increased expression of T cell exhaustion markers (PD-1, CD160, and CD244). In addition, $CD8^+$ T cells showed lower cytotoxicity and proliferative capacity [191].

The *ex vivo* expansion and production of CAR-T cells from CLL patients and healthy controls showed a clear difference in terms of T cell phenotype and activity. Clearly, patient-derived T cells displayed lower expansion of the naïve T cell subset endowed with long-term persistence compared to healthy donors. In addition, the expression of exhaustion markers in naïve $CD4^+$ T cell subsets was significantly higher in patients compared to healthy donors [192].

These observations support the need for developing allogeneic CAR-T cells, where T cell expansion and activity are not modified by the disease pathogenesis. In this regard, ibrutinib, a permanent selective bruton's tyrosine kinase inhibitor, demonstrated its ability to enhance the engraftment and therapeutic efficacy of CAR-T cells with CLL. Indeed, this effect was reported in three studies. Fraietta et al. performed a phenotypic and functional characterization of T cells in CLL patients treated with ibrutinib. The results showed that five cycles of ibrutinib therapy resulted in an enhanced CD19 CAR-T cell expansion and lowered expression of PD-1 on T cells and CD200 on B cells [193]. Two other groups reported promising results for CLL patients

receiving CAR-T cells in combination with ibrutinib with an overall response rate of 80% and above [194, 195].

2.3.4 Multiple myeloma (MM)

MM is a B-cell malignancy characterized by the rapid proliferation and accumulation of plasma cells in the bone marrow. MM is ranked as the second most common hematological malignancy accounting for 1% of all human cancers [196]. Despite the availability of several treatment modalities and the introduction of novel agents, MM remains incurable due to its poor prognosis and complicated pathogenesis [197]. Adoptive T cell therapy represents a promising approach for treating MM. This proposed role is mostly due to the fact that graft versus myeloma effect after allogeneic bone marrow transplantation is curative even with minimal or absence of concomitant chemotherapy [198, 199].

Although CD19 is a widely expressed tumor cell antigen in hematological malignancies, the expression of CD19 in myeloma cells is very limited. Thus, the effect of anti-CD19 CAR-T cells therapy is actually more harmful rather than curative where it targets and kills healthy tissues instead of tumor cells [200]. However, targeting of myeloma propagating cells with lower expression of CD19 has been shown to be clinically effective when treating patients with the chemotherapy drug melphalan, and in the setting of post autologous stem cell transplantation [201]. In addition, the CR has been registered upon treatment with a higher dose of melphalan despite the absence of CD19 expression in patients' cancer cells [202]. Despite of these encouraging results, the most common potential targeted molecules on myeloma cells are CD38, CD56, and CD138 [203]. CD138 is a heparan sulfate large glycoprotein that belongs to the syndecan family (syndecan-1) that shed from myeloma tumor cells and accumulate in

the BM promoting tumor growth [204]. CD138 is widely expressed in human cells and tissues such as in hematopoietic tissues, malignant plasma cells, neoplastic epithelial cells, and other healthy tissues [205, 206]. The use of anti-CD138 CAR-T cells in five patients with advanced MM showed a reduction of myeloma cells in one patient and stable disease in four patients [207]. However, immune escape by antigen loss or shedding has been observed with myeloma chemotherapy leading to relapse and tumor progression [208].

CD56 on the other hand, is only expressed on tumor cells and absent in non-neoplastic tissues [209]. However, the expression of this marker on cells from the central and peripheral nervous system can enhance the risks of having a neurologic toxic effect. Indeed, in a phase I clinical trial, the use of antibody-drug- conjugate orvotuzumab mertansine targeting CD56 in MM patients resulted in stable disease in 42.9% of patients. However, drug-associated peripheral neuropathy occurred in 50% of patients [210]. In terms of using CD56 as a target for CAR-T cell therapy, the use of second-generation anti-CD57 CAR-T cells demonstrated anti-tumor efficacy in a pre-clinical study [211]. Other molecules identified as a potential target is CD38. However, this marker has been shown to be expressed in solid tissues, myeloid precursors, and hematopoietic stem cells, leading to the risk of on-target and off-tumor toxicity. The use of light-chain exchange technology to produce low affinity anti-CD38 CAR-T cells with the ability to distinguish tumor cells from normal cells has been investigated [212, 213].

B cell maturation antigen (BCMA) is another target for MM also identified on normal and malignant plasma cells. Studies conducted to evaluate the efficacy of CAR-T cells targeting the BCMA has been performed. Upon receiving this cell-therapy, all subjects received the lymphodepletion chemotherapy with fludarabine and

cyclophosphamide beforehand. The Phase-I study included 33 patients with aggressively pre-treated MM. 85% of overall response rate and 45% of CR were registered. Although the incidence of cytokine release syndrome was high (25 patients), the severe form of CRS was only developed by two patients [214]. The second trial, named KarMMa, determined the efficacy of the CAR-T cells Idecabtagene vicleucel. The trial enrolled 128 patients who received lymphodepletion chemotherapy followed by the CAR-T product at the dose range of 150 to 450 $x10^6$ cells. The overall response and CR were estimated at 73% and 33%, respectively, coupled with minimal residual disease of 33% [215].

2.4 *In vitro* isolation and characterization of Allogenic CD19-CAR-T cells

The development of CAR-T cell therapy has drastically changed the therapeutic landscape for the treatment of hematological malignancies. The production of clinical-grade autologous CAR-T cells is complex and is associated with several challenges. In this regard, the development of allogeneic "off the shelf" CAR-T cells represents a promising strategy to overcome these limitations.

The manufacturing of allogenic CAR-T cells from healthy donors is currently under development, with several ongoing clinical trials for some hematological malignancies [216]. The aim of these clinical trial is to evaluate the safety and efficacy of allogeneic CAR-T cells. Earlier trials utilizing anti-CD19 CAR-T cells in patients with relapsed B-ALL achieved minimal residual disease negative remission [217]. However, the use of allogeneic CAR-T cells is associated with the risk of rejection or GVHD.

UCB represents a good source of antigen naïve allogenic T cells for CAR-T cell production. Pre-clinical studies of UCB-derived CD19 CAR-T cells in animal model of lymphoma showed anti-tumor efficacy upon treatment with second and third

generation allogenic CAR-T cells. Unfortunately, no comparison has been made to PBMC- derived CAR-T cells in these studies [218]. The safety and efficacy of using UCB-derived CAR-T cells are still under investigation. Moreover, the optimization of the procedures for the generation of UCB-derived CAR-T cells is still undergoing. To this regard, we aim in this study to optimize the *in vitro* manufacturing of CAR-T cells utilizing UCB as source of immune cells and to obtain a comprehensive characterization of these cells.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell lines

1061-, 1869- and 1076-EBV-B cell lines, were previously generated from the peripheral blood of subject through the immortalization with deactivated EBV as described [219]. Raji, T2, CEM, and K562 were obtained from (American Type Culture Collection, USA). The cell lines were cultured *in vitro* in RPMI 1640 media (Gibco, 12633012, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Sigma, F9665), and 1% HEPES (Gibco, 15630080, USA), L-glutamine (Gibco, 35050061, USA), penicillin and streptomycin (Corning, 30-001-Cl, USA). The cell lines were incubated at 37^0C and 5% CO2.

3.2 Plasmid preparation and bacterial transformation

Plasmids encoding for LVs for the CD19-CAR bearing the mutated nerve growth factor receptor (p75mut or delta LNGFR; ΔNGFR) in a bidirectional orientation, that represented a tool to detect the expression of CARs, were produced together with plasmids encoding for helper vectors. ΔNGFR lacks the ability to translate signaling and serves as a probe of transduction only. These plasmids have been kindly provided by Drs. A. Bondanza and M. Casucci, San Raffele Scientific Institute, Milan, Italy [220].

The bacterial transformation with plasmids was performed using DH10α cells (One-Shot Top10 chemically competent cells; Invitrogen, C404003, USA). The agar powder (20g; Sigma, L2897-250G, USA) was dissolved in LB medium and then sterilized with autoclave at 121^0C for 20 minutes. After the cooling of the medium, ampicillin (100 μg/mL) was added and then the solution was poured into petri dishes for solidification.

Competent DH10α cells were thawed on ice and 1 μg of plasmid was added to the cells. The cells were then incubated on ice for thirty minutes and then placed at 42°C for thirty seconds. Then, 250 μl of S.O.C media (Invitrogen, 15544034, USA) were added. The transformed cells were incubated for one hour in a shaking incubator at 37°C at 225 rpm. The transformation reaction was then spread on agar plates followed by the incubation upside down at 37°C. The day after, the colonies were collected and added to LB broth (Teknova, L8000, USA) plus 100 μg/mL ampicillin in a 15 ml tube for 3-5 hours of incubation in the bacterial shaker.

Then, the content was transferred to an Erlenmeyer flask with 200 ml of LB broth plus ampicillin and incubated overnight at 37^0C in the bacterial shaker. The bacteria culture was collected, and the purification of the plasmids was performed using the Purelink hiPure Plasmid Filter maxiprep kit (Invitrogen, K210017, USA) following the manufacturer instruction. The final yield and purity of plasmids was determined using the nanodrop spectrometer. These plasmids were then utilized to prepare the LVs.

293T cells were transfected with the plasmids and the produced LV were purified by high-speed ultracentrifugation of the cell culture medium (the preparation of LVs was performed by team members in the lab of Dr. C. Maccalli). Viral titration was performed through the transduction of 293T cells in a 6-well plate ($7.4x10^4$ cells/well) with serial dilution of LVs encoding for CAR-T cells. The efficiency of transduction was determined by immunofluorescence and flow cytometry analysis using CD19-PE and NGFR-PE antibodies.

3.3 UCB T cell isolation, expansion, and retroviral transduction

Fresh UCBs were obtained from newborn babies with cesarean section procedures performed at Sidra Medicine. The inclusion criteria of the study were limited to healthy women without pathogenic or genetic conditions. The study was approved by the Sidra Medicine Institutional Review Board (#1812044429).

3.3.1 Isolation of mononucleated cells from UCB samples

Mononuclear cells were isolated by gradient centrifugation with ficoll plaque (Cytiva, 17144002, Sweden) and SepMate™ isolation tubes (StemCell Technologies, 85450, Canada). UCB samples were diluted at 1:1 ratio with phosphate buffer saline (PBS) (Gibco, 14190136, USA) for the gradient centrifugation. The sample was centrifuged at 300 x g for 10 minutes without the brake. After the centrifugation, the mononuclear cells localized in the interface between red cells and plasma where collected.

The cells were then centrifuged twice with PBS, then the cell counts were performed. In the case of the presence of residual red blood cell (RBCs) in the fraction of mononucleated cells, RBC lysis buffer (Gibco, A1049201, USA) was added, and the sample was incubated for 10 minutes at 4 degree. After incubation, the sample was centrifuged with PBS and utilized for cell counting.

3.3.2 Cell counting

All cell counts were performed using the Scepter™ automated cell counter (Millipore, PHCCOOOOO, USA). Briefly, an aliquot of cell suspension was diluted at 1:20 in PBS. T cells and the aforementioned cell lines were counted using the 40 (Millipore, PHCC40050, USA) and 60 μm (Millipore, PHCC60050, USA) sensors,

respectively. The cell count was performed with appropriate gating based on the size of the cells. The instrument was able to discriminate live and dead cells.

3.3.3 Isolation of T lymphocytes

T lymphocytes were isolated from UCB by magnetic separation using Pan T cell isolation kit (Miltenyi Biotech, 130-096-535, Germany). After the determination of the cell numbers, they were resuspended in AutoMacs buffer (Miltenyi Biotech, 130-091-221, Germany) at the concentration of $10x10^6$ cells/40 μL. The cells were labeled with 10 μL of biotin antibody cocktail and incubated for 5 minutes at 4ºC followed by a second incubation for 10 minutes at 4ºC in the presence of 30 μL of buffer and 20 μL of microbeads cocktail solution.

A LS column (Miltenyi Biotech, 130-042-401, Germany) was placed on the MACS MultiStand with a MiniMACS Separator. The column was reconstituted through rinsing with 3 ml of AutoMacs buffer. The cell suspension was added to the column and the flow-through containing the enriched T lymphocytes was collected. The column was further washed with 3 ml buffer. The cell fraction containing T cells was then counted as described above.

3.3.4 Generation of CAR-T cells

The purified T cells were activated *in vitro* for 48 hours using anti-CD3 and anti-CD28 agonistic monoclonal antibodies either conjugated to magnetic beads (Dynabeads) (Gibco, 40203D, Norway) or to a colloidal polymeric nanomatrix (T cell TransAct) (Miltenyi Biotech, 130-111-160, Germany).

Dynabeads: 3 beads/ T cell were used. The estimated number of beads was added to a 15 ml tube with 5 ml of AutoMacs buffer. The tube was placed on the DynaMag

magnet for three minutes and then the supernatant was discarded. The cells ($20x10^6$ cells/ml) in buffer were added to the beads and incubated in the magnet. Then, the cells/beads mixture was transferred to 1.5 ml tubes (800 ul/tube; Eppendorf) and incubated with continuous rotation for two hours. The cells bound to the beads were transferred to a 15 ml tube in 3 ml of buffer and placed on the DynaMag magnet for three minutes to separate the cells from the buffer. The cells where then re-suspended in X-VIVO15 (Lonza, 04-418Q, USA) plus 2% of human serum (HS) (Gemini bio-products, 100-512, USA) culture medium and plated in a 24-well plate at the concentration of $1x10^6$ cells/ml in 2 ml of medium/well.

TransAct: T cell were plated at the density of $1x10^6$ cells per cm^2 in 24-well plate in 2 ml of culture medium/well in the presence of 20 μl of TransAct.

3.3.5 Transduction of T cells with LVs

The transduction of T cells with LVs was performed utilizing different methods including spinoculation, Vectofusin (Miltenyi, 130-111-163, Germany), or RetroNectin (Takara). T cells activated *in vitro* were seeded in a 24-well plate ($2x10^6$cells/well) in 1 ml of medium and then the volume of LVs corresponding to the desired Multiplicity of Infection (MOI; 5-25) was added. These cultures were centrifuged for 2 hours at 2400 RPM. Then, the plate was incubated for 4 hours at 37^{o}C, followed by a centrifugation for 6 minutes at 2400 RPM. Half of the medium was removed and replaced with fresh medium plus rh-IL7 (Miltenyi, 170-076-111, Germany) and rh-IL-15 (Miltenyi, 170-076-114, Germany) with (5ng/ml each).

Alternatively, RetroNectin (20 μg/ml) coated plates were used to culture the T cells in the presence of the LVs. The usage of soluble Vectofusin (20 μg/ml) for the transduction with LVs of T cells was also utilized. The following day of transduction 1

ml of fresh medium plus cytokines was added to the transduced cells with a final volume of 2 ml/well.

3.4 Flow cytometry analysis

The deep phenotype characterization of T cells transduced with LVs encoding for CARs was performed at different time points following their transduction *in vitro* (Day +9 and Day +14). The phenotypic analysis was performed using ad hoc designed panels developed by Dr. Cristina Maccalli (lead principal investigator of CAR-T cell project at Sidra Medicine) that allowed to determine the composition of T cell subsets representing different stages of differentiation of the T cells (Table 2). These panels include markers of activatory and immune checkpoint molecules. Details of fluorochrome-conjugated antibodies included in the panel are provided in (Table 1).

Briefly, $2x10^5$ cells/tube were resuspended with the staining buffer (PBS plus 2% FBS) and incubated with the mix of antibodies. Following 30 minutes of incubation in the dark at 4^oC,cells were then centrifuged with staining buffer to eliminate the excess of unbound Abs and resuspended in staining buffer with DRQ7. Following incubation for 15 minutes at room temperature, cells were acquired with the flow cytometer Navios (Beckman Coulter, B83535, USA). Data were analyzed using the software Kaluza (Beckman Coulter, B83535, USA).

Table 1: Antibodies used for the phenotype analyses of CAR-T cells

Antibody	Fluorochrome	Catalog number	Company	Country
CD57	FITC	IM0466U	Beckman coulter	USA
HLA-DR	FITC	IM1638U	Beckman coulter	USA
CD127	FITC	B25366	Biolegend	USA
CD28	FITC	IM1236U	Beckman coulter	USA
CD152	FITC	349907	Biolegend	USA
CD134	FITC	350006	Biolegend	USA
TIM3	FITC	345022	Biolegend	USA
CD127	FITC	B25366	Beckman coulter	USA
CD28	FITC	IM1236U	Beckman coulter	USA
CD152	FITC	IM2282	Beckman coulter	USA
CD62L	FITC	IM1231U	Beckman coulter	USA
CD137	PerCP-Cy5.5	309814	Biolegend	USA
CCR7	PerCP-Cy5.5	353220	Biolegend	USA
CD69	PerCP-Cy5.5	310926	Biolegend	USA
CD278	PerCP-Cy5.5	313518	Biolegend	USA
CD279	PerCP-Cy5.5	329914	Biolegend	USA
CD25	PerCP-Cy5.5	302626	Biolegend	USA
CD223	APC	369212	Biolegend	USA
CD272	APC	344510	Biolegend	USA
CD56	APC	IM2474	Beckman coulter	USA
CD27	APC	B09983	Beckman coulter	USA
NKG2D	APC	A22329	Beckman coulter	USA
NGFR	PE	557196	BD Pharmingen	USA
CD45RO	PE-Cy7	B13648	Beckman coulter	USA
CD8	PB	B49182	Beckman coulter	USA
CD45RA	ECD	IM2711U	Beckman coulter	USA
CD4	APC	A94685	Beckman coulter	USA
CD3	VioGreen	130-113-128	Miltenyi Biotech	Germany
DRQ7	-	424001	Biolegend	USA

FITC, fluorescein isothiocyanate ; PerCP-Cy5.5, Peridinin chlorophyll protein-Cyanine5.5 ; APC, Allophycocyanin ; PE, Phycoerythrin ; PE-Cy7, Phycoerythrin cyanine dye 7 ; PB, Pacific blue ; ECD, phycoerythrin-Texas Red conjugate

Table 2: Combinations of antibodies for the staining of CAR-T cell phenotypic characterization

Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9
CD134	CD57	CD28	CD62L	CD28	HLA-DR	CD152	TIM3	CD127
NGFR	NGFR	NGFR	NGFR	NGFR	NGFR	NGFR	NGFR	NGFR
CD45RA	CD45RA	CD45RA	CD45RA	CD45RA	CD45RA	CD45RA	CD45RA	CD45RA
CD137	CCR7	CCR7	CCR7	CD69	CD278	CD279	CD279	CD25
CD45RO	CD45RO	CD45RO	CD45RO	CD45RO	CD45RO	CD45RO	CD45RO	CD45RO
NKG2D	CD56	CD27	LAG3	CD27	LAG3	CD272	CD272	LAG3
DRQ7	DRQ7	DRQ7	DRQ7	DRQ7	DRQ7	DRQ7	DRQ7	DRQ7
CD4	CD4	CD4	CD4	CD4	CD4	CD4	CD4	CD4
CD8	CD8	CD8	CD8	CD8	CD8	CD8	CD8	CD8
CD3	CD3	CD3	CD3	CD3	CD3	CD3	CD3	CD3

3.5 **Cytokine release and cytotoxicity assay**

3.5.1 EliSpot and FluroSpot assays

Cytokine release assays have been performed upon the co-culture of CAR-T cells ($1X10^4$ cells/well) with either $CD19^+$ (Raji, EBVB and T2 cell lines) or $CD19^-$ (CEM and K562 cell lines) target cells ($1.7X10^4$ cells/well). T cells were plated in antibody coated 96 well plates in duplicate overnight with the following conditions: T cell alone, or in the presence of $CD19^+$ or $CD19^-$ cells. The release of cytokines, granzyme B and perforin was measured using an enzymatic colorimetric ELISPOT (Mabtech, 3420-4APT-2, Sweden or CTL, hIFNgIL4-1M/10, USA) or fluorescent-based FluoroSpot kits (CTL, hT2014F, hT08, hT66, 10, USA). IFN-γ or dual color IFN-γ and IL-4 were detected by colorimetric EliSpot assay (Mabtech and CTL, respectively).

The simultaneous detection of IFN-γ, Granzyme B and Perforin was assessed by florescence-based EliSpot. The experimental protocol was performed following the manufacturer instructions as described below.

Day 0: The PVDF membrane of the 96 well Multiscree-HTS plate was hydrated with 70% ethanol for one minute and then washed three times with PBS. The Capture Solution (monoclonal antibody) was then diluted according to the manufacture guideline and 100 μl were added/well for an overnight incubation at 4ºC.

Day 1: Wells containing the capture solution were emptied and washed three times with PBS. The cells are then re-suspended with either RPMI plus 5% FBS or CTL media, depending on the type of assay, and seeded according to the different culture conditions as described above.

Day 2: The plate was washed either 5 times with PBS or 3 times with PBS followed by two washes with PBS plus 0.05% tween. The detection solution, composed by the

secondary antibodies was then added followed by 2 hours of incubation at RT, and then by washing with the appropriate washing buffer. The tertiary solution, including either streptavidin HRP or ALP for colorimetric assays or fluorochrome conjugated reagents for the FluoroSpot assay were then added. Following 1 hr of incubation, the plate was washed three times with PBS/PBS-TWEEN (Sigma, P9416, USA) and once with distilled water. For the colorimetric assay, the plate was washed five times with PBS and then incubated with the development colorimetric solution. The reaction was blocked through the washing with tap water and dried overnight at RT in the dark. The developed EliSpot or FluoroSpot were then scanned with either visible or UV lights, respectively with the ImmunoSpot® analyzer (CTL, USA).

3.6 Data analysis

Statistical analysis of the immunofluorescence assays of CAR-T cells was performed with the Mann-Whitney' test followed by the correction for multiple hypothesis testing using a Benjamini-Hochberg procedure (analyses were performed by Dr. Cristina Maccalli and Dr. Mohammed Elanbari). Data from EliSpot and FluroSpot assays were analyzed by GraphPad Prism and results were represented as mean± SD. The statistical analyses of *in vitro* expansion experiments and functional assays were performed with the unpaired T-test to compare CAR-T cells stimulated with different conditions. P-value < 0.005 was considered for statistical significance.

CHAPTER 4: RESULTS

4.1 Generation of UCB-derived CAR-T cells

T cells were isolated from UCB through the first step of gradient centrifugation followed by the magnetic negative enrichment of T cells. In order to optimize the experimental procedures to generate CAR-T cells two methods of activation *in vitro* for T cells were evaluated: anti-CD3/anti-CD28 mAbs conjugated to either magnetic beads (B) or bound to a polymeric nanomatrix structure, TransAct (T). The cellular yields of mononuclear cells (MNC) following the gradient separation and the T cell enrichment are shown in Table 1. The total yield of T cells isolated from UCB ranged from $1.6x10^7$ to $5.7x10^7$ cells (Table 3). In Table 3 the yield of the isolation of T cells from the peripheral blood of healthy donors is represented. T cells in the range of $1.9x10^7$ to $2.2x10^7$ cells were obtained from 30 ml of peripheral blood (Table 4). These results highlighted that T cells could be efficiently isolated from UCB with a concentration of about $5.6x10^5$ cells/ml that is compatible with the *in vitro* processing of CAR-T cells.

Table 3: Total yield of cells isolated from UCB samples

Sample	MNC count	Yield after T cell enrichment	UCB volume
UCB1	3.80E+07	2.30E+07	45ml
UCB2	3.56E+07	1.62E+07	45ml
UCB3	1.42E+08	4.05E+07	60ml
UCB4	2.00E+08	5.68E+07	75ml
UCB5	1.29E+08	2.93E+07	60ml

MNC, mononuclear cells; UCB, umbilical cord blood.

Table 4: Total yield cells isolated from PBMC samples:

Sample	PBMC count	Yield after T cell enrichment
PBMC 1	1.17E+08	1.90E+07
PBMC 2	4.88E+07	2.24E+07

PBMC, peripheral blood mononuclear cells.

These T cells have been utilized for the generation of CAR-T cells, through the activation *in vitro* followed by the transduction with LVs. These cells were either untransduced (UT) or transduced with LVs encoding for CD19-CD28ζ, or CD19-4-1BBζ CARs. The comparison of the engineering of T cells with LVs bearing two different co-stimulatory molecules had the purpose to determine the influence of these signaling domains on CAR-T cell phenotype, anti-tumor activity and *in vitro* expansion. Overall, the manufacturing timeline and *in vitro* expansion of CAR-T cells was approximately of 14 days. T cells derived from PBMC represented the reference for the optimization of the workflow for UCB-CAR-T cells. The experimental process to isolate T cells from UCB and the generation of CAR-T cell *in vitro* is shown in (Figure 6).

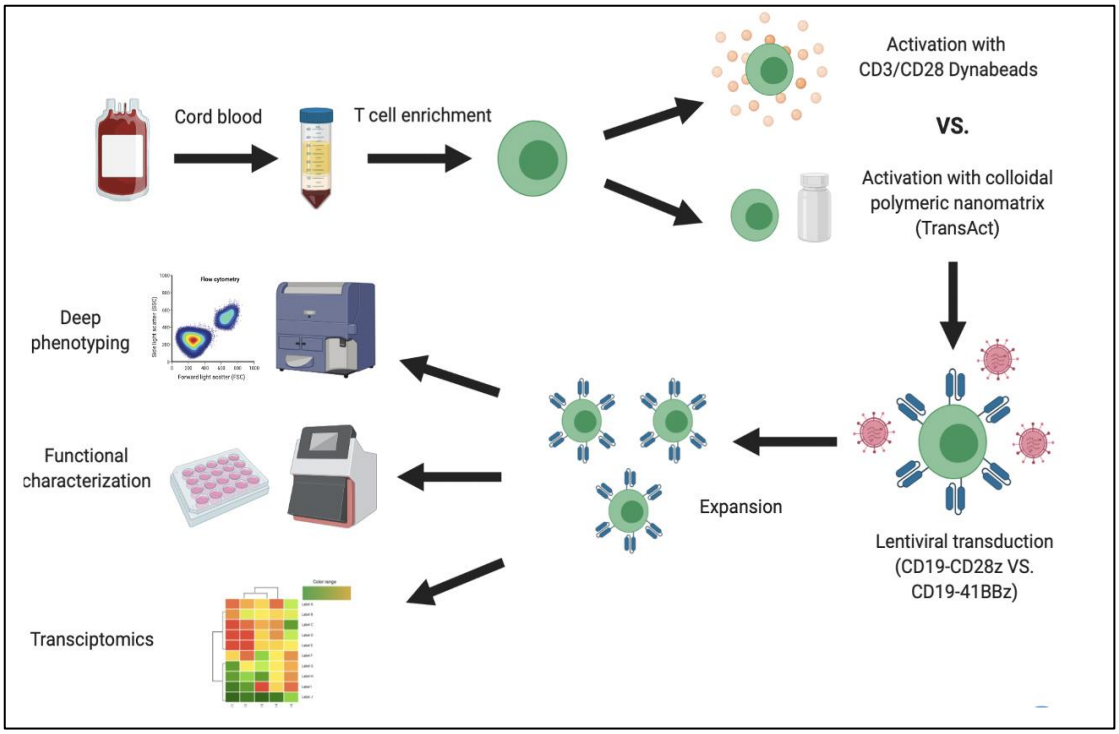

Figure 6: Schematic representation of the process of generating UCB-derived CAR-T cells and their manufacturing validation.

4.2 Efficiency of the transduction of T cells with CD19-LV

Three different procedures for the transduction *in vitro* of the T cells were utilized and compared: two commercial compounds, retronectin and vectofusin, and the usage of spinoculation following the addition of LVs to the cell cultures. CD19-CD28ζ-CAR-T cells generated from N=1 UCBs and N=1 PBMCs were utilized for this pilot experiment.

The efficiency of transduction of T cells was assessed by flow cytometry assays, through the staining of the cells with either a NGFR specific mAb or fluorochrome labelled CD19 protein at day +9 following the engineering of the T cells. These two reagents were directed to either the ΔNGFR encoded by the LVs, to assess the overall efficiency of transduction or the expression of CAR bearing the Ab domain specific for CD19, respectively.

When comparing different methods of transduction in UCB only (Table 4), the results demonstrated that the overall % of transduced cells was 49.93-96.25 as shown by the % of cells expressing NGFR (Figure 7, Panels B, C and D, and Table 5). The expression of CD19- CD28ζ-CAR ranged between 29.39-85.19% (Figure 8, Panels B, C and D, and Table 5).

Notably, superior efficiency of CD19-CAR$^+$ T lymphocytes was achieved through the usage of vectofusin as compared to spinoculation or retronectin (85.19% of positive cells as compared to 38.21% and 50.52%, respectively) (Figure 8, panel B, C and D). Similar results have been obtained for the expression of NGFR (96.25% vs. 64.27%, or 69.46% of positive cells) following the transduction with vectofusin, spinoculation or retronectin, respectively (Figure 7, panel B, C and D).

On the contrary, the methods of transduction of T cells isolated from PBMCs did not affect the efficiency of cells expressing either NGFR or CD19-CARs. Interestingly, when comparing the different methods of transduction in PBMC (Table 5), the results showed that the overall % of transduction of cells ranged between 83.73 - 93.63% as indicated by staining for NGFR (Figure 9, panel B, C and D, and Table 5. Moreover, the expression of CD19-CD28ζ-CAR ranged between 23.94–44.56% (Figure 10, Panels B, C and D, and Table 5).

In addition, we have also evaluated the effect of the method of transduction comparing the activation *in vitro* of T cells with beads vs. TransAct. The activation of T cells with beads led to higher expression of CD19-CAR compared to the usage of TransAct for UCB (69.46% compared to 49.92% and 96.25% compared to 67.55%) with retronectin and vectofusin, respectively as shown by NGFR staining, and (50.52% compared to 29.39% and 85.19% compared to 42.23%, respectively) as shown by staining of CD19 (Figures 7, panel C and D, and Figure 8, Panel C and D).

Similar results have been obtained in PBMC (93.62% compared to 88.54% and 91.95% compared to 86.08%) with retronectin and vectofusin, respectively as shown by NGFR staining, and (44.56% compared to 23.94% and 41.14% compared to 24.94%) for the staining of CD19 (Figures 9, Panel C and D, and Figure 10, panel C and D).

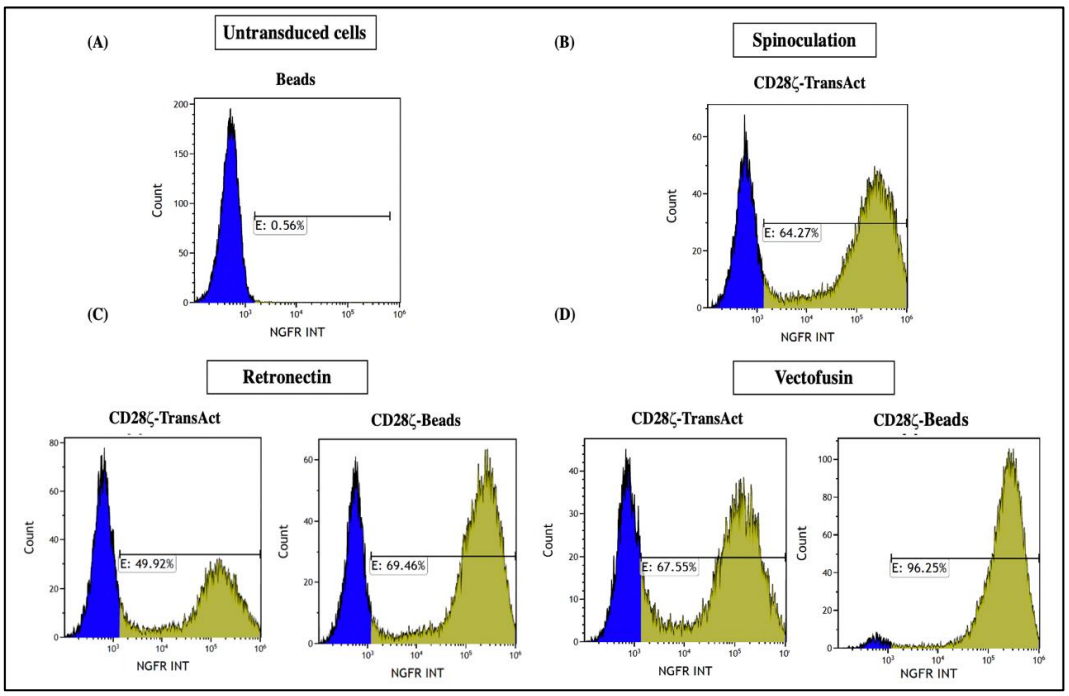

Figure 7: Flow cytometry analysis showing the efficiency of transduction by NGFR staining in T lymphocytes isolated from UCB and transduced with LVs encoding for CD19-CD28ζ CAR. Untransduced cells (A), cells transduced with spinoculation (B), retronectin (C) or vectofusin (D). The results shown here are from N=1 UCB-derived CD19-CAR-T cells. CD28ζ, CD19-CD28ζ-CAR-T cells.

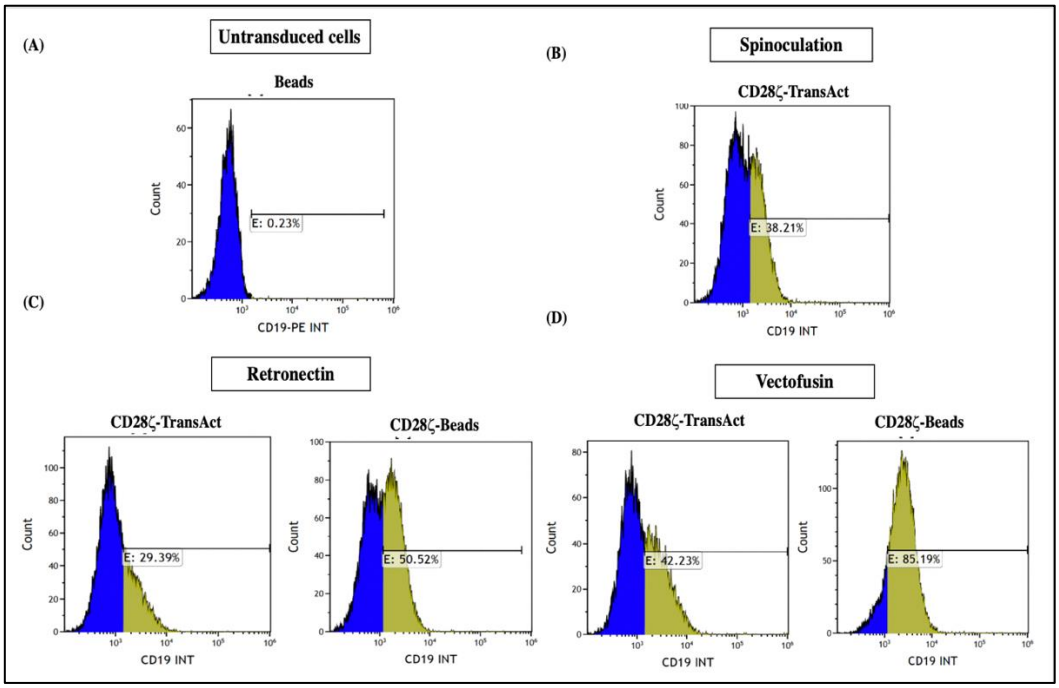

Figure 8: Flow cytometry analysis showing the efficiency of transduction by the staining of CD19 in T lymphocytes isolated from UCB and transduced with LVs encoding for CD19-CD28ζ CAR. Untransduced cells (A), cells transduced with spinoculation (B), retronectin (C) or vectofusin (D). The results shown here are from N=1 UCB-derived CD19-CAR-T cells. CD28ζ, CD19-CD28ζ-CAR-T cells.

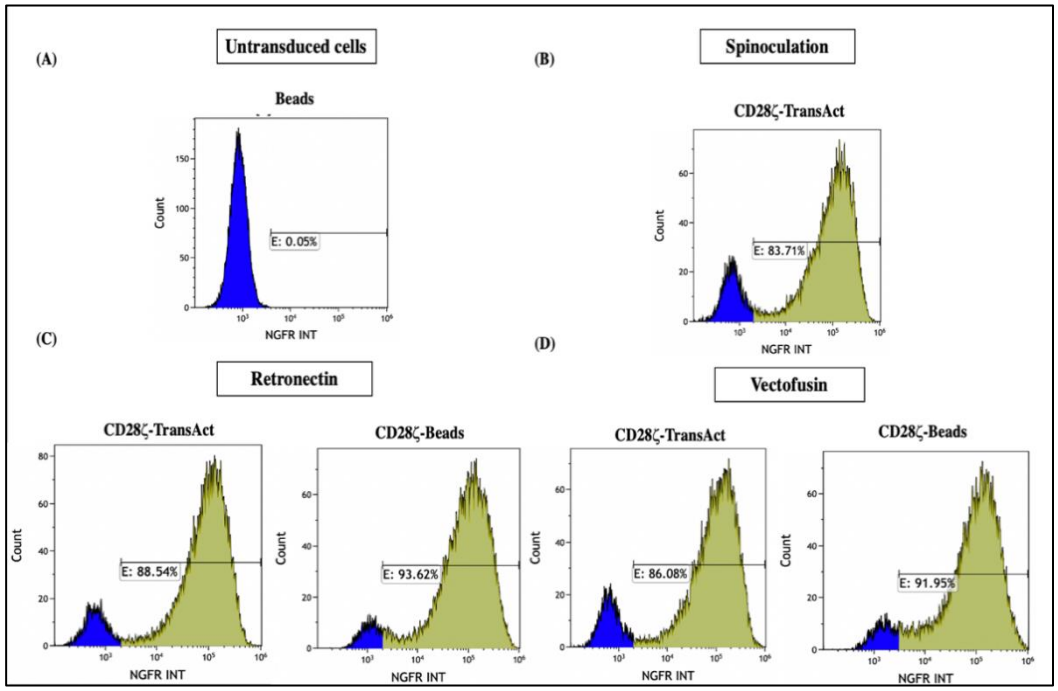

Figure 9: Flow cytometry analysis showing the efficiency of transduction by the staining of NGFR in T lymphocytes isolated from PBMC and transduced with LVs encoding for CD19-CD28ζ CAR. Showing untransduced cells (A), cells transduced with spinoculation (B), retronectin (C) or vectofusin (D). The results shown here are from N=1 PBMC- derived CD19-CAR-T cells. CD28ζ, CD19-CD28ζ-CAR-T cells.

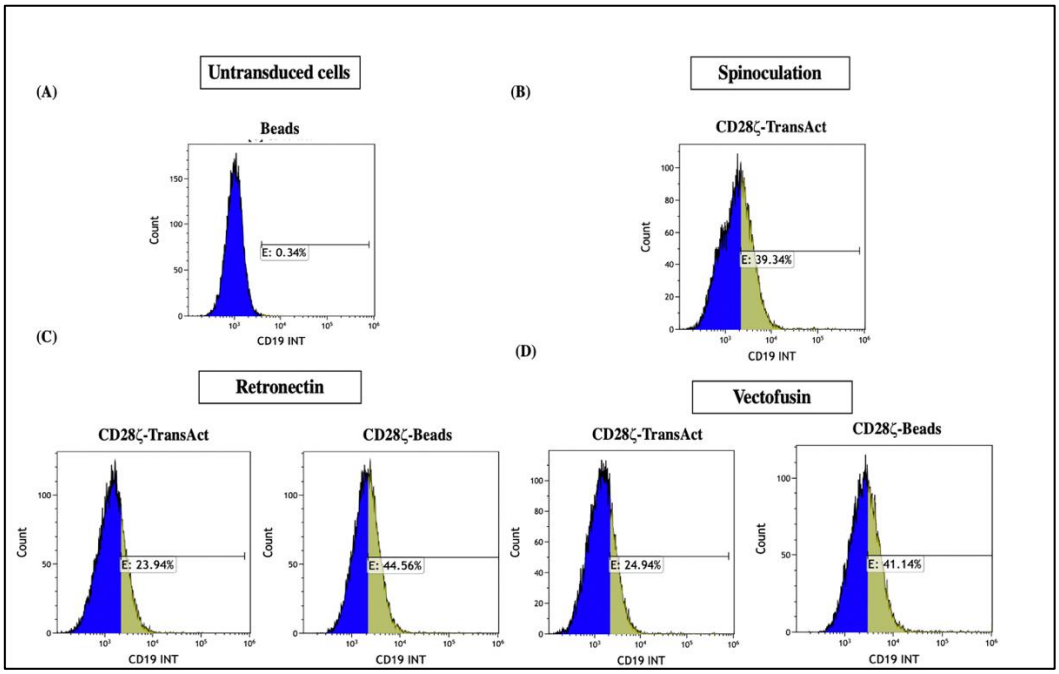

Figure 10: Flow cytometry analysis showing efficiency of transduction by CD19 staining in T lymphocytes isolated from PBMC and transduced with lentiviral vector encoding CD19-CD28ζ CAR. Untransduced cells (A), cells transduced with spinoculation (B), retronectin (C) or vectofusin (D). The results shown here are from N=1 PBMC-derived CD19-CAR-T cells. CD28ζ, CD19-CD28ζ-CAR-T cells.

Table 5: Summary of the comparison of the transduction of T cells with LVs using different methods in UCB and PBMC:

		UCB		PBMC	
Method	**Marker**	**CD28ζ-T**	**CD28ζ-B**	**CD28ζ-T**	**CD28ζ-B**
Spinoculation	**NGFR**	64.27%	NA	83.72%	NA
Retronectin	**NGFR**	49.92%	69.46%	88.54%	93.62%
Vectofusin	**NGFR**	67.55%	96.25%	86.08%	91.94%
Spinoculation	**CD19**	38.21%	NA	39.34%	NA
Retronectin	**CD19**	29.39%	50.52%	23.94%	44.56%
Vectofusin	**CD19**	42.23%	85.19%	24.94%	41.14%

The results shown here are from N=1 UCB-derived CD19-CAR-T cells and N=1 PBMC-derived CD19-CAR-T cells. UCB, umbilical cord blood; PBMC, peripheral blood mononuclear cells; T, TransAct; B, beads. NA, not available; CD28ζ, CD19-CD28ζ–CAR-T cells.

Taken together, these results suggested that performing the transduction of UCB-T cells with CD19-LVs in the presence of vectofusin can lead to superior efficiency of expression of CARs by T lymphocytes. Moreover, higher efficiency of transduction can be achieved in T lymphocytes upon activation with beads compared to TransAct.

4.3 Expansion of CD19-CAR-T cells from UCB and PBMC

To further optimize the process of the isolation *in vitro* of CAR-T cells, the expansion of T cells was assessed and monitored over 14 days of culture (Figures 11 and 12). The expansion of CAR-expressing T cells had a range of 28.5-105.7 and 92.2–165-fold increase in UCB and PBMC, respectively.

Notably, the most effective expansion of UCB-derived CAR-T cells was observed upon the usage of retronectin for their transduction with a fold-increase of 105.7 compared to 61.5 and 45.1 with vectofusin and spinoculation, respectively (Figure 11, panel B). Importantly, the efficiency of expansion *in vitro* of both UCB- and PBMC-derived CAR-T cells was superior when beads vs. TransAct were used for the activation of the cells for all the methods of transduction (Figure 11, panel B and Figure 12, panel B). Suggesting that the tools used for the activation of T cells can be more relevant for the expansion *in vitro* of CAR-T cells as compared to the procedure used for the transduction of the cells.

The comparison between UCB- and PBMC-derived CD19-CAR-T cells highlighted that the latest at day +14 achieved higher *in vitro* expansion, 105.7-165-fold-increase, VS. 25-110-fold-increase in UCB (Figures 11 and 12). $2x10^6$ cells were used at day 0 for all the types of T cell cultures described above.

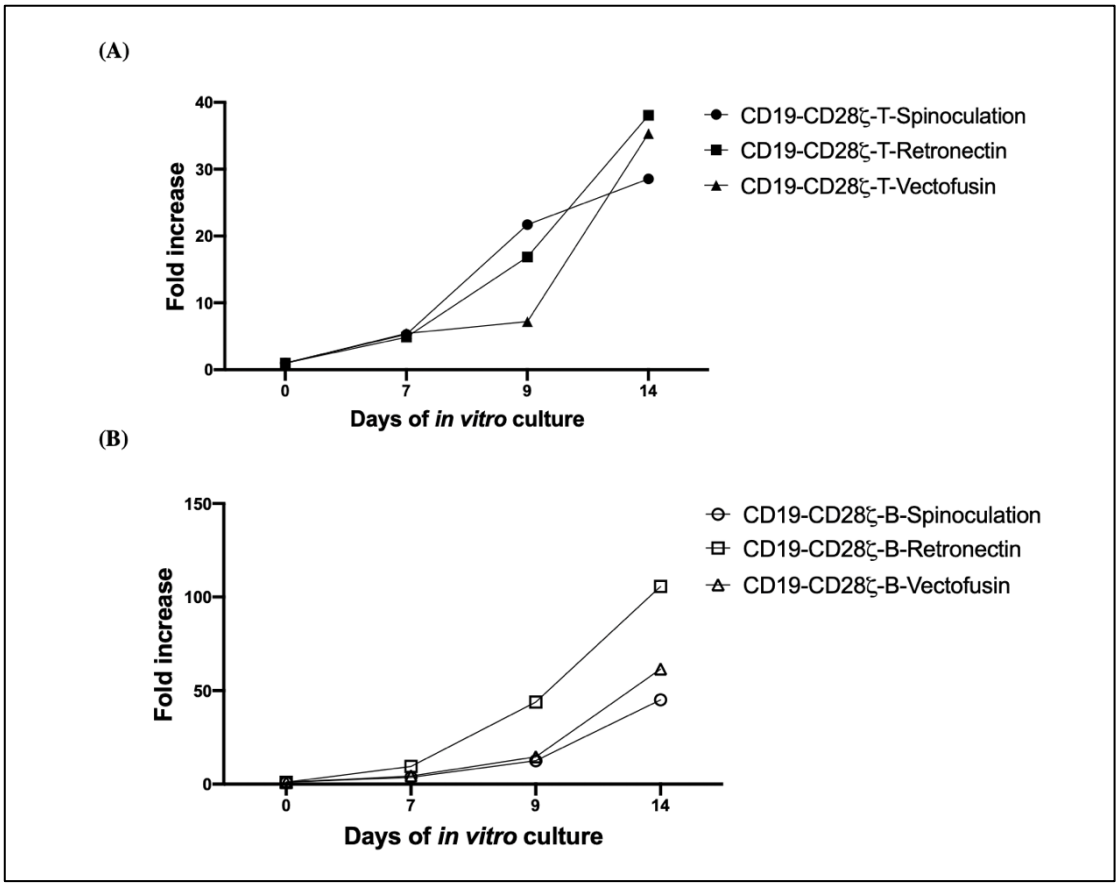

Figure 11: Fold expansion of CD19-CAR-T cells derived from UCB activated with either TransAct (panel A) or beads (Panel B) at different time points of the *in vitro* culture (day 0, +7, +9, and +14). The results shown here are from N=1 UCB-derived CD19-CAR-T cells. T, TransAct; B, beads.

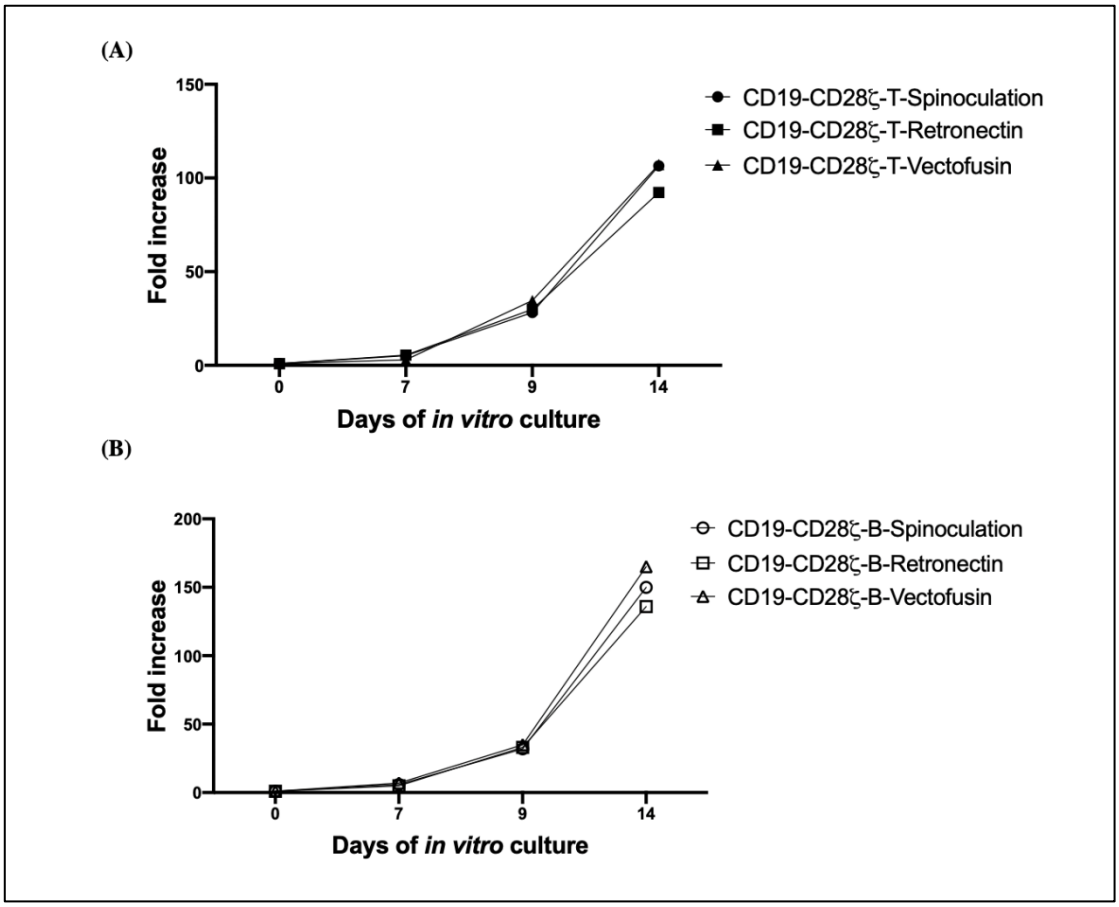

Figure 12: Fold expansion of CD19-CAR-T cells derived from PBMC activated with either TransAct (panel A) or beads (Panel B) at different time points of the *in vitro* culture (day 0, +7, +9, and +14). The results shown here are from N=1 PBMC-derived CD19-CAR-T cells. T, TransAct; B, beads.

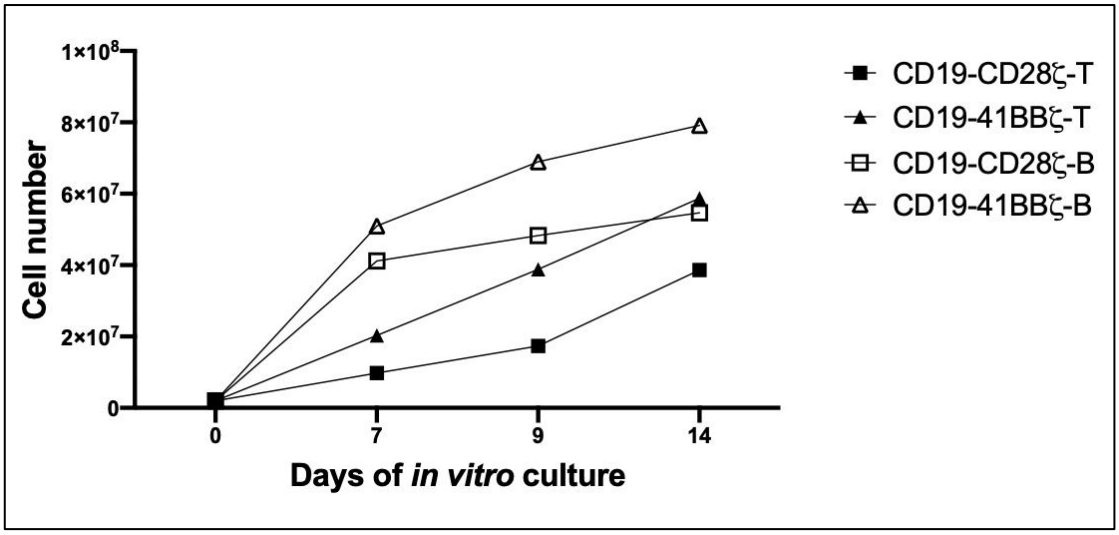

Figure 13: Expansion of UCB derived CD19-CAR-T cells activated with either TransAct or beads at different time points of the *in vitro* culture (day 0, +7, +9, and +14). Representative data showing one out of three UCB-derived CD19-CAR-T cells is shown. T, TransAct; B, beads.

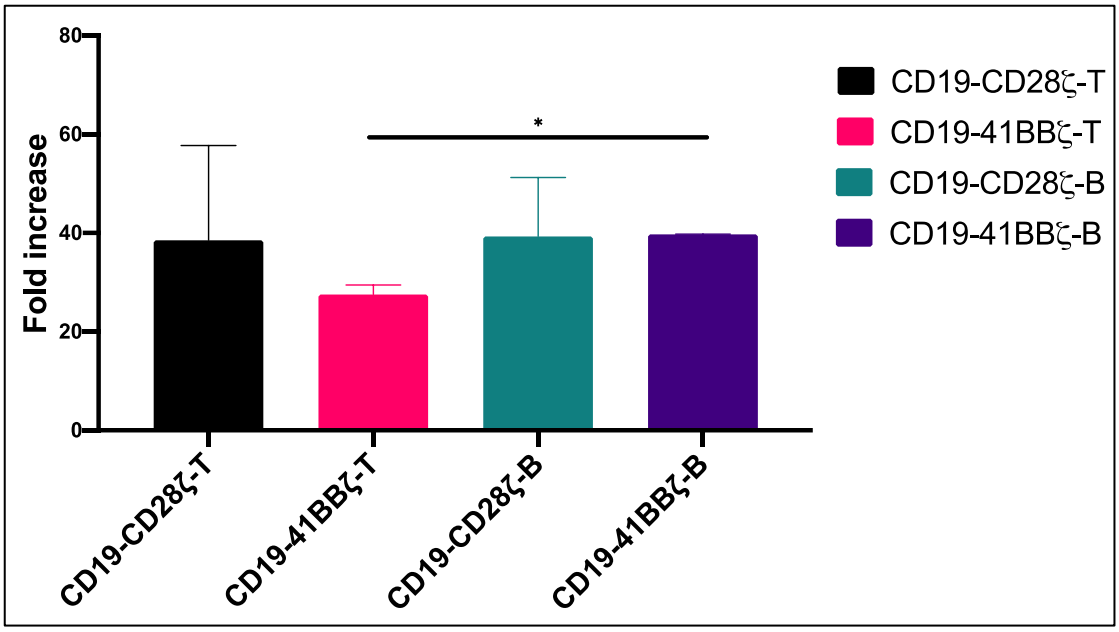

Figure 14: Fold expansion of UCB derived CD19-CAR-T cells at day +14 of *in vitro* culture. The data shown in the figure are means ± SEM of N=3 UCB-derived CD19-CAR-T cells. * indicates $p<0.05$. T, TransAct; B, beads.

We have also evaluated the influence of CD28ζ and 4-1BBζ co-stimulation in the LVs upon activation with TransAct or beads in N=3 UCB-CD19-CAR-T cells. We did not observe any variation in the influence of different signaling domains or method of activation on cell proliferation. However, we have noticed that cells activated with beads, particularly in CD19-41-BBζ–CAR-T cells, show an overall increased *in vitro* expansion compared to TransAct. A higher range of expansion was also observed in CD19-CD28ζ–CAR-T ranging from 19.35–57.7 and 27.39-51.20 in TransAct and Beads respectively (Figures 13 and 14). Overall, the obtained data suggest that activation with beads can lead to superior *in vitro* expansion of CAR^+-T cells for both UCB and PBMCs. In addition, although lower compared to PBMCs, the expansion of UCB-CAR-T cells that was obtained starting from $2x10^6$ of T cells and ending with $2x10^8$ of cells at day +14 of *in vitro* culture suggests that indeed the methodology of generation of CAR-T cells is allowing the expansion of large number of cells.

4.4 Phenotypic characterization of UCB and PBMC-derived CD19-CAR-T cells

A deep phenotypic characterization of the UCB- and PBMC-derived CD19-CAR-T cells was performed through flow cytometry both at day +9 and +14 of the *in vitro* culture. The staining of the cells was performed using ad hoc designed panels to assess the expression of 29 markers associated with T cell activation, differentiation, and immune checkpoints (Table 2). CD19-CAR-T cells generated from N=3 UCBs and N=3 PBMCs were utilized for the phenotypic analyses. T cells from each of the aforementioned samples were activated *in vitro* with either beads or TransAct and transduced with LVs encoding for CD19-CD28ζ or CD19-4-1BBζ. Untransduced T cells activated with either TransAct or beads were used as negative control. In total 36 different CAR-T cells populations have been utilized for the phenotypic analyses and comparisons of the phenotype have been performed according to the information reported in (Table 6). A representative illustration of the gating strategy and flow cytometry analysis of UCB- or PBMC- derived CD19-CAR-T cells is provided in (Figure 15).

Descriptive statistical analysis has been performed with the Mann-Whitney' test. The analysis included about five hundred combinations of markers. The obtained results highlighted high complexity and heterogeneity in the composition of T cell subpopulations within the CD19-CAR-T cells. Representative data of statistically significant differentially expressed T cell subpopulations are shown in (Figures 16-21).

Table 6: CD19-CAR-T cells analyzed for phenotype

Source	Activation	CARs	Time point
UCB	Beads	CD19-CD28ζ	Day +9
		CD19-4-1BBζ	
	TransAct	CD19-CD28ζ	Day +14
		CD19-4-1BBζ	
PBMC	Beads	CD19-CD28ζ	Day +9
		CD19-4-1BBζ	
	TransAct	CD19-CD28ζ	Day +14
		CD19-4-1BBζ	

UCB, umbilical cord blood; PBMC, peripheral blood mononuclear cells; CD19-4-1BBz, CD19-4-1BBz-CAR-T cells; CD19-CD28z, CD19-CD28z-CAR-T cells; Day+9 and Day+14: time point of cell culture *in vitro.*

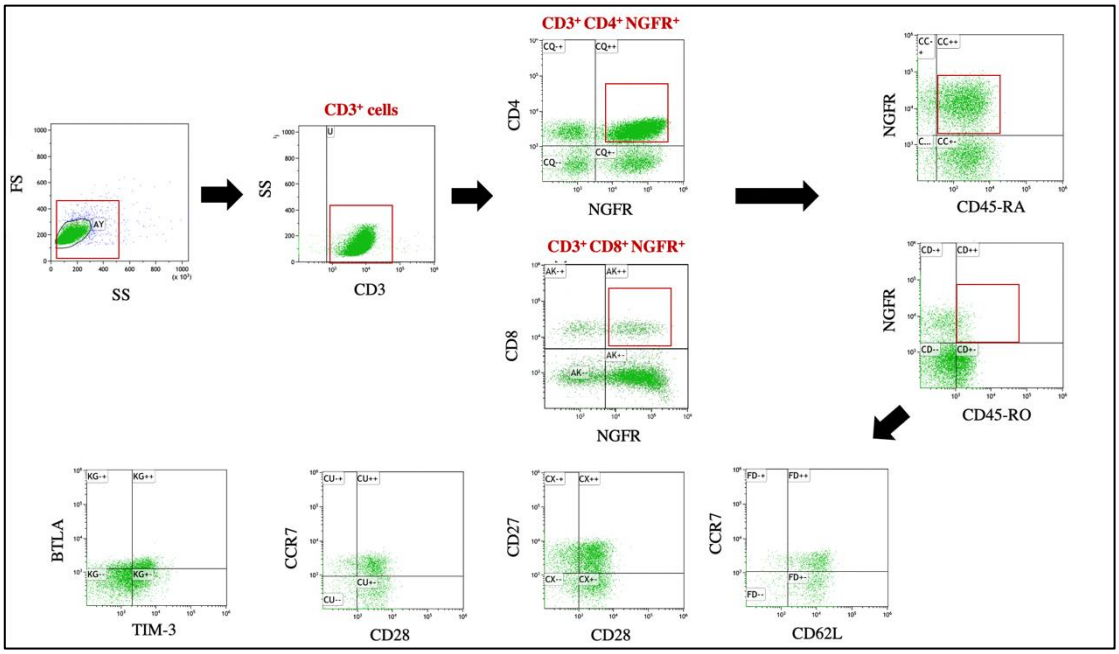

Figure 15. The strategy of gating used for the phenotype analysis of UCB- and PBMC-derived CD19-CAR-T cells. Flow cytometry analysis was performed on N=36 CD19-CAR-T cells isolated from N=3 UCBs and N=3 PBMCs. FS, forward scatter; SS, side scatter; NGFR, nerve growth factor receptor; BTLA, B and T lymphocyte attenuator; TIM3, T-cell immunoglobulin domain and mucin domain 3.

CD19-CAR-T cells population based on the physical parameters forward scatter (FS) and side scatter (SS) was then selected for the expression of CD3. The $CD4^+$ $NGFR^+$ or $CD8^+NGFR^+$ T lymphocytes were gated to identify the T cells expressing the CARs. The subsequent gating occurred for the cells expressing either CD45RA or CD45RO. The gating strategy resulted in the following populations of interest: $CD3^+$ $CD4^+NGFR^+CD45RA^+$, $CD3^+CD8^+NGFR^+CD45RA^+$, $CD3^+CD4^+NGFR^+CD45RO^+$, $CD3^+CD8^+NGFR^+CD45RO^+$ that have been used to identify the expression of the remaining markers included in the staining panels (Figure 15).

The composition of $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes isolated from UCB after the enrichment of T cells has been assessed prior to the *in vitro* processing for the generation of CD19-CAR-T cells. A $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes were found with the range of 75-80% and 6-15% of positive cells, respectively. Both populations of T cell subsets expressed either CD45RA or CD45RO with a range of 66-78.3% vs. 12-35% and 85-90% vs. 6-7% of positive cells for either $CD4^+$ or $CD8^+$ T lymphocytes, respectively (Table 7).

The same T cell subsets have been determined in either $CD4^+$ or $CD8^+$ CD19-CAR-T cells. UCB-derived cells showed that $CD4^+$ CD19-CAR-T cells represented 44-65% of positive cells while $CD8^+$ CD19-CAR-T cells were 20-40% of positive cells. 40-62.3% and 20-48% of positive cells were found for PBMC-derived $CD4^+$ CD19-CAR-T cells or $CD8^+$ CD19-CAR-T cells, respectively (Table 8).

Table 7: Composition of $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes in UCB at day 0

T cell subset	Percentage (%)
$CD3^+CD4$	75-80
$CD3^+CD4^+CD45RA^+$	66-78.3
$CD3^+CD4^+CD45RO^+$	12-35
$CD3^+CD8^+$	6-15
$CD3^+CD8^+CD45RA^+$	85-90
$CD3^+CD8^+CD35RO^+$	6-7

Data presented are from N=3 UCBs.

Table 8: Proportions of $CD4^+$ and $CD8^+$ UCB- and PBMC- derived CD19-CAR-T cells

	UCB	PBMC
$CD3^+CD4^+$ CD19-CAR-T cells	44-65%	40-62.3%
$CD3^+CD8^+$ CD19-CAR-T cells	20-40%	20-48%

Data presented are of N=3 UCB-derived CD19-CAR-T cells and N=3 PBMC-derived CD19-CAR-T cells. UCB, umbilical cord blood; PBMC, peripheral blood mononuclear cells.

The comparison of the CD19-CAR-T cells activated either with TransAct or Beads highlighted that a preferential enrichment of $CD3^+$ T cells at early stage of differentiation, as shown by the co-expression of T cell markers CD3 and NGFR in association with CD45RA, occurred in T cells activated with beads ($P<0.005$; Figure 16, Panels A and C). These cells could co-express either CD27 or BTLA (showing Figure 16, Panels B and C) confirming the presence of T cell subsets belonging to T stem memory/central memory subsets that can express also activatory markers (e.g., BTLA). However, even though not statistically significant, also T cell subsets co-expressing CD8, CD45RA or CD45RO and CD69 or CD27 ($P=0.058$ and 0.057, respectively) could also be detected among the T lymphocytes $NGFR^+$. In addition, $CD3^+CD8^+CD45RA^+$ lymphocytes were found to express either LAG3 ($P=0.057$) or

both CCR7 and LAG3 (p=0.055) which represent early-stage of differentiation of T cell subsets expressing molecules indicative of the activatory status (data not shown). These observations occurred similarly in both UCB and PBMCs (Figure 16). However, superior grade of heterogeneity in the detection of sub-populations of CD19-CAR-T cells occurred in PBMC. Other T cell subsets that were detected in PBMC-derived CD19-CAR-T cells such as $CD3^{+}CD8^{+}CD45RO^{+}$ cells expressing CD57, a marker of late differentiated effector memory cells ($P<0.05$), or $CD3^{+}CD8^{+}CD45RO^{+}$ cells expressing CTLA-4 ($P<0.05$) representing central memory lymphocyte subsets (data not shown).

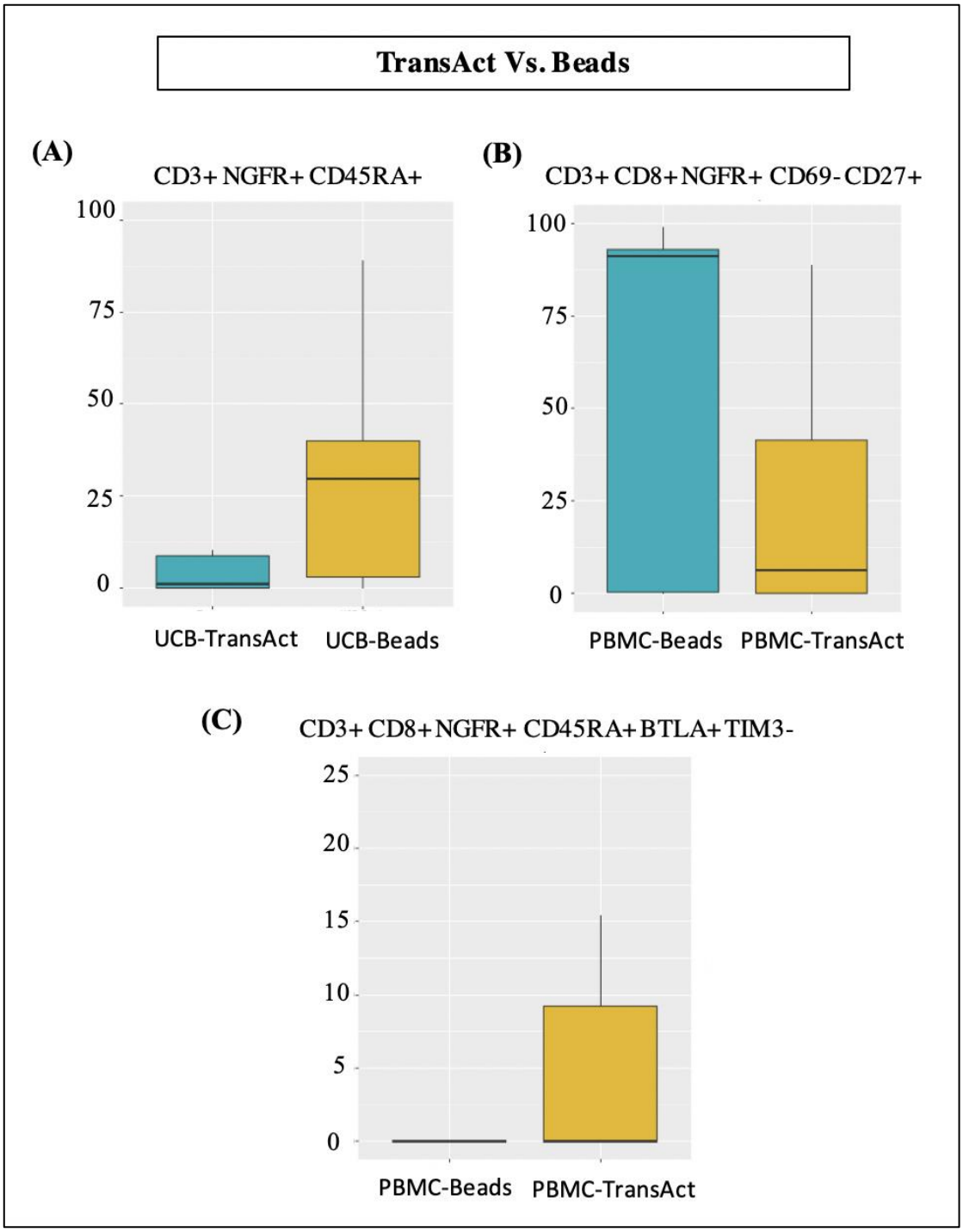

Figure 16: Phenotypic characterization of UCB- and PBMC-derived CD19-CAR-T cells comparing TransAct and Beads. The data that are shown in this figure are from N=3 UCB-derived and N=3 PBMC-derived CD19-CAR-T cells ($P<0.005$). UCB, umbilical cord blood; PBMC, peripheral mononuclear cells; NGFR, nerve growth factor receptor; BTLA, B and T lymphocyte attenuator; TIM3, T-cell immunoglobulin domain and mucin domain 3.

The comparison of CAR-T cells isolated from UCB and engineered with CARs bearing different co-stimulatory molecules, CD19-CD28ζ or CD19-4-1BBζ showed that an enrichment of $CD3^+CD4^+CD45RA^+$ T cells was observed in CD19-4-1BBζ-CAR-T cells. These cells co-expressed the immune checkpoint molecule LAG3 (Figure 17, Panel A). The $CD3^+CD4^+CD45RO^+CD62L^+$ T cell subsets were also over-expressed in CD19-4-1BBζ as compared to CD19-CD28ζ ($p<0.05$; Figure 17, Panel B), indicating the presence of central memory phenotype. In contrast, CD19-CD28ζ could be enriched either in $CD3^+CD8^+NGFR^+CD45RO^+$ effector T cells co-expressing CCR7 and CD56 (Figure 17, Panel C) or $CD3^+CD8^+$NGFR co-expressing CD45RA and the immune checkpoint markers CTLA-4 (Figure 17, Panel D), suggesting that in CD19-CAR-CD28ζ the enrichment of CD19-CAR-T cells at early stages of differentiation occurred . Moreover, the expression of CD45RA in association with the immune checkpoint marker CTLA-4 indicates the status of activation the cells rather than the exhaustion of T lymphocytes.

On the other hand, PBMC-derived CAR-T cells $CD3^+CD8^+CD45RA^+LAG3^+$ were enriched in CD19-CD28ζ vs 4-1BBζ-CAR-T cells indicating the presence of central memory phenotype (Figure 18, Panel A and B).

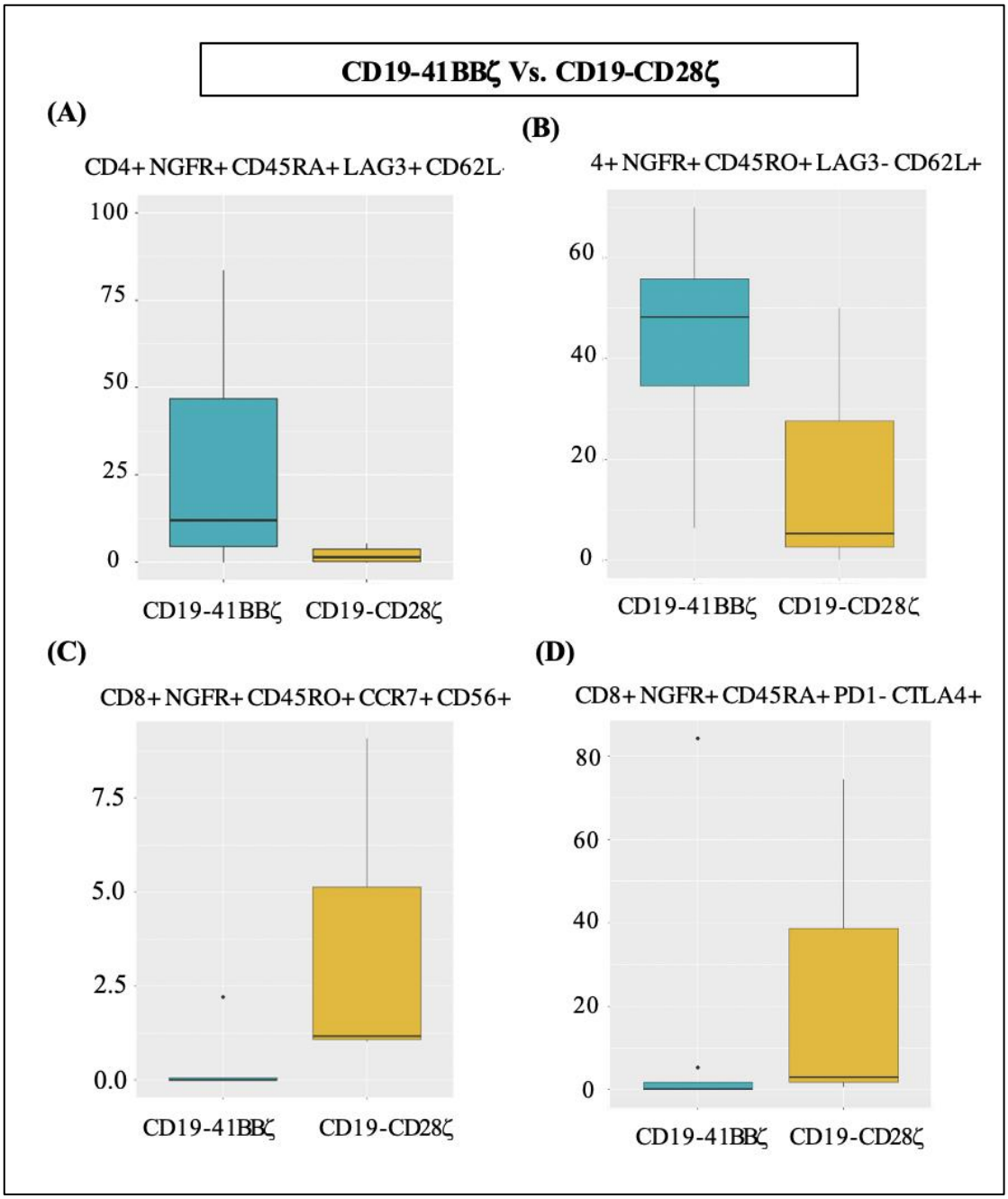

Figure 17: Phenotypic characterization of UCB-derived CD19-CAR-T cells comparing the expression of CD19-4-1BBζ and CD19-CD28ζ-CARs. Data presented shows the statistical analysis of the means of N=3 UCB-derived CD19-CAR-T cells ($P<0.005$). NGFR, nerve growth factor receptor; LAG3, lymphocyte activation gene 3 protein; CCR7, C-C chemokine receptor type 7; PD-1, programmed cell death protein 1; CTLA-4, cytotoxic T lymphocyte associated protein 4.

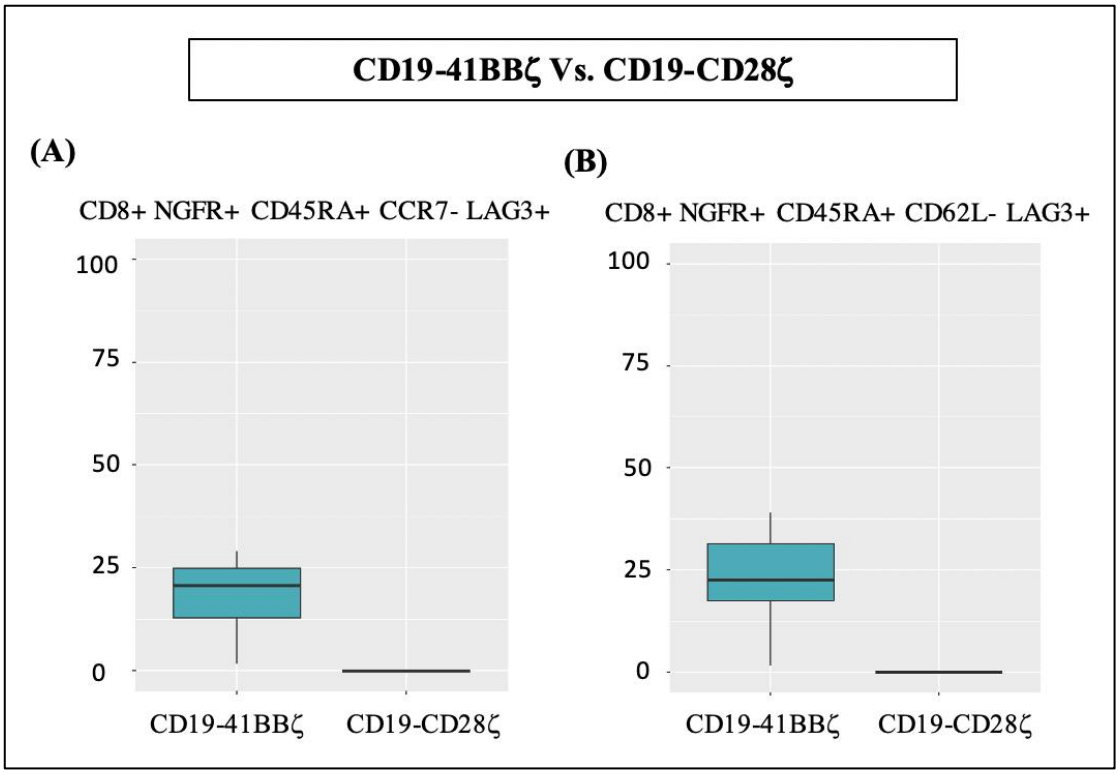

Figure 18: Phenotypic characterization of PBMC-derived CD19-CAR-T cells comparing the different CARs CD19-4-1BBζ and CD19-CD28ζ. Data presented shows statistical analysis of the means of N=3 PBMC-derived CD19-CAR-T cells (P<0.005). NGFR, nerve growth factor receptor; LAG3, lymphocyte activation gene 3 protein; CCR7, C-C chemokine receptor type 7.

When comparing the CD19-4-1BBζ-CAR in UCB and PBMC, the selection of early differentiated T cells subsets ($CD45RA^+$) in UCB, compared to PBMC-derived CD19-CAR-T cells was observed (P<0.005; Figure19). For instance, CD19-4-1BBζ induced a higher frequency of effector $CD3^+CD8^+CD45RA^+$ cells expressing either BTLA or CTLA-4 but not TIM3 or PD-1 (Figure 19, Panels A and B). However, in PBMC-derived CAR-T cells expressing the CD19-4-1BBζ-CAR belonged to central memory T lymphocytes co-expressing CCR7 and CD57 (Figure 19, Panel C).

The CD19-CD28ζ-CAR-T cells isolated from UCBs vs. PBMCs revealed a heterogeneous composition of T cell subsets. Mostly, of $CD3^+CD4^+CD45RA^+$ either co-expressing the activatory /immune checkpoint molecules BTLA and CTLA-4

($p<0.05$; Figure 20 Panel A). The levels of $CD3^+CD8^+NGFR^+CD45RA^+CCR7^+CD56^+$ were also significantly different in UCB- vs. PBMC-CD19-CAR-T cells ($p<0.05$; Figure 20, Panel B). $CD3^+CD8^+NGFR^+CD45RO^+BTLA^+TIM\text{-}3^+$ T lymphocytes resembling the late stage of differentiation of T cell subset ($p<0.05$; Figure 20, Panel D). Of note, early stage of differentiation of CAR-T cells expressing TIM-3 were enriched in PBMC- vs. UCB-derived CAR-T cells ($p<0.05$; Figure 20, Panel C). TIM-3 expressing T cell subpopulation was not found as statistically significant over-expressed in UCB-CAR-T cells (data not shown).

Even though late differentiated cells expressing immune checkpoint markers were not found as statistically significant in UCB-CAR-T cells as compared to PBMC, lower frequency of $CD8^+CD45RO^+$ cells co-expressing BTLA and TIM-3 in UCB was detected (Figure 20, Panel C). However, this population of late differentiated cells constitute a very low percentage of the total populations in UCB.

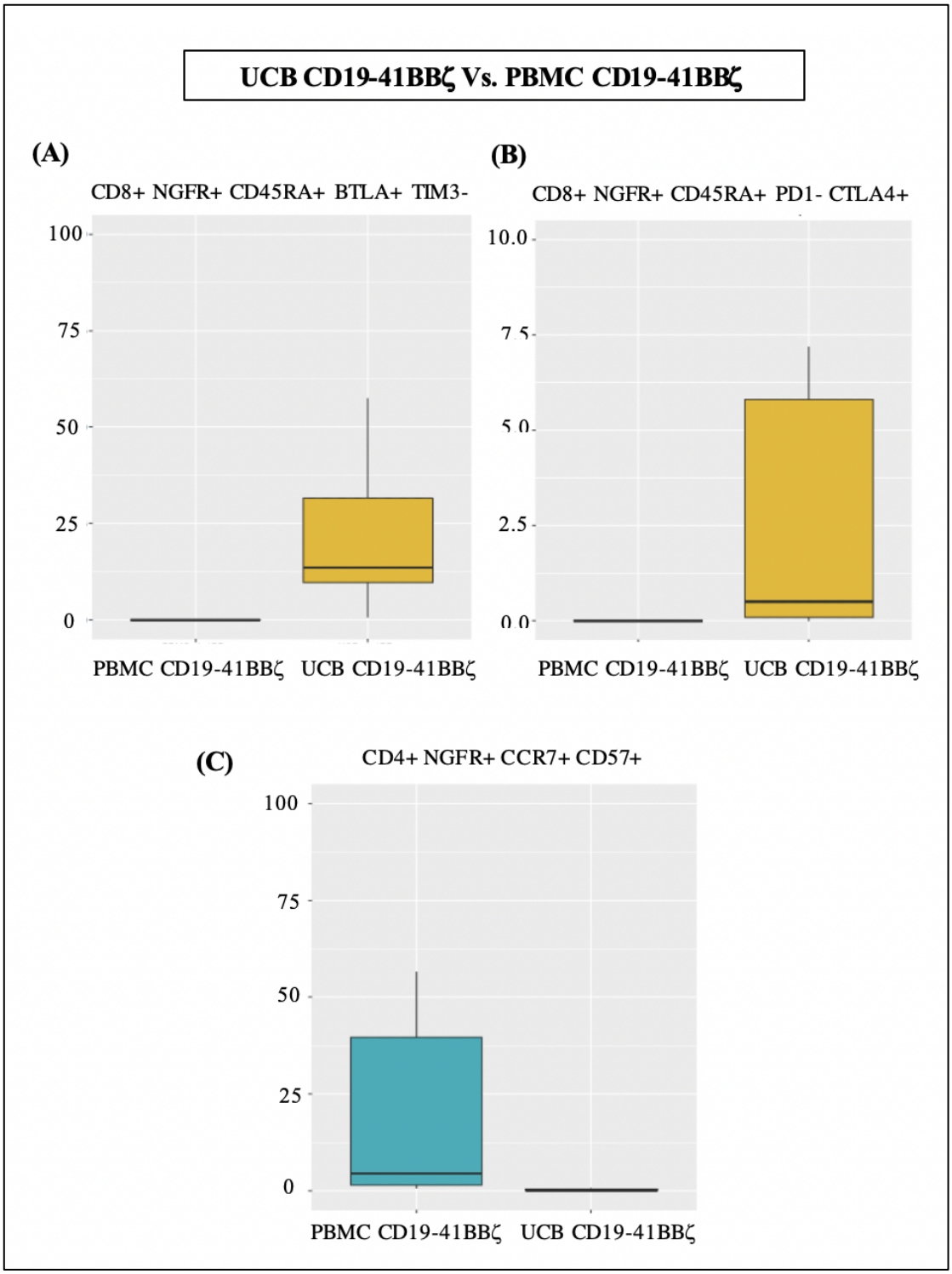

Figure 19: Phenotypic characterization of UCB- and PBMC-derived CD19-4-1BBζ-CAR-T cells. The statistical analysis of means of N=3 UCB-derived CD19-CAR-T cells and N=3 PBMC-derived CD19-CAR-T cells ($P<0.005$) is shown. UCB, umbilical cord blood; PBMC, peripheral blood mononuclear cells; CD19-4-1BBζ, CD19-4-1BBζ-CAR-T cells. NGFR, nerve growth factor receptor; BTLA, B and T lymphocyte attenuator; TIM3, T-cell immunoglobulin domain and mucin domain 3; PD-1, programmed cell death protein 1; CTLA4, cytotoxic T lymphocyte associated protein 4; CCR7, C-C chemokine receptor type 7.

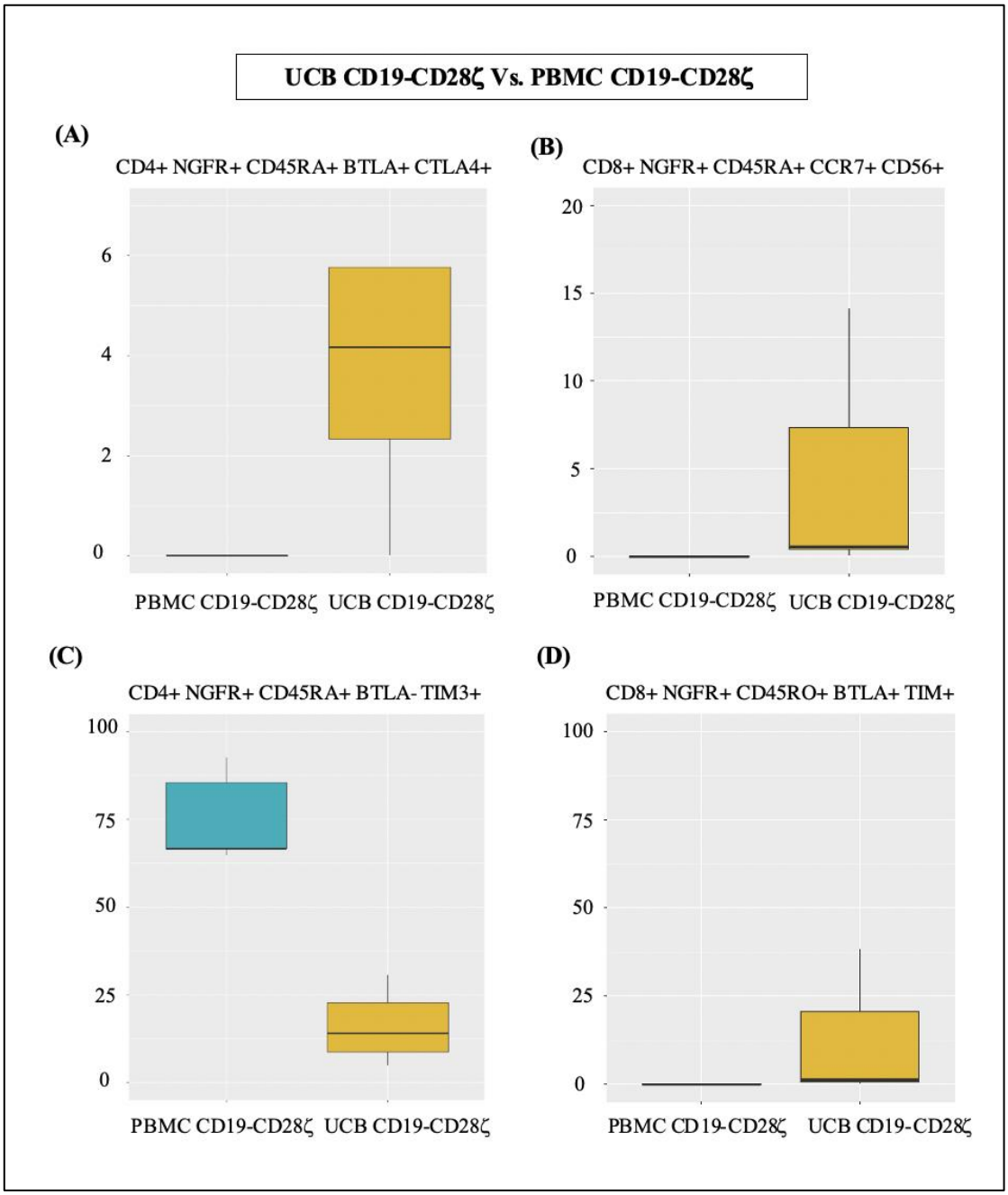

Figure 20: Phenotypic characterization of UCB- and PBMC-derived CD19-CD28ζ-CAR-T cells. The statistical analysis of the means of N=3 UCB-derived CD19-CAR-T cells and N=3 PBMC-derived CD19-CAR-T cells ($P<0.005$) is shown. UCB, umbilical cord blood; PBMC, peripheral blood mononuclear cells; CD19-CD28ζ, CD19-CD28ζ-CAR-T cells. NGFR, nerve growth factor receptor; BTLA, B and T lymphocyte attenuator; CTLA4, cytotoxic T lymphocyte associated protein 4; CCR7, C-C chemokine receptor type 7; TIM3, T-cell immunoglobulin domain and mucin domain 3.

The phenotype of CD19-CAR-T cells at different time points (Day +9 and day +14) of *in vitro* culture were compared. Representative results of UCB-CD19-CAR-T and PBMC-CD19-CAR-T cells are shown in (Figure 21). Overall, an enrichment of both $CD3^{+}CD8^{+}$NGFR+$CD45RA^{+}$ (Figure 21, Panel A) and $CD3^{+}CD4^{+}$NGFR+$CD45RA^{+}$ (data not shown) T cells were detected at day +9 and they were further selected at day +14 of culture in UCB-CD19-CAR-T cells. An enrichment of $CD3^{+}CD8^{+}NFGR^{+}CD45RO^{+}$ was also detected at lower frequencies at day +14 of *in vitro* culture (Figure 21, panel B). Nevertheless, $CD3^{+}CD4^{+}NGFR^{+}CD45RO^{+}CCR7^{+}$ T lymphocytes, belonging to T effector memory subsets at day +14 of the *in vitro* culture was detected in PBMC (Figure 21, Panel C).

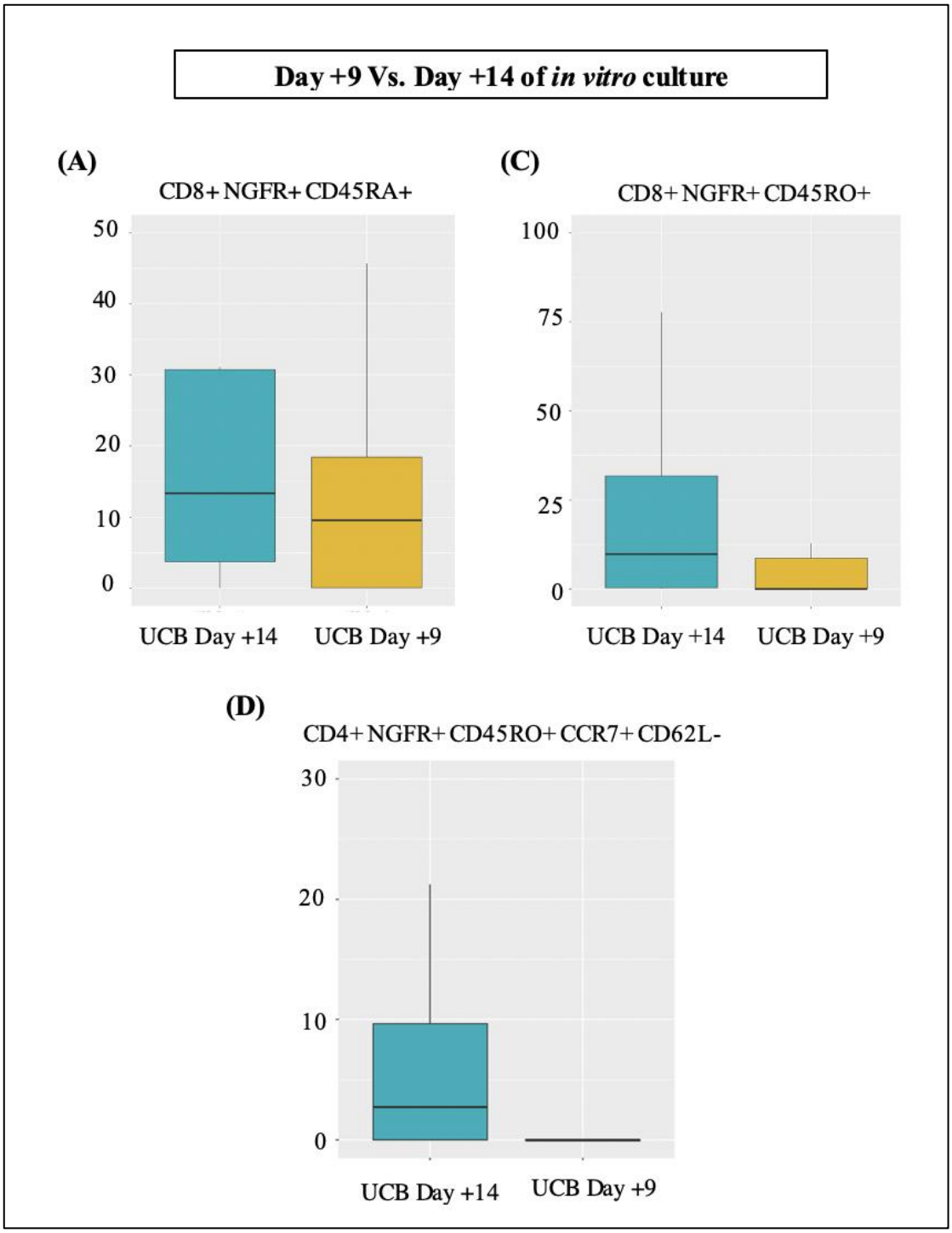

Figure 21: Phenotypic characterization of UCB- and PBMC-derived CD19-CAR-T cells at day+9 and day+14 of *in vitro* culture. In the figure representative data are shown. The statistical analysis has been calculated with the means of N=3 UCB-derived CD19-CAR-T cells and N=3 PBMC-derived CD19-CAR-T cells ($P<0.005$). UCB, umbilical cord blood; PBMC, peripheral mononuclear cells. Day+9 and Day+14: time point of cell culture *in vitro*. NGFR, nerve growth factor receptor; CCR7, C-C chemokine receptor type 7.

Overall, our results highlighted high level of heterogeneity in terms of different sub-populations of CD19-CAR-T cells. Of note, UCB-derived CD19-CAR-T cells showed statistically significant superior frequency of T stem cell memory or central memory T cells in T lymphocytes engineered with CD19-CARs isolated from UCB vs. PBMCs. This trend was maintained along the course of the *in vitro* culture and expansion of CD19-CAR-T cells. The differentially detected T cell subsets might display different functions in terms of *in vivo* persistence and anti-tumor activity.

4.5 Flow cytometry analyses of the expression of CD19 in the target cell lines

Before performing the functional analysis of CD19-CAR-T cells against target cell lines, we performed a Flow cytometry analysis to confirm the expression of CD19 marker in the $CD19^+$ cell leukemic cell line Raji, EBVB 1061, T2 and $CD19^-$ cell line CEM. High expression of CD19 was detected in $CD19^+$ cell lines Raji, EBVB 1061 and T2, although with different levels of the intensity of fluorescence (Figure 22, Panels A, B and C). No expression of CD19 was detected in CEM cell line (Figure 22, Panel D).

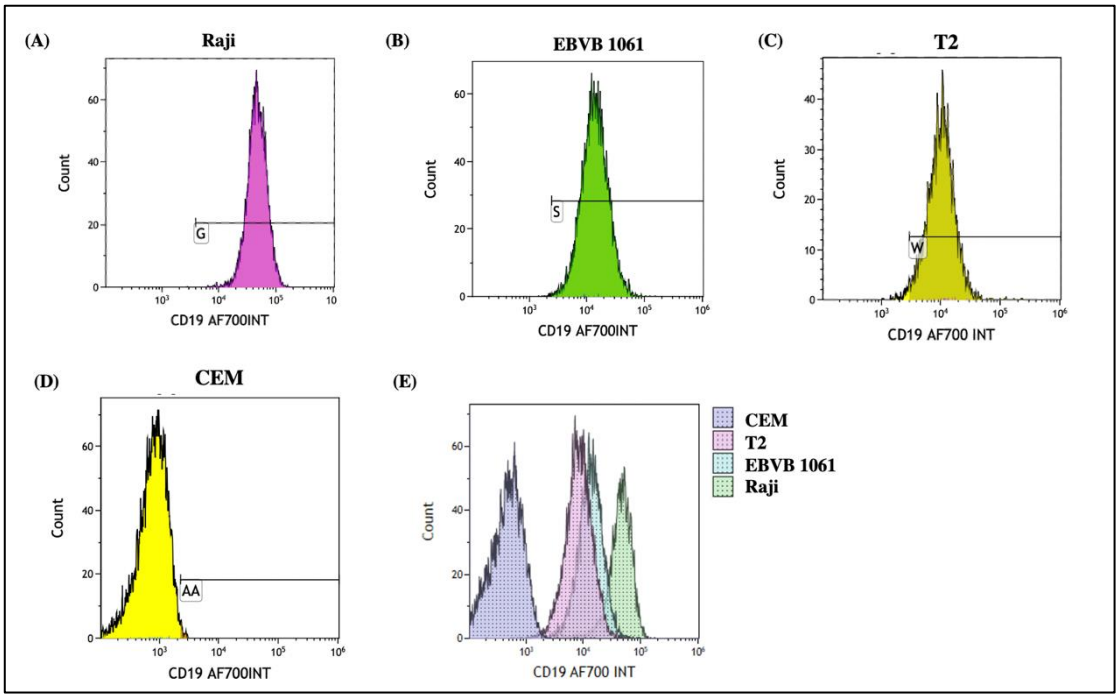

Figure 22: Flow cytometry analysis for the expression of CD19 in the cell lines Raji, EBVB 1061, T2 and CEM. (A, B and C) show high expression of CD19 in Raji, EBVB 1061 and T2 target cells. (D) shows lack of CD19 marker expression on CEM target cells. (E) Overlay of histograms showing CD19 expression in CEM, T2, EBVB 1061 and Raji target cells.

4.6 Anti-tumor reactivity of CD19-CAR-T cells

The specific reactivity of CD19-CAR-T cells was assessed by the detection of IFN-γ release with EliSpot assay upon an overnight co-culture with $CD19^+$ (the leukemic cell line Raji, 1869-, 1076- and 1061-EBV-B and T2) and $CD19^-$ (CEM and K562) cell lines (Figure 23).

IFN-γ release was observed following the incubation of CD19-CAR-T isolated from UCB with all $CD19^+$ target cells (# of spot 157 - 470). CD19-CD28ζ-CAR-T cells activated with both TransAct or beads displayed higher release of the cytokine (# of spots 181 - 470) as compared to CD19-41BBζ-CAR-T cells (# of spots 157 - 315) (Figure 23, Panel A and B).

Higher release of IFN-γ was observed in CD19-CAR-T cells activated with TransAct (Figure 23, Panel A, # of spots 470) as compared to beads (Figure 23, Panel

B, # of spots 253). Overall, the IFN-γ release occurring upon the co-culture with all the $CD19^+$ cell lines was statistically significant as compare to the release of the cytokine in the presence of either medium alone or CD19- target cells (only representative statistical analyses is represented in the Figures). Moreover, we did not observe any statistically significant difference in the release of IFN-γ between different CD19 expressing cell lines or signaling domains.

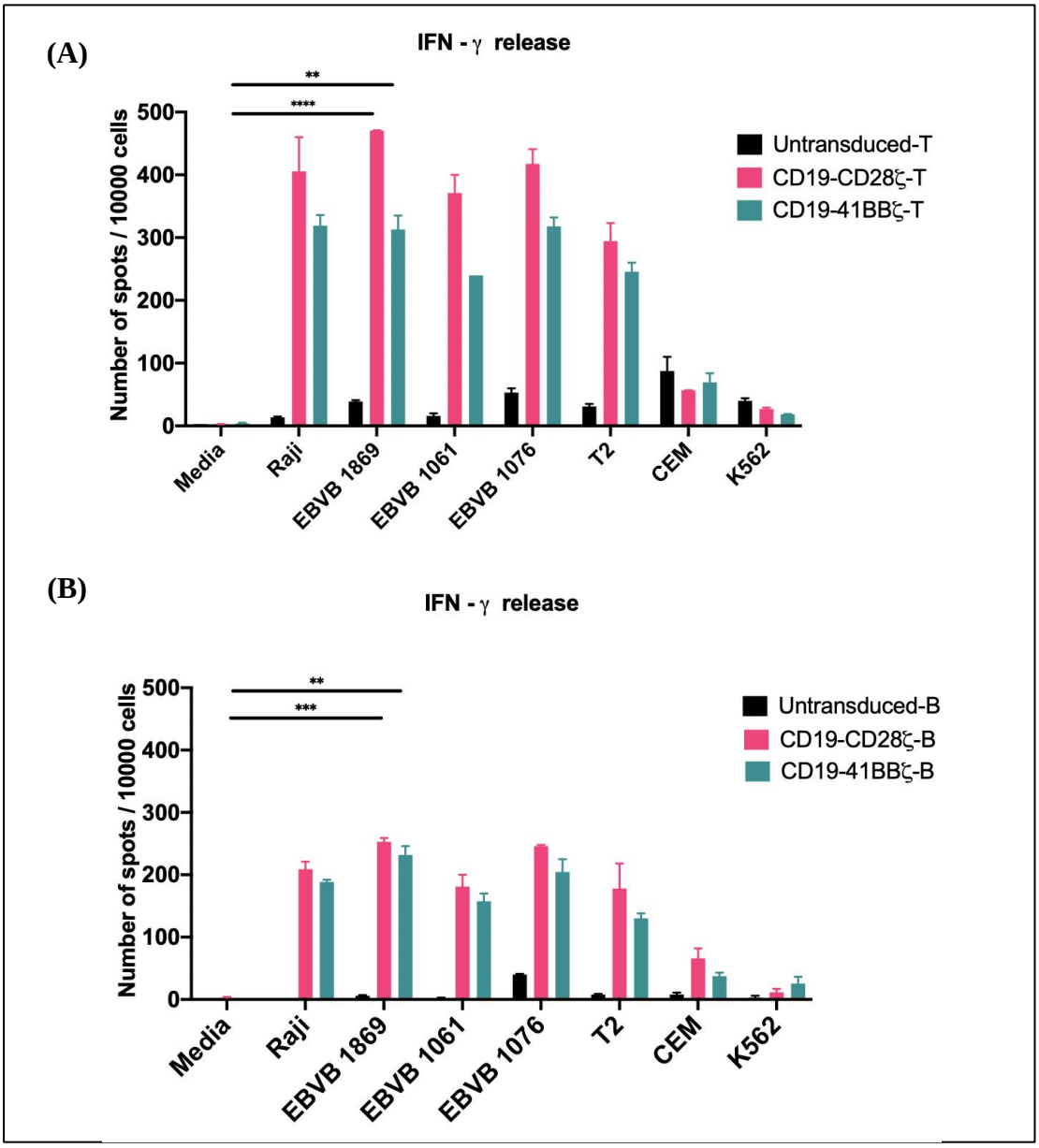

Figure 23: IFN-γ release assay of UCB CD19-CAR-T cells co-cultured with $CD19^+$ (Raji, EBVB 1869, EBVB 1061, EBVB 1076, T2) or CD19- (CEM, K562) target cell lines. Showing cells activated with TransAct (A) or Beads (B). Representative data showing two experiments performed with samples isolated from one UCB. Similar results were obtained for CD19-CAR-T cells isolated from other UCBs (data not shown). Data are represented as mean ± SEM of N= 2 replicates. ** indicates $p<0.01$ and *** indicates $p<0.001$. T, TransAct; B, beads.

The simultaneous detection of the release of IFN-γ and IL-4 was also assessed through dual color EliSpot assay to assess whether effector cell activity and TH2 type immune responses could be present in UCB-CD19-CAR-T cell. The ani-tumor reactivity was determined following the co-culture of either UCB- or PBMC-derived CD19-CAR-T cells with Raji, 1061 EBV-B, K562 or CEM cell lines.

Specific anti-tumor reactivity was detected for both UCB- and PBMC-derived CAR-T cells with (# of spots 77 - 299 vs. 9 - 117) in UCB and PBMC respectively as assessed by IFN-γ release in response to co-culture with Raji and EBVB 1061 $CD19^+$ cell lines (Figure 24, 25, 26 and 27, Panel A and C). Notably, Higher release of IFN-γ has been detected upon co-culture with the leukemic cell line Raji compared to EBVB 1061 in both UCB (# of spots 298 vs. 77 respectively) and PBMC (# of spots 117 vs. 9 respectively). Moreover, our CD19-CAR-T cells demonstrated diminished activity towards K562 and CEM $CD19^-$ target cells.

No statistically significant difference was detected between UCB-derived CD19-CD28ζ-CAR -T cells or CD19-41-BBζ-CAR-T cells in term of IFN-γ release upon activation with TransAct (Figure 24, Panel A; # of spots 88 vs. 77, respectively) or beads (Figure 24, Panel; # of spots 298 vs. 284, respectively). However, T cells activated with beads showed a higher anti-tumor reactivity compared to TransAct (Figure 25, panel A and C; # of spots 298 vs. 88). Similar results have been obtained for PBMC-derived CD19-CAR-T cells (Figure 25, Panel A&C, # of spots 117 vs. 21 in beads and TransAct).

The production of IL-4 was also detected in some cases according to antigen-specific reactivity in both UCB- and PBMC-derived CAR-T cells (Figures 25 and 27; # of spots 129 vs. 82). The reactivity of UCB-CD19-CAR-T cells activated with TransAct was superior compared to the same type of T cells activated with beads (Figure 25, Panel B

and D, # of spots 129 vs. 69). Similar results were obtained for CAR-T cells isolated from PBMC (Figure 27, Panel B and D; # of spots 82 vs. 39). Of note, this type of EliSpot assay allowed the simultaneous detection upon antigen stimulation of these two cytokines by the different type of CD19-CAR-T cells, allowing to discriminate the presence of TH1 and TH2 type of T cells since the two types of reactivity couldn't be detected in the same co-culture (Data not shown). The cytokine release occurred in antigen-specific manner, since no statistically significant cytokine release was detectable upon the co-culture of CD19-CAR-T cells with the $CD19^-$ K562 cells or in the presence of medium alone (Figures 25 and 27).

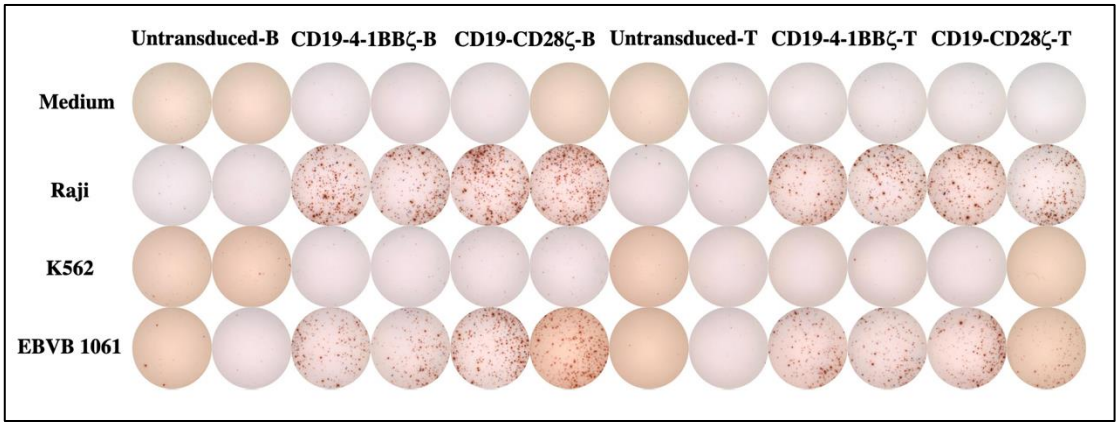

Figure 24: Elispot assays to detect both IFN-γ and IL-4 release by UCB-derived CD19-CAR-T cells upon the co-culture with $CD19^+$ (Raji, EBVB 1061) or $CD19^-$ (K562) target cell lines. Representative data showing one out of three UCB-derived CD19-CAR-T cells is shown.

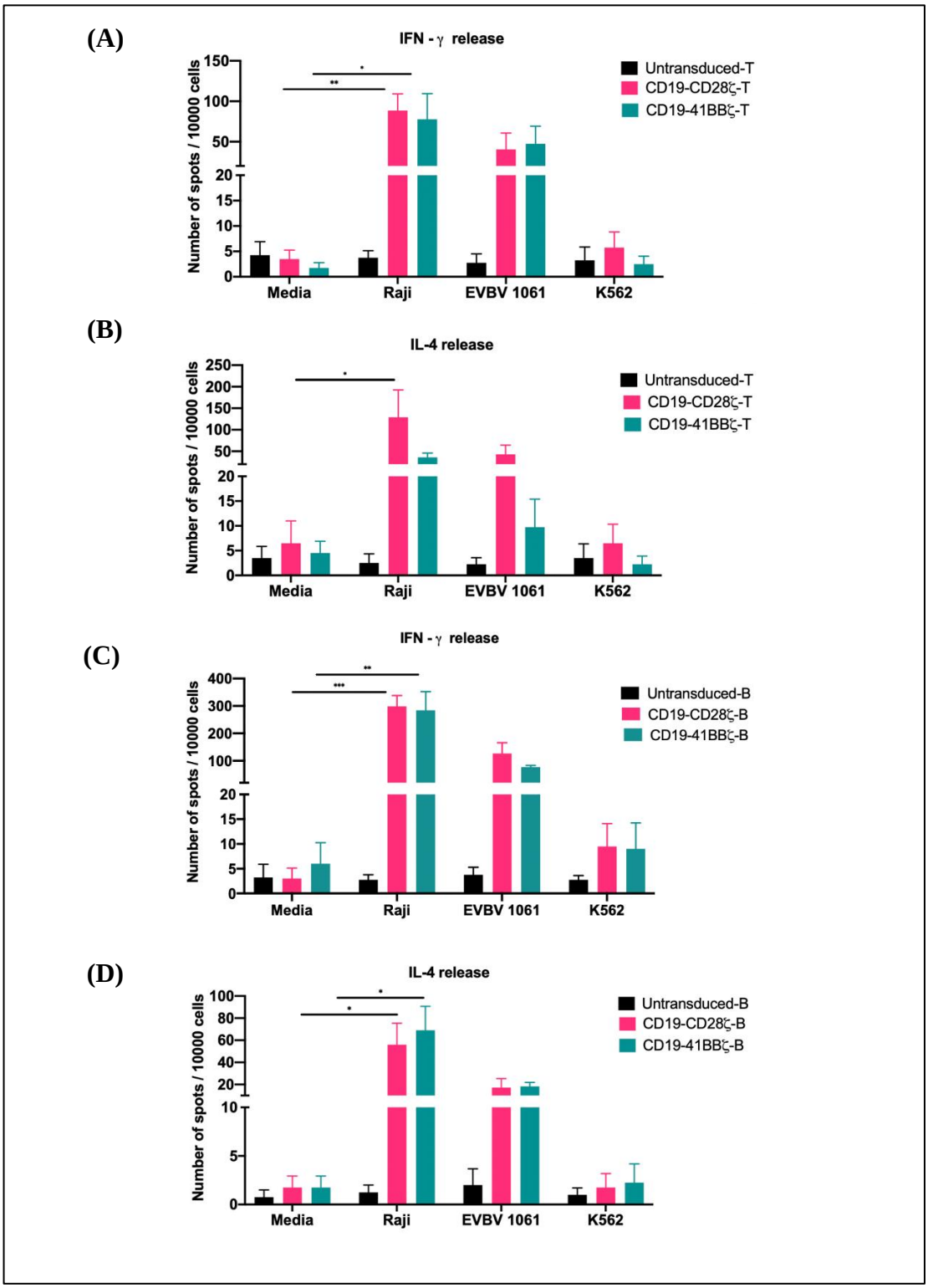

Figure 25: Elispot assays to detect both IFN-γ and IL-4 release by UCB-derived CD19-CAR-T cells upon the co-culture with $CD19^+$ (Raji, EBVB 1061) or $CD19^-$ (K562) target cell lines. T cells were activated with either TransAct (A and B) or Beads (C and D). Data are shown as mean ± SEM of N=3 UCB-derived CD19-CAR-T cells with N=2 replicates. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$. T, TransAct; B, beads.

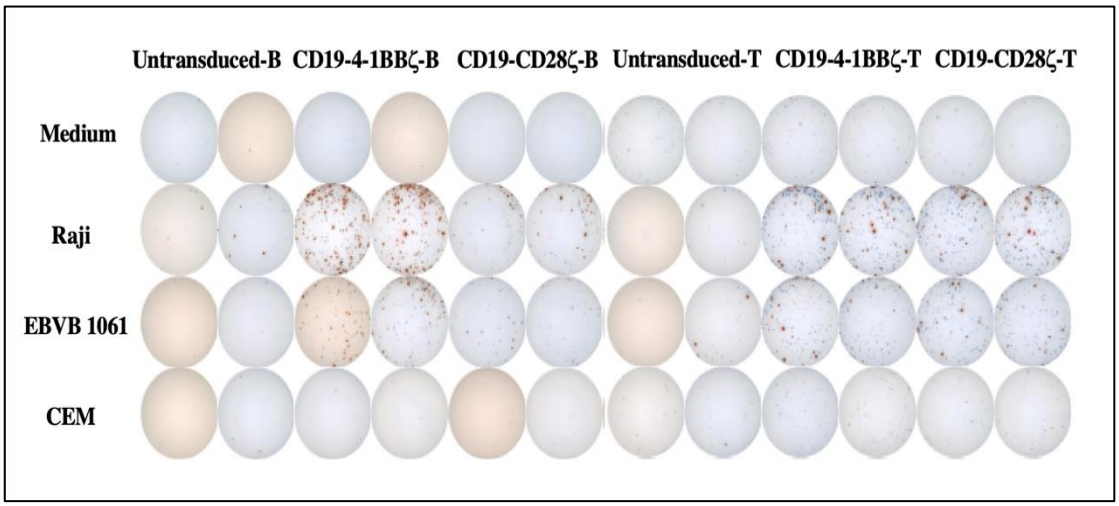

Figure 26: Elispot assays to detect both IFN-γ and IL-4 release by PBMC-derived CD19-CAR-T cells upon the co-culture with $CD19^+$ (Raji, EBVB 1061) or $CD19^-$ (CEM) cell lines. Representative data showing one out of three PBMC-derived CD19-CAR-T cells is shown.

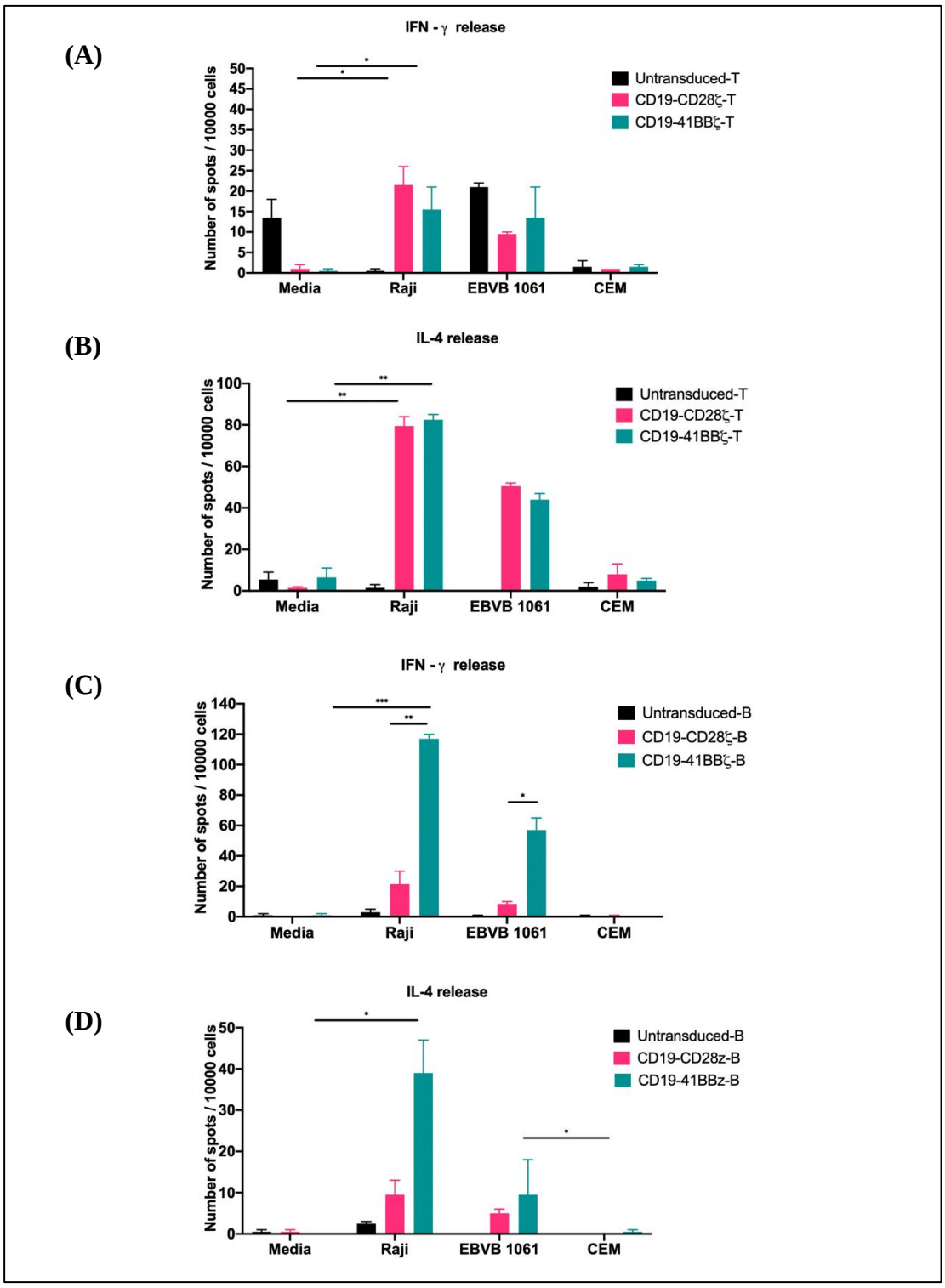

Figure 27: Elispot assays to detect both IFN-γ and IL-4 release by PBMC-derived CD19-CAR-T cells upon the co-culture with $CD19^+$ (Raji, EBVB 1061) or $CD19^-$ (CEM) cell lines. T cells were activated with TransAct (A&B) or Beads (C&D). Representative data showing one out of three PBMC-derived CD19-CAR-T cells is shown. Data are represented as mean ± SEM of N=2 replicates. Similar results were obtained for CD19-CAR-T cells isolated from other PBMCs (data not shown). * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$. T, TransAct; B, beads.

4.7 Detection of antigen-specific cytotoxic activity of CD19-CAR-T cells

To determine the cytotoxic ability of CD19-CAR-T cells, the release of granzyme-B and perforin was measured upon co-culture with the aforementioned target cells (Figure 28 and 29). Granzyme B release was detected upon co-culture with Raji and EBVB 1061 cell lines of all the CD19-CAR-T cells (Figure 29, Panel A and C; N. of spots 33.5 vs. 59.5 for TransAct and beads).

Furthermore, the difference in term of anti-tumor activity between the methods of transduction or different co-stimulatory molecules was detected. The release of perforin was lower in cells activated with beads compared to TransAct (Figure 29, Panel B and D; N. of spots 10 vs. 182.5, respectively). Moreover, the production of granzyme B was higher in CD19-4-1BBζ-B CAR-T cells compared to CD19-4-1BBζ-T CAR-T cells (Figure 29, Panel A and C; N. of spots 59.5 vs. 24,.

Overall, our data confirm that the CD19-CAR-T cells exerted specific anti-tumor activity as observed by the release of IFN-γ, Granzyme B and Perforin, indicating the prevalence of effector cells in these CAR-T cells. Moreover, the extent of the cytotoxic activity correlated with the expression of CD19 antigen on target cells as shown in (Figure 22).

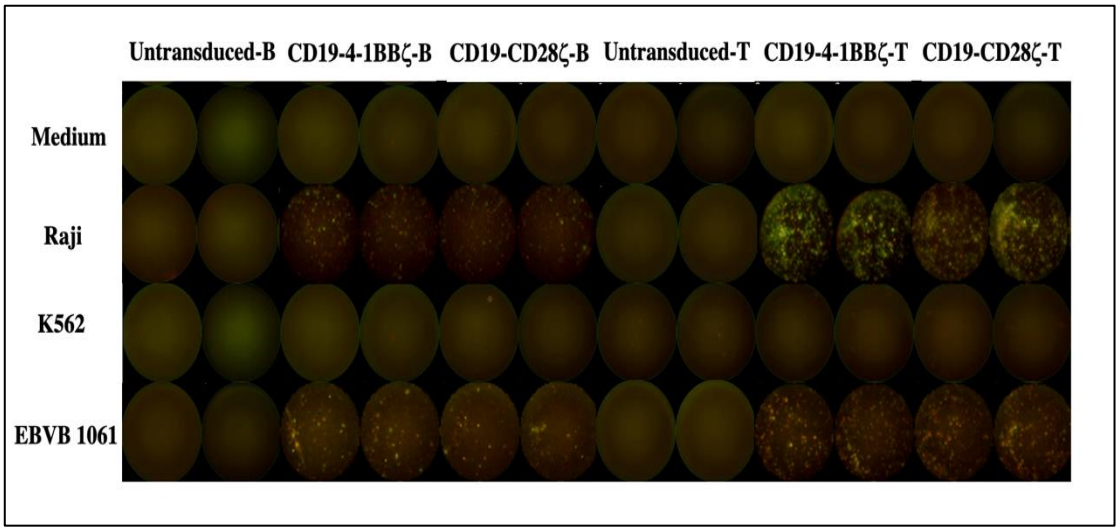

Figure 28: Cytotoxicity of UCB-derived CD19-CAR-T cells assessed by granzyme B and perforin assays upon the co-culture with $CD19^+$ (Raji and EBVB 1061) or $CD19^-$ (K562) target cells. Antigen-specific release of granzyme B (red spots) and perforin (green spots) was detected all CD19-CAR-T cells. Representative data showing one out of three UCB-derived CD19-CAR-T cells is shown.

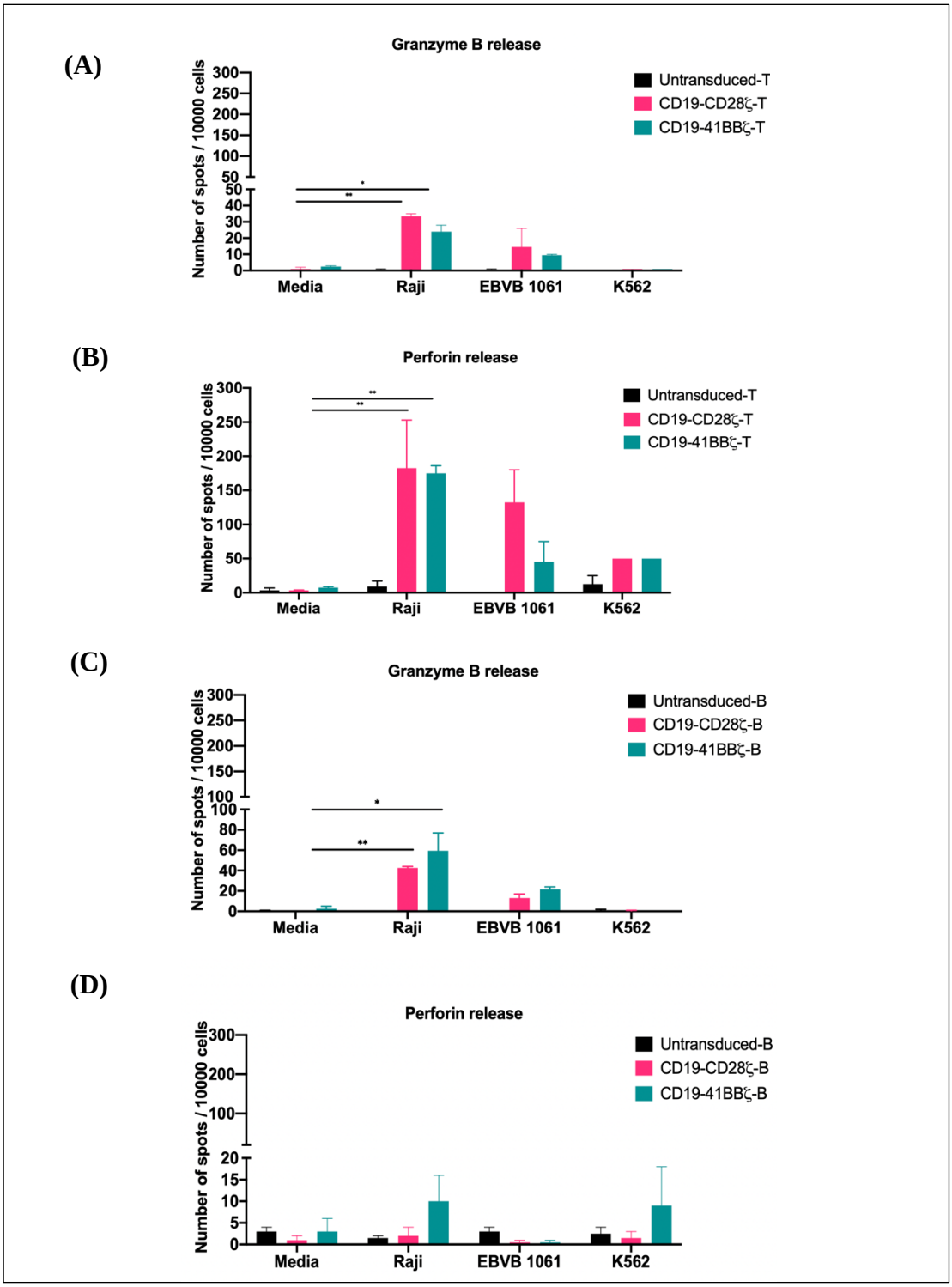

Figure 29: Cytotoxicity of UCB-CD19-CAR-T cells assessed by granzyme B and perforin assays upon the co-culture with $CD19^+$ (Raji and EBVB 1061) or $CD19^-$ (K562) target cells. Antigen-specific release of granzyme B and perforin was detected by T cells activated with TransAct (A&B) or beads (C and D). Representative data showing one out of three UCB-derived CD19-CAR-T cells is shown. Data are represented as mean ± SEM of N=2 replicates. Similar results were obtained for CD19-CAR-T cells isolated from other UCBs (data not shown). * indicates $p<0.05$ and ** indicates $p<0.01$. T, TransAct; B, beads.

CHAPTER 5: DISCUSSION

This study achieved the major goal to optimize pre-clinical *in vitro* manufacturing of UCB–derived CD19-CAR-T cells. Moreover, a novel comprehensive phenotypic characterization of these cells was performed.

Although that the frequency of T cells expressing CD3 was lower in UCB compared to PBMC [221], the yield of $CD3^+$ T lymphocytes was obtained from UCB was suitable for the manufacturing of CAR-T cells ($1.62x10^7$ - $5.68x10^7$) as compared to the numbers of $CD3^+$ cells obtained from PBMC ($1.90\ x10^7$- $2.24\ x10^7$). A positive correlation between the yield of T lymphocytes and the volume of UCB was found. These results indicate that similar to PBMC, T lymphocytes can be efficiently isolated from UCB for the *in vitro* processing of CD19-CAR-T cells.

The development of CD19-CAR-T cells using UCB has been previously established [218, 222-224]. However, conclusive procedures have not been yet identified. Moreover, the comparison of UCB- vs PBMC- derived CAR-T cells is not available.

In this study, similarly to PBMC, CD19-CAR T lymphocytes were successfully expanded *in vitro* when UCB was used as source of T cells. The expansion could reach total number of cells of 20-110-fold, that is compatible with the translation of the methods for large scale expansion and number of cells required for the clinical application ($5x10^8$-$1x10^9$ cells).

The *in vitro* generation of UCB-CD19-CAR-T cells showed that these cells expressed homogeneously the CD19-CARs with the highest percentage of transduction, associated with the expression of the CARs achieved through use of vectofusin during the process of transduction and upon activation with beads (85.19% and 96.25%, respectively as indicated by CD19 and NGFR staining in UCB).

Interestingly, the usage of vectofusin as a method of transduction of T cells has not been previously applied for the production of UCB-derived CAR-T cells. Other studies have utilized as enhancers of the transaction, fibronectin, polybrene or non-viral gene transfer methods such as piggyBac-mediated gene transfer or sleeping beauty transposon system [15, 218, 223, 224]. In contrast with the results obtained from the aforementioned methods, the efficiency of transduction of T cells was superior in the presence of vectofusin. Moreover, the discrepancy exhibited between stimulation with TransAct and beads in terms of efficiency of transduction might be the result from the different activation mechanisms which is expected to be milder with polymeric nanomatrix compared to magnetic beads .

Indeed, vectofusin-1, a cationic peptide belonging to the LAH4 peptide family, has been recently shown to enhance the transduction of human $CD34^+$ hematopoietic stem cells, progenitor cells, and activated T cells [225]. Similar to our results, a study by Fenard *et al.* demonstrated that the addition of vectofusin peptide (12ug/ml) have enhanced the transduction of UCB-derived hematopoietic stem cells to a higher extent compared to the widely used transduction enhancer retronectin and with no evidence of toxicity associated with the use of vectofusin [226].

A recent study by Radek *et al.* tested the possibility of implementing the use of vectofusin in CAR-T cell manufacturing showed that similar to recombinant fibronectin (retronectin), vectofusin can also augment the transduction of donor derived CAR-T cells [227]. In term of manufacturing UCB-derived CAR-T cells, vectofusin is easy to use since it can be added as a soluble factor to the *in vitro* cell culture.

Next, we cultured our activated UCB CAR-T cells in combination with 5ng/ml IL-7 and IL-15 to assess their expansion *in vitro* . Initial experiments with engineered UCB T cells using both OKT3 and IL-2 have resulted in 39-fold expansion only [222]. Later

on, an improvement of 140-fold increase has been shown with the use of CD3 and CD28 magnetic beads. However, the expanded UCB T cells where enriched with an effector memory phenotype [228]. In contrast, T cell activation with GMP grade TransAct seems to efficiently prime naïve T cells and have the tendency to preserve CCR7 expression [229, 230]. Based on that, we speculated that the use of TranAct will help in priming and activating our naïve UCB T cells more effectively than beads. In this study, CD19-41-BBζ–CAR-T cells activated with beads demonstrated a statistically significant increase *in vitro* expansion compared to TransAct, leading to a reproducible superior *in vitro* expansion of both UCB and PBMC. This study is the first report of the comparison of different co-stimulatory molecules or methods of T cell activation in UCB-derived CAR-T cell. However, higher expansion of CD19-CAR-T cells upon activation with beads compared to TransAct has been previously demonstrated in PBMC [231, 232].

In this work, even though the stimulation with CD3/CD28 beads has been proved to be the optimal method for CAR-T cell expansion, the results obtained from T cell TransAct are still comparable. Recently, the use of T cell TransAct has been described as in line with automated generation of CAR-T cells in a closed system (CliniMACS Prodigy) [233, 234]. Thus, TransAct might be suitable for a simplified clinical grade manufacturing of UCB-derived CAR-T cells .

The available results on UCB-derived-CAR-T cells, limited the evaluation of exogenous cytokines on their *in vitro* growth. In 2011, Hollie *et al.* showed that the *in vitro* culture of UCB T cells in the presence of IL-12 plus either IL-2 or IL-15 resulted in >150 fold expansion [235]. Later it was shown that combination of 10 ng/ml IL-7 and IL-15 resulted in 120-fold expansion after 12 days of culture only [13]. Our observations are in line with the latest results.

Importantly, UCB-derived CD19 CAR-T cells isolated in the context of this study exerted antigen-specific recognition of $CD19^+$ target cells as shown by the detection of IFN-γ and IL-4 in the co-cultures. These findings are in agreement with previous reports showing the anti-tumor function of UCB CAR-T cells towards its $CD19^+$ target cell lines [13, 224]. Moreover, granzyme-B and perforin, although the latest with some variability, could be detected upon the co-culture of UCB-CD19-CAR-T cells with $CD19^+$ cell lines. These results are a support of the efficiency of the method of manufacturing we have utilized for UCB-CAR-T cells. Overall, CD19-CAR-T cell bearing CD28ζ as co-stimulatory signaling and isolated in the presence of CD3/CD28 beads displayed slightly higher anti-tumor reactivity as compared to either the activation of the cells with TranAct or CD19-4-1BBζ-CAR.

In this study, a comprehensive phenotypic characterization of UCB-derived CD19-CAR-T cells as compared to the usage of PBMC for the engineering of T cells has been performed. Previous reports described the antigen naïve status of UCB-T cells, showing higher proportion of naïve $CD45RA^+$ and low memory $CD45RO^+$ T cells compared to that of PBMC [221, 236-238]. Based on that, we speculated that a higher proportion of early differentiated T cell subsets expressing CD19-CARs could be found in the CD19-CAR-T cells that were isolated in this study.

Our findings highlighted a high level of heterogeneity in term of different sub-populations of CD19-CAR-T cells that could be detected. Nevertheless, UCB-derived CD19-CAR-T cells clearly demonstrated a superior enrichment of Tscm and Tcm CD19-CAR-T cells as compared to PBMC-CAR-T cells. A preferential enrichment of $CD3^+CD45RA^+CD19^+$CAR-T cells was found upon the usage of CD3/CD28 beads as method of activation of the T lymphocytes. These results might explain the higher

proliferative capacity observed by UCB-derived CAR-T cells upon the stimulations with beads compared to TransAct.

The phenotypic comparison of UCB-derived CAR-T cells bearing different co-stimulatory molecules demonstrated a higher enrichment of effector $CD8^+$ cells in CD19-CD28ζ compared to CD19-41BBζ, in line also with the pattern of anti-tumor activity that was observed for these T cells.

Both the two type of CD19-CAR-T cells displayed an enrichment of $CD45RA^+$ T cells that could co-express either CCR7 or immune checkpoint molecules, e.g., BTLA, CTL-4, TIM-3. However, these molecules represent the activatory status of the T cells and not the exhaustion as occur in the late stage of differentiation of the T cells (the latest subset of T cells co-express $CD45RO^+$ and immune checkpoint). However, the heterogeneity of UCB-CAR-T cells, especially for CD19-CD28ζ–CAR-T cells, was shown by the presence of both $CD4^+$ and $CD8^+$ T cell subsets co-expressing either CD45RA or CD45RO. Overall, the extent of the presence of CD19-CAR-T cells at early stage of maturation (CD45RA+) was higher in T cells derived from UCB as compared to PBMC. Of note, the phenotype of these cells was maintained at the two different time points (day + 9 and +14) along with the *in vitro* culture and expansion of the T lymphocytes.

The ability of UCB derived-CD19-CAR-T cells to maintain a population of Tscm and Tcm cells might represent a benefit in term of the anti-tumor activity and survival *in vivo*. This point will represent the object of the future studies that will be performed. Klebanoffet al. demostrated that the ACT of $CD8^+$ Tcm cells exhibited superior anti-tumor activity compared to $CD8^+$ Tem cells [239]. Other studies showed that effector $CD8^+$ cells derived from naïve T cell subsets displayed enhanced proliferative potential, engraftment, survival, and resistant to terminal differentiation, making them preferable

for ACT compared to memory T cell subsets [240, 241]. Moreover, the frequency of memory T cell subsets in the cell product used for the administration to patients has been shown to be associated with clinical response in CAR-T cell therapy. For instance, the percentage of central memory phenotype $CD8^{+}CD45RO^{-}CD27^{+}$ T cells detected in the initial leukapheresis following the induction therapy in MM patients was associated with the clinical activity [242].

Overall, our work indicates that UCB T lymphocytes can be efficiently transduced and expanded *in vitro* in association with the selection of early differentiated UCB-derived CD19-CAR-T cells endowed with anti-tumor reactivity and cytotoxicity. However, some differences have been observed depending on the usage of the either TransAct or beads for the activation can represent an efficient method for clinical grade production of UCB-derived CAR-T cells. Moreover, superior anti-tumor activity and selection of effector $CD8^{+}$ cells upon the use of CD19-CD28ζ CAR vs. CD19-4-1BBζ CAR indicate that CD28 co-stimulatory signaling might be required for the production of UCB-derived CAR-T cells endowed with higher anti-tumor capacity. The mixture of two populations of CAR-T cells bearing either one of the two different co-stimulatory molecules might also represent an option to implement the quality of the engineered T cells.

In the future study, we aim at investigating the contribution of the different T cell subsets we detected in CD19-CAR-T cells in terms of anti-tumor activity and *in vivo* persistence. This will be achieved through the cell sorting of the T cell subsets and to assess their functional properties.

In addition, our study aims at the implementation of the characterization of UCB-CD19-CAR-T cells through the metabolomic and transcriptomic profiling. These analyses are currently ongoing.

Taken together, the obtained results allowed to identify the optimal *in vitro* procedures to isolate CD19-CAR-T cells from UCB. An integrated deep phenotype and functional characterization has also been obtained. Our data demonstrate that UCB represent a promising source for the production of CAR-T cells to be exploited for ACT studies.

CHAPTER 6: CONCLUSION

This study provides the proof of principle that UCB represents an efficient source of T lymphocytes to generate “off the shelf” CAR-T cells. The CD19-CAR-T cells that were generated in the context of this study showed antigen-specific recognition and cytotoxic activity against $CD19^+$ target cells, including leukemia and transformed cell lines. A differential phenotype subsets of CD19-CAR-T cells could be observed depending on the methods utilized for the *in vitro* activation of the T lymphocytes and the origin of these cells. These results contribute to identify the optimal procedures and to generate allogeneic CD19-CAR-T cells and the optimal target T cell subpopulations to be exploited for further analyses and for the future clinical grade manufacturing.

REFERENCES

1. Moller, G. and E. Moller, *The concept of immunological surveillance against neoplasia.* Transplant Rev, 1976. **28**: p. 3-16.
2. Thomas, L., *Discussion. In Cellular and Humoral Aspects of the Hypersensitive States.* New York: Hoeber- Harper, 1959: p. 529–532.
3. Schreiber, R.D., L.J. Old, and M.J. Smyth, *Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion.* Science, 2011. **331**(6024): p. 1565-70.
4. Koebel, C.M., et al., *Adaptive immunity maintains occult cancer in an equilibrium state.* Nature, 2007. **450**(7171): p. 903-7.
5. Dunn, G.P., et al., *Cancer immunoediting: from immunosurveillance to tumor escape.* Nat Immunol, 2002. **3**(11): p. 991-8.
6. Dunn, G.P., L.J. Old, and R.D. Schreiber, *The immunobiology of cancer immunosurveillance and immunoediting.* Immunity, 2004. **21**(2): p. 137-48.
7. Zhang, Y. and Z. Zhang, *The history and advances in cancer immunotherapy: understanding the characteristics of tumor-infiltrating immune cells and their therapeutic implications.* Cell Mol Immunol, 2020. **17**(8): p. 807-821.
8. Rosenberg, S.A. and N.P. Restifo, *Adoptive cell transfer as personalized immunotherapy for human cancer.* Science, 2015. **348**(6230): p. 62-8.
9. Maude, S.L., et al., *Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia.* N Engl J Med, 2018. **378**(5): p. 439-448.
10. Neelapu, S.S., et al., *Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma.* N Engl J Med, 2017. **377**(26): p. 2531-2544.
11. Abramson, J.S., et al., *Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study.* Lancet, 2020. **396**(10254): p. 839-852.
12. Kohl, U., et al., *CAR T Cells in Trials: Recent Achievements and Challenges that Remain in the Production of Modified T Cells for Clinical Applications.* Hum Gene Ther, 2018. **29**(5): p. 559-568.
13. Pegram, H.J., et al., *IL-12-secreting CD19-targeted cord blood-derived T cells for the immunotherapy of B-cell acute lymphoblastic leukemia.* Leukemia, 2015. **29**(2): p. 415-22.
14. Ma, Q., et al., *A novel TCR-like CAR with specificity for PR1/HLA-A2 effectively targets myeloid leukemia in vitro when expressed in human adult peripheral blood and cord blood T cells.* Cytotherapy, 2016. **18**(8): p. 985-994.
15. Liu, E., et al., *Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent antitumor activity.* Leukemia, 2018. **32**(2): p. 520-531.
16. Janeway, C.A., Jr., *How the immune system works to protect the host from infection: a personal view.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7461-8.
17. Zinkernagel, R.M., *On natural and artificial vaccinations.* Annu Rev Immunol, 2003. **21**: p. 515-46.
18. Dranoff, G., *Cytokines in cancer pathogenesis and cancer therapy.* Nat Rev Cancer, 2004. **4**(1): p. 11-22.

19. Albert, M.L., et al., *Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes.* J Exp Med, 1998. **188**(7): p. 1359-68.
20. Basu, S., et al., *CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin.* Immunity, 2001. **14**(3): p. 303-13.
21. Diefenbach, A. and D.H. Raulet, *The innate immune response to tumors and its role in the induction of T-cell immunity.* Immunol Rev, 2002. **188**: p. 9-21.
22. Correale, P., et al., *In vitro generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen.* J Natl Cancer Inst, 1997. **89**(4): p. 293-300.
23. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-52.
24. Boon, T. and P. van der Bruggen, *Human tumor antigens recognized by T lymphocytes.* J Exp Med, 1996. **183**(3): p. 725-9.
25. Mahnke, Y.D., et al., *The who's who of T-cell differentiation: human memory T-cell subsets.* Eur J Immunol, 2013. **43**(11): p. 2797-809.
26. Golubovskaya, V. and L. Wu, *Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy.* Cancers (Basel), 2016. **8**(3).
27. Nurieva, R.I., X. Liu, and C. Dong, *Molecular mechanisms of T-cell tolerance.* Immunol Rev, 2011. **241**(1): p. 133-44.
28. Leibson, P.J., *The regulation of lymphocyte activation by inhibitory receptors.* Curr Opin Immunol, 2004. **16**(3): p. 328-36.
29. Chen, L., *Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity.* Nat Rev Immunol, 2004. **4**(5): p. 336-47.
30. Wang, N., et al., *CD150 is a member of a family of genes that encode glycoproteins on the surface of hematopoietic cells.* Immunogenetics, 2001. **53**(5): p. 382-94.
31. Boles, K.S., et al., *2B4 (CD244) and CS1: novel members of the CD2 subset of the immunoglobulin superfamily molecules expressed on natural killer cells and other leukocytes.* Immunol Rev, 2001. **181**: p. 234-49.
32. Zhu, C., A.C. Anderson, and V.K. Kuchroo, *TIM-3 and its regulatory role in immune responses.* Curr Top Microbiol Immunol, 2011. **350**: p. 1-15.
33. Huard, B., et al., *Cellular expression and tissue distribution of the human LAG-3-encoded protein, an MHC class II ligand.* Immunogenetics, 1994. **39**(3): p. 213-7.
34. Denoeud, J. and M. Moser, *Role of CD27/CD70 pathway of activation in immunity and tolerance.* J Leukoc Biol, 2011. **89**(2): p. 195-203.
35. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.
36. Hutloff, A., et al., *ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28.* Nature, 1999. **397**(6716): p. 263-6.
37. Chambers, C.A. and J.P. Allison, *Costimulatory regulation of T cell function.* Curr Opin Cell Biol, 1999. **11**(2): p. 203-10.
38. Greenwald, R.J., G.J. Freeman, and A.H. Sharpe, *The B7 family revisited.* Annu Rev Immunol, 2005. **23**: p. 515-48.
39. Sedy, J.R., et al., *B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator.* Nat Immunol, 2005. **6**(1): p. 90-8.

40. Chen, G., et al., *Increased proliferation of CD8+ T cells in SAP-deficient mice is associated with impaired activation-induced cell death.* Eur J Immunol, 2007. **37**(3): p. 663-74.
41. Sakuishi, K., et al., *Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity.* J Exp Med, 2010. **207**(10): p. 2187-94.
42. Fourcade, J., et al., *Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients.* J Exp Med, 2010. **207**(10): p. 2175-86.
43. Stratton, M.R., P.J. Campbell, and P.A. Futreal, *The cancer genome.* Nature, 2009. **458**(7239): p. 719-24.
44. Podlaha, O., et al., *Evolution of the cancer genome.* Trends Genet, 2012. **28**(4): p. 155-63.
45. Trapani, J.A. and P.K. Darcy, *Immunotherapy of cancer.* Aust Fam Physician, 2017. **46**(4): p. 194-199.
46. Coley, W.B., *The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893.* Clin Orthop Relat Res, 1991(262): p. 3-11.
47. Dougan, M. and G. Dranoff, *Immune therapy for cancer.* Annu Rev Immunol, 2009. **27**: p. 83-117.
48. Mellman, I., G. Coukos, and G. Dranoff, *Cancer immunotherapy comes of age.* Nature, 2011. **480**(7378): p. 480-9.
49. Hodi, F.S., et al., *Improved survival with ipilimumab in patients with metastatic melanoma.* N Engl J Med, 2010. **363**(8): p. 711-23.
50. Waldmann, T.A., *Cytokines in Cancer Immunotherapy.* Cold Spring Harb Perspect Biol, 2018. **10**(12).
51. Yron, I., et al., *In vitro growth of murine T cells. V. The isolation and growth of lymphoid cells infiltrating syngeneic solid tumors.* J Immunol, 1980. **125**(1): p. 238-45.
52. Cheever, M.A., et al., *Interleukin 2 administered in vivo induces the growth of cultured T cells in vivo.* J Immunol, 1984. **132**(5): p. 2259-65.
53. Rosenberg, S.A., et al., *Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer.* N Engl J Med, 1985. **313**(23): p. 1485-92.
54. Italian Cooperative Study Group on Chronic Myeloid Leukemia; Sante Tura, M.B., Eliana Zuffa, Domenico Russo, Renato Fanin, Alfonso Zaccaria, Mauro Fiacchini, *Interferon alfa-2a as compared with conventional chemotherapy for the treatment of chronic myeloid leukemia.* The New England Journal od Medicine, 1994.
55. Kirkwood, J.M., et al., *Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684.* J Clin Oncol, 1996. **14**(1): p. 7-17.
56. van der Bruggen, P., et al., *A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma.* Science, 1991. **254**(5038): p. 1643-7.
57. Melero, I., et al., *Therapeutic vaccines for cancer: an overview of clinical trials.* Nat Rev Clin Oncol, 2014. **11**(9): p. 509-24.
58. Parmiani, G., et al., *A pilot Phase I study combining peptide-based vaccination and NGR-hTNF vessel targeting therapy in metastatic melanoma.* Oncoimmunology, 2014. **3**(11): p. e963406.

59. Pilla, L., S. Ferrone, and C. Maccalli, *Methods for improving the immunogenicity and efficacy of cancer vaccines.* Expert Opin Biol Ther, 2018. **18**(7): p. 765-784.
60. Choi, A.H., et al., *From Benchtop to Bedside: A Review of Oncolytic Virotherapy.* Biomedicines, 2016. **4**(3).
61. Russell, L. and K.W. Peng, *The emerging role of oncolytic virus therapy against cancer.* Chin Clin Oncol, 2018. **7**(2): p. 16.
62. Ramsay, A.G., *Immune checkpoint blockade immunotherapy to activate anti-tumour T-cell immunity.* Br J Haematol, 2013. **162**(3): p. 313-25.
63. Pardoll, D.M., *The blockade of immune checkpoints in cancer immunotherapy.* Nat Rev Cancer, 2012. **12**(4): p. 252-64.
64. Buder-Bakhaya, K. and J.C. Hassel, *Biomarkers for Clinical Benefit of Immune Checkpoint Inhibitor Treatment-A Review From the Melanoma Perspective and Beyond.* Front Immunol, 2018. **9**: p. 1474.
65. Pilla, L. and C. Maccalli, *Immune Profiling of Cancer Patients Treated with Immunotherapy: Advances and Challenges.* Biomedicines, 2018. **6**(3).
66. Rosenberg, S.A. and N.P. Restifo, *Adoptive cell transfer as personalized immunotherapy for human cancer.* Science, 2015. **348**(6230).
67. Eberlein, T.J., M. Rosenstein, and S.A. Rosenberg, *Regression of a disseminated syngeneic solid tumor by systemic transfer of lymphoid cells expanded in interleukin 2.* J Exp Med, 1982. **156**(2): p. 385-97.
68. Chang, A.E., et al., *Phase II trial of autologous tumor vaccination, anti-CD3-activated vaccine-primed lymphocytes, and interleukin-2 in stage IV renal cell cancer.* J Clin Oncol, 2003. **21**(5): p. 884-90.
69. Smith, K.A. and F.W. Ruscetti, *T-cell growth factor and the culture of cloned functional T cells.* Adv Immunol, 1981. **31**: p. 137-75.
70. Guillerey, C., N.D. Huntington, and M.J. Smyth, *Targeting natural killer cells in cancer immunotherapy.* Nat Immunol, 2016. **17**(9): p. 1025-36.
71. Jinek, M., et al., *A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity.* Science, 2012. **337**(6096): p. 816-21.
72. Rosenberg, S.A., et al., *Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report.* N Engl J Med, 1988. **319**(25): p. 1676-80.
73. Stevanovic, S., et al., *Complete regression of metastatic cervical cancer after treatment with human papillomavirus-targeted tumor-infiltrating T cells.* J Clin Oncol, 2015. **33**(14): p. 1543-50.
74. Tran, E., et al., *Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer.* Science, 2014. **344**(6184): p. 641-5.
75. Zacharakis, N., et al., *Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer.* Nat Med, 2018. **24**(6): p. 724-730.
76. Khong, H.T., Q.J. Wang, and S.A. Rosenberg, *Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression.* J Immunother, 2004. **27**(3): p. 184-90.
77. Dudley, M.E., et al., *Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes.* Science, 2002. **298**(5594): p. 850-4.
78. Hinrichs, C.S. and S.A. Rosenberg, *Exploiting the curative potential of adoptive T-cell therapy for cancer.* Immunol Rev, 2014. **257**(1): p. 56-71.

79. Gross, G., T. Waks, and Z. Eshhar, *Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity.* Proc Natl Acad Sci U S A, 1989. **86**(24): p. 10024-8.
80. Wang, M., et al., *KTE-X19 CAR T-Cell Therapy in Relapsed or Refractory Mantle-Cell Lymphoma.* N Engl J Med, 2020. **382**(14): p. 1331-1342.
81. Zhao, Z., et al., *The application of CAR-T cell therapy in hematological malignancies: advantages and challenges.* Acta Pharm Sin B, 2018. **8**(4): p. 539-551.
82. Hudecek, M., et al., *Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells.* Clin Cancer Res, 2013. **19**(12): p. 3153-64.
83. Chmielewski, M., et al., *T cell activation by antibody-like immunoreceptors: increase in affinity of the single-chain fragment domain above threshold does not increase T cell activation against antigen-positive target cells but decreases selectivity.* J Immunol, 2004. **173**(12): p. 7647-53.
84. Liu, X., et al., *Affinity-Tuned ErbB2 or EGFR Chimeric Antigen Receptor T Cells Exhibit an Increased Therapeutic Index against Tumors in Mice.* Cancer Res, 2015. **75**(17): p. 3596-607.
85. Haso, W., et al., *Anti-CD22-chimeric antigen receptors targeting B-cell precursor acute lymphoblastic leukemia.* Blood, 2013. **121**(7): p. 1165-74.
86. De Genst, E., et al., *Antibody repertoire development in camelids.* Dev Comp Immunol, 2006. **30**(1-2): p. 187-98.
87. Lanitis, E., et al., *Redirected antitumor activity of primary human lymphocytes transduced with a fully human anti-mesothelin chimeric receptor.* Mol Ther, 2012. **20**(3): p. 633-43.
88. Ellebrecht, C.T., et al., *Reengineering chimeric antigen receptor T cells for targeted therapy of autoimmune disease.* Science, 2016. **353**(6295): p. 179-84.
89. Kong, S., et al., *Suppression of human glioma xenografts with second-generation IL13R-specific chimeric antigen receptor-modified T cells.* Clin Cancer Res, 2012. **18**(21): p. 5949-60.
90. Guedan, S., et al., *Engineering and Design of Chimeric Antigen Receptors.* Mol Ther Methods Clin Dev, 2019. **12**: p. 145-156.
91. Guest, R.D., et al., *The role of extracellular spacer regions in the optimal design of chimeric immune receptors: evaluation of four different scFvs and antigens.* J Immunother, 2005. **28**(3): p. 203-11.
92. James, S.E., et al., *Antigen sensitivity of CD22-specific chimeric TCR is modulated by target epitope distance from the cell membrane.* J Immunol, 2008. **180**(10): p. 7028-38.
93. Hombach, A., A.A. Hombach, and H. Abken, *Adoptive immunotherapy with genetically engineered T cells: modification of the IgG1 Fc 'spacer' domain in the extracellular moiety of chimeric antigen receptors avoids 'off-target' activation and unintended initiation of an innate immune response.* Gene Ther, 2010. **17**(10): p. 1206-13.
94. Almasbak, H., et al., *Inclusion of an IgG1-Fc spacer abrogates efficacy of CD19 CAR T cells in a xenograft mouse model.* Gene Ther, 2015. **22**(5): p. 391-403.
95. Jonnalagadda, M., et al., *Chimeric antigen receptors with mutated IgG4 Fc spacer avoid fc receptor binding and improve T cell persistence and antitumor efficacy.* Mol Ther, 2015. **23**(4): p. 757-68.

96. Bridgeman, J.S., et al., *The optimal antigen response of chimeric antigen receptors harboring the CD3zeta transmembrane domain is dependent upon incorporation of the receptor into the endogenous TCR/CD3 complex.* J Immunol, 2010. **184**(12): p. 6938-49.
97. Kochenderfer, J.N., et al., *Construction and preclinical evaluation of an anti-CD19 chimeric antigen receptor.* J Immunother, 2009. **32**(7): p. 689-702.
98. Imai, C., et al., *Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia.* Leukemia, 2004. **18**(4): p. 676-84.
99. Jensen, M., et al., *CD20 is a molecular target for scFvFc:zeta receptor redirected T cells: implications for cellular immunotherapy of CD20+ malignancy.* Biol Blood Marrow Transplant, 1998. **4**(2): p. 75-83.
100. Savoldo, B., et al., *CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients.* J Clin Invest, 2011. **121**(5): p. 1822-6.
101. Huang, R., et al., *Recent advances in CAR-T cell engineering.* J Hematol Oncol, 2020. **13**(1): p. 86.
102. Guedan, S., et al., *Enhancing CAR T cell persistence through ICOS and 4-1BB costimulation.* JCI Insight, 2018. **3**(1).
103. Till, B.G., et al., *Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells.* Blood, 2008. **112**(6): p. 2261-71.
104. Croft, M., *Costimulation of T cells by OX40, 4-1BB, and CD27.* Cytokine Growth Factor Rev, 2003. **14**(3-4): p. 265-73.
105. Song, D.G., et al., *CD27 costimulation augments the survival and antitumor activity of redirected human T cells in vivo.* Blood, 2012. **119**(3): p. 696-706.
106. Pule, M.A., et al., *A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells.* Mol Ther, 2005. **12**(5): p. 933-41.
107. Paulos, C.M., et al., *The inducible costimulator (ICOS) is critical for the development of human T(H)17 cells.* Sci Transl Med, 2010. **2**(55): p. 55ra78.
108. Maude, S.L., et al., *Chimeric antigen receptor T cells for sustained remissions in leukemia.* N Engl J Med, 2014. **371**(16): p. 1507-17.
109. Finney, H.M., et al., *Chimeric receptors providing both primary and costimulatory signaling in T cells from a single gene product.* J Immunol, 1998. **161**(6): p. 2791-7.
110. Chmielewski, M. and H. Abken, *TRUCKs: the fourth generation of CARs.* Expert Opin Biol Ther, 2015. **15**(8): p. 1145-54.
111. Milone, M.C., et al., *Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo.* Mol Ther, 2009. **17**(8): p. 1453-64.
112. Brentjens, R.J., et al., *Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias.* Blood, 2011. **118**(18): p. 4817-28.
113. Fraietta, J.A., et al., *Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia.* Nat Med, 2018. **24**(5): p. 563-571.
114. Levine, B.L., *Performance-enhancing drugs: design and production of redirected chimeric antigen receptor (CAR) T cells.* Cancer Gene Ther, 2015. **22**(2): p. 79-84.

115. Allen, E.S., et al., *Autologous lymphapheresis for the production of chimeric antigen receptor T cells.* Transfusion, 2017. **57**(5): p. 1133-1141.
116. Powell, D.J., Jr., et al., *Efficient clinical-scale enrichment of lymphocytes for use in adoptive immunotherapy using a modified counterflow centrifugal elutriation program.* Cytotherapy, 2009. **11**(7): p. 923-35.
117. Riddell, S.R., et al., *Adoptive therapy with chimeric antigen receptor-modified T cells of defined subset composition.* Cancer J, 2014. **20**(2): p. 141-4.
118. BL., L., *Personalized Cell-Based Medicine: Activated and Expanded T Cells for Adoptive Immunotherapy.* BioProcess J, 2007. **6**(2).
119. McGarrity, G.J., et al., *Patient monitoring and follow-up in lentiviral clinical trials.* J Gene Med, 2013. **15**(2): p. 78-82.
120. Montini, E., et al., *The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy.* J Clin Invest, 2009. **119**(4): p. 964-75.
121. MacLeod, D.T., 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. **25**(4): p. 949-961.
122. Singh, H., et al., *Redirecting specificity of T-cell populations for CD19 using the Sleeping Beauty system.* Cancer Res, 2008. **68**(8): p. 2961-71.
123. Boissel, L., et al., *Transfection with mRNA for CD19 specific chimeric antigen receptor restores NK cell mediated killing of CLL cells.* Leuk Res, 2009. **33**(9): p. 1255-9.
124. Hollyman, D., et al., *Manufacturing validation of biologically functional T cells targeted to CD19 antigen for autologous adoptive cell therapy.* J Immunother, 2009. **32**(2): p. 169-80.
125. Bajgain, P., et al., *Optimizing the production of suspension cells using the G-Rex "M" series.* Mol Ther Methods Clin Dev, 2014. **1**: p. 14015.
126. Kaiser, A.D., et al., *Towards a commercial process for the manufacture of genetically modified T cells for therapy.* Cancer Gene Ther, 2015. **22**(2): p. 72-8.
127. Dai, X., et al., *Standardizing CAR-T therapy: Getting it scaled up.* Biotechnol Adv, 2019. **37**(1): p. 239-245.
128. Wang, X. and I. Riviere, *Clinical manufacturing of CAR T cells: foundation of a promising therapy.* Mol Ther Oncolytics, 2016. **3**: p. 16015.
129. Thommen, D.S. and T.N. Schumacher, *T Cell Dysfunction in Cancer.* Cancer Cell, 2018. **33**(4): p. 547-562.
130. Salmikangas, P., N. Kinsella, and P. Chamberlain, *Chimeric Antigen Receptor T-Cells (CAR T-Cells) for Cancer Immunotherapy - Moving Target for Industry?* Pharm Res, 2018. **35**(8): p. 152.
131. Lin, J.K., et al., *Cost Effectiveness of Chimeric Antigen Receptor T-Cell Therapy in Multiply Relapsed or Refractory Adult Large B-Cell Lymphoma.* J Clin Oncol, 2019. **37**(24): p. 2105-2119.
132. Depil, S., et al., *'Off-the-shelf' allogeneic CAR T cells: development and challenges.* Nat Rev Drug Discov, 2020. **19**(3): p. 185-199.
133. Graham, C., et al., *Allogeneic CAR-T Cells: More than Ease of Access?* Cells, 2018. **7**(10).
134. Torikai, H., et al., *Toward eliminating HLA class I expression to generate universal cells from allogeneic donors.* Blood, 2013. **122**(8): p. 1341-9.
135. Ren, J., et al., *Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition.* Clin Cancer Res, 2017. **23**(9): p. 2255-2266.

136. Poirot, L., et al., *Multiplex Genome-Edited T-cell Manufacturing Platform for "Off-the-Shelf" Adoptive T-cell Immunotherapies.* Cancer Res, 2015. **75**(18): p. 3853-64.
137. Qasim, W., et al., *Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells.* Sci Transl Med, 2017. **9**(374).
138. Eapen, M., et al., *Effect of graft source on unrelated donor haemopoietic stem-cell transplantation in adults with acute leukaemia: a retrospective analysis.* Lancet Oncol, 2010. **11**(7): p. 653-60.
139. Kwoczek, J., et al., *Cord blood-derived T cells allow the generation of a more naive tumor-reactive cytotoxic T-cell phenotype.* Transfusion, 2018. **58**(1): p. 88-99.
140. Kadereit, S., et al., *Reduced NFAT1 protein expression in human umbilical cord blood T lymphocytes.* Blood, 1999. **94**(9): p. 3101-7.
141. Tchao, N.K. and L.A. Turka, *Lymphodepletion and homeostatic proliferation: implications for transplantation.* Am J Transplant, 2012. **12**(5): p. 1079-90.
142. Fry, T.J., et al., *A potential role for interleukin-7 in T-cell homeostasis.* Blood, 2001. **97**(10): p. 2983-90.
143. Sandau, M.M., C.J. Winstead, and S.C. Jameson, *IL-15 is required for sustained lymphopenia-driven proliferation and accumulation of CD8 T cells.* J Immunol, 2007. **179**(1): p. 120-5.
144. Maus, M.V. and C.H. June, *Making Better Chimeric Antigen Receptors for Adoptive T-cell Therapy.* Clin Cancer Res, 2016. **22**(8): p. 1875-84.
145. Ying, Z., et al., *Parallel Comparison of 4-1BB or CD28 Co-stimulated CD19-Targeted CAR-T Cells for B Cell Non-Hodgkin's Lymphoma.* Mol Ther Oncolytics, 2019. **15**: p. 60-68.
146. Opelz, G. and B. Dohler, *Effect of human leukocyte antigen compatibility on kidney graft survival: comparative analysis of two decades.* Transplantation, 2007. **84**(2): p. 137-43.
147. Johnson, R.J., et al., *Factors influencing outcome after deceased heart beating donor kidney transplantation in the United Kingdom: an evidence base for a new national kidney allocation policy.* Transplantation, 2010. **89**(4): p. 379-86.
148. Kurtzberg, J., et al., *Results of the Cord Blood Transplantation Study (COBLT): clinical outcomes of unrelated donor umbilical cord blood transplantation in pediatric patients with hematologic malignancies.* Blood, 2008. **112**(10): p. 4318-27.
149. Taylor, C.J., et al., *Generating an iPSC bank for HLA-matched tissue transplantation based on known donor and recipient HLA types.* Cell Stem Cell, 2012. **11**(2): p. 147-52.
150. O'Reilly, R.J., et al., *Virus-specific T-cell banks for 'off the shelf' adoptive therapy of refractory infections.* Bone Marrow Transplant, 2016. **51**(9): p. 1163-72.
151. Benjamin R, G.C., Yallop D, et al. , *Preliminary data on safety, cellular kinetics and anti-leukemic activity of UCART19, an allo- geneic anti-CD19 CAR T-cell product, in a pool of adult and pe- diatric patients with high-risk CD19+ relapsed/refractory B-cell acute lymphoblastic leukemia.* American Society of Hematology, 2018. **132**.
152. Valton, J., et al., *A Multidrug-resistant Engineered CAR T Cell for Allogeneic Combination Immunotherapy.* Mol Ther, 2015. **23**(9): p. 1507-18.

153. Wang, D., et al., *Targeted Disruption of the beta2-Microglobulin Gene Minimizes the Immunogenicity of Human Embryonic Stem Cells.* Stem Cells Transl Med, 2015. **4**(10): p. 1234-45.
154. Lanza, R., D.W. Russell, and A. Nagy, *Engineering universal cells that evade immune detection.* Nat Rev Immunol, 2019. **19**(12): p. 723-733.
155. Lanza R, R.D., Nagy A., *Induction of long-term allogeneic cell acceptance and formation of immune privileged tissue in immunocompetent hosts.* bioRxiv, 2019.
156. Raphael, I., et al., *T cell subsets and their signature cytokines in autoimmune and inflammatory diseases.* Cytokine, 2015. **74**(1): p. 5-17.
157. Sadelain, M., I. Riviere, and S. Riddell, *Therapeutic T cell engineering.* Nature, 2017. **545**(7655): p. 423-431.
158. Sommermeyer, D., et al., *Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity in vivo.* Leukemia, 2016. **30**(2): p. 492-500.
159. Rosenblum, M.D., S.S. Way, and A.K. Abbas, *Regulatory T cell memory.* Nat Rev Immunol, 2016. **16**(2): p. 90-101.
160. Berger, C., et al., *Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates.* J Clin Invest, 2008. **118**(1): p. 294-305.
161. Gattinoni, L., et al., *A human memory T cell subset with stem cell-like properties.* Nat Med, 2011. **17**(10): p. 1290-7.
162. Turtle, C.J., et al. , *Immunotherapy with CD19-specific chimeric antigen receptor (CAR)-modified T cells of defined subset composition.* Journal of Clinical Oncology, 2015. **33**(15).
163. Turtle, C.J., et al., *CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients.* J Clin Invest, 2016. **126**(6): p. 2123-38.
164. Louis, C.U., et al., *Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma.* Blood, 2011. **118**(23): p. 6050-6.
165. Shedlock, D.J. and H. Shen, *Requirement for CD4 T cell help in generating functional CD8 T cell memory.* Science, 2003. **300**(5617): p. 337-9.
166. Xu, Y., et al., *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, 2014. **123**(24): p. 3750-9.
167. Cieri, N., et al., *IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors.* Blood, 2013. **121**(4): p. 573-84.
168. Geiger, R., et al., *L-Arginine Modulates T Cell Metabolism and Enhances Survival and Anti-tumor Activity.* Cell, 2016. **167**(3): p. 829-842 e13.
169. Barnett, B.E.e.a., *piggyBacTM-produced CAR-T cells exhibit stem-cell memory phenotype.* . Blood, 2016. **128**(22).
170. Li, Y.S., K. Hayakawa, and R.R. Hardy, *The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver.* J Exp Med, 1993. **178**(3): p. 951-60.
171. Davila, M.L., et al., *Chimeric antigen receptors for the adoptive T cell therapy of hematologic malignancies.* Int J Hematol, 2014. **99**(4): p. 361-71.
172. Lee, D.W., et al., *T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial.* Lancet, 2015. **385**(9967): p. 517-528.

173. Brentjens, R.J., et al., *CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia.* Sci Transl Med, 2013. **5**(177): p. 177ra38.
174. Davila, M.L., et al., *Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia.* Sci Transl Med, 2014. **6**(224): p. 224ra25.
175. Davila, M.L. and M. Sadelain, *Biology and clinical application of CAR T cells for B cell malignancies.* Int J Hematol, 2016. **104**(1): p. 6-17.
176. Maude SL, T.D., Rheingold SR, et al. , *Sustained remissions with CD19-specific chimeric antigen receptor (CAR)-modified T cells in children with relapsed/refractory ALL.* Journal of Clinical Oncology 2016. **34**(15).
177. Gardner, R.A., et al., *Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults.* Blood, 2017. **129**(25): p. 3322-3331.
178. Zah, E., et al., *T Cells Expressing CD19/CD20 Bispecific Chimeric Antigen Receptors Prevent Antigen Escape by Malignant B Cells.* Cancer Immunol Res, 2016. **4**(6): p. 498-508.
179. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2018.* CA Cancer J Clin, 2018. **68**(1): p. 7-30.
180. Coiffier, B., et al., *CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma.* N Engl J Med, 2002. **346**(4): p. 235-42.
181. Younes, A., et al., *Brentuximab vedotin (SGN-35) for relapsed CD30-positive lymphomas.* N Engl J Med, 2010. **363**(19): p. 1812-21.
182. Turtle, C.J., et al., *Immunotherapy of non-Hodgkin's lymphoma with a defined ratio of CD8+ and CD4+ CD19-specific chimeric antigen receptor–modified T cells.* Science translational medicine, 2016.
183. Locke, F.L., et al., *Phase 1 Results of ZUMA-1: A Multicenter Study of KTE-C19 Anti-CD19 CAR T Cell Therapy in Refractory Aggressive Lymphoma.* Mol Ther, 2017. **25**(1): p. 285-295.
184. Locke, F.L., et al., *Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial.* Lancet Oncol, 2019. **20**(1): p. 31-42.
185. Schuster, S.J., et al., *Tisagenlecleucel in Adult Relapsed or Refractory Diffuse Large B-Cell Lymphoma.* N Engl J Med, 2019. **380**(1): p. 45-56.
186. Chiorazzi, N., K.R. Rai, and M. Ferrarini, *Chronic lymphocytic leukemia.* N Engl J Med, 2005. **352**(8): p. 804-15.
187. Gribben, J.G., *How and when I do allogeneic transplant in CLL.* Blood, 2018. **132**(1): p. 31-39.
188. Porter, D.L., et al., *Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia.* N Engl J Med, 2011. **365**(8): p. 725-33.
189. Cruz, C.R., et al., *Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase 1 study.* Blood, 2013. **122**(17): p. 2965-73.
190. Brudno, J.N., et al., *Allogeneic T Cells That Express an Anti-CD19 Chimeric Antigen Receptor Induce Remissions of B-Cell Malignancies That Progress After Allogeneic Hematopoietic Stem-Cell Transplantation Without Causing Graft-Versus-Host Disease.* J Clin Oncol, 2016. **34**(10): p. 1112-21.

191. Riches, J.C., et al., *T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production.* Blood, 2013. **121**(9): p. 1612-21.
192. Hoffmann, J.M., et al., *Differences in Expansion Potential of Naive Chimeric Antigen Receptor T Cells from Healthy Donors and Untreated Chronic Lymphocytic Leukemia Patients.* Front Immunol, 2017. **8**: p. 1956.
193. Fraietta, J.A., et al., *Ibrutinib enhances chimeric antigen receptor T-cell engraftment and efficacy in leukemia.* Blood, 2016. **127**(9): p. 1117-27.
194. Gill, S.I., et al., *Prospective Clinical Trial of Anti-CD19 CAR T Cells in Combination with Ibrutinib for the Treatment of Chronic Lymphocytic Leukemia Shows a High Response Rate.* Blood, 2018. **132**(1).
195. Gauthier, J., et al. , *Comparison of efficacy and toxicity of CD19-specific chimeric antigen receptor T-cells alone or in combination with ibrutinib for relapsed and/or refractory CLL.* Blood, 2018. **132**(1).
196. Kumar, S.K., et al., *Multiple myeloma.* Nat Rev Dis Primers, 2017. **3**: p. 17046.
197. Chung, C., *Role of Immunotherapy in Targeting the Bone Marrow Microenvironment in Multiple Myeloma: An Evolving Therapeutic Strategy.* Pharmacotherapy, 2017. **37**(1): p. 129-143.
198. Bellucci, R., et al., *Immunologic effects of prophylactic donor lymphocyte infusion after allogeneic marrow transplantation for multiple myeloma.* Blood, 2002. **99**(12): p. 4610-7.
199. Verdonck, L.F., et al., *Graft-versus-myeloma effect in two cases.* Lancet, 1996. **347**(9004): p. 800-1.
200. Atanackovic, D., et al., *Chimeric Antigen Receptor (CAR) therapy for multiple myeloma.* Br J Haematol, 2016. **172**(5): p. 685-98.
201. Garfall, A.L., et al., *Anti-CD19 CAR T cells with high-dose melphalan and autologous stem cell transplantation for refractory multiple myeloma.* JCI Insight, 2018. **3**(8).
202. Garfall, A.L., et al., *Chimeric Antigen Receptor T Cells against CD19 for Multiple Myeloma.* N Engl J Med, 2015. **373**(11): p. 1040-7.
203. Lin, P., et al., *Flow cytometric immunophenotypic analysis of 306 cases of multiple myeloma.* Am J Clin Pathol, 2004. **121**(4): p. 482-8.
204. Akhmetzyanova, I., et al., *Dynamic CD138 surface expression regulates switch between myeloma growth and dissemination.* Leukemia, 2020. **34**(1): p. 245-256.
205. O'Connell, F.P., J.L. Pinkus, and G.S. Pinkus, *CD138 (syndecan-1), a plasma cell marker immunohistochemical profile in hematopoietic and nonhematopoietic neoplasms.* Am J Clin Pathol, 2004. **121**(2): p. 254-63.
206. Kambham, N., et al., *Utility of syndecan-1 (CD138) expression in the diagnosis of undifferentiated malignant neoplasms: a tissue microarray study of 1,754 cases.* Appl Immunohistochem Mol Morphol, 2005. **13**(4): p. 304-10.
207. Bo Guo, M.C., Qingwang Han, Fan Hui, Hanren Dai, Wenying Zhang, Yajing Zhang, Yao Wang, Hongli Zhu, Weidong Han, *CD138-directed adoptive immunotherapy of chimeric antigen receptor(CAR)-modified T cells for multiple myeloma.* Journal of cellular Immunotherapy, 2016. **2**(1): p. 28-35.
208. Ramani, V.C. and R.D. Sanderson, *Chemotherapy stimulates syndecan-1 shedding: a potentially negative effect of treatment that may promote tumor relapse.* Matrix Biol, 2014. **35**: p. 215-22.

209. Van Camp, B., et al., *Plasma cells in multiple myeloma express a natural killer cell-associated antigen: CD56 (NKH-1; Leu-19).* Blood, 1990. **76**(2): p. 377-82.
210. Ailawadhi, S., et al., *A Phase I Study to Assess the Safety and Pharmacokinetics of Single-agent Lorvotuzumab Mertansine (IMGN901) in Patients with Relapsed and/or Refractory CD-56-positive Multiple Myeloma.* Clin Lymphoma Myeloma Leuk, 2019. **19**(1): p. 29-34.
211. Reuben Benjamin, M.C., Gertrude Gunset and Michel Sadelain, *CD56 targeted chimeric antigen receptors for immunotherapy of multiple myeloma.* Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research, 2012. **72**(8).
212. de Weers, M., et al., *Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors.* J Immunol, 2011. **186**(3): p. 1840-8.
213. Drent, E., et al., *A Rational Strategy for Reducing On-Target Off-Tumor Effects of CD38-Chimeric Antigen Receptors by Affinity Optimization.* Mol Ther, 2017. **25**(8): p. 1946-1958.
214. Raje, N., et al., *Anti-BCMA CAR T-Cell Therapy bb2121 in Relapsed or Refractory Multiple Myeloma.* N Engl J Med, 2019. **380**(18): p. 1726-1737.
215. Munshi, N.C., et al., *Idecabtagene vicleucel (ide-cel; bb2121), a BCMA-targeted CAR T-cell therapy, in patients with relapsed and refractory multiple myeloma (RRMM): Initial KarMMa results.* Journal of Clinical Oncology 2020. **38**(15).
216. Aftab, B.T., et al., *Toward "off-the-shelf" allogeneic CAR T cells.* Advances in Cell and Gene Therapy 2020. **3**(3).
217. Chen, Y., et al. , *Donor-derived CD 19-targeted T cell infusion induces minimal residual disease-negative remission in relapsed B-cell acute lymphoblastic leukaemia with no response to donor lymphocyte infusions after haploidentical haematopoietic stem cell transplantation.* British journal of haematology 2017. **179**(4).
218. Tammana, S., et al., *4-1BB and CD28 signaling plays a synergistic role in redirecting umbilical cord blood T cells against B-cell malignancies.* Hum Gene Ther, 2010. **21**(1): p. 75-86.
219. Mazzarella, T., et al., *Ex vivo enrichment of circulating anti-tumor T cells from both cutaneous and ocular melanoma patients: clinical implications for adoptive cell transfer therapy.* Cancer Immunol Immunother, 2012. **61**(8): p. 1169-82.
220. Norelli, M., et al., *Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells.* Nat Med, 2018. **24**(6): p. 739-748.
221. Paloczi, K., *Immunophenotypic and functional characterization of human umbilical cord blood mononuclear cells.* Leukemia, 1999. **13 Suppl 1**: p. S87-9.
222. Serrano, L.M., et al., *Differentiation of naive cord-blood T cells into CD19-specific cytolytic effectors for posttransplantation adoptive immunotherapy.* Blood, 2006. **107**(7): p. 2643-52.
223. Dolnikov, A., et al., *Antileukemic potency of CD19-specific T cells against chemoresistant pediatric acute lymphoblastic leukemia.* Exp Hematol, 2015. **43**(12): p. 1001-1014 e5.

224. Huang, X., et al., *Sleeping Beauty transposon-mediated engineering of human primary T cells for therapy of CD19+ lymphoid malignancies.* Mol Ther, 2008. **16**(3): p. 580-9.
225. Piovan, C., et al., *Vectofusin-1 Promotes RD114-TR-Pseudotyped Lentiviral Vector Transduction of Human HSPCs and T Lymphocytes.* Mol Ther Methods Clin Dev, 2017. **5**: p. 22-30.
226. Fenard, D., et al., *Vectofusin-1, a new viral entry enhancer, strongly promotes lentiviral transduction of human hematopoietic stem cells.* Mol Ther Nucleic Acids, 2013. **2**: p. e90.
227. Radek, C., et al., *Vectofusin-1 Improves Transduction of Primary Human Cells with Diverse Retroviral and Lentiviral Pseudotypes, Enabling Robust, Automated Closed-System Manufacturing.* Hum Gene Ther, 2019. **30**(12): p. 1477-1493.
228. Okas, 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**(1): p. 96-105.
229. Kaiser, D.M.N.M.-T.K.D.C.L.I.J.H.B.M.A.A., *Potent polyclonal T cell activation and expansion through GMP-grade transact nano-matrices.* Cytotherapy, 2014. **16**(4).
230. Casati, A., et al., *Clinical-scale selection and viral transduction of human naive and central memory CD8+ T cells for adoptive cell therapy of cancer patients.* Cancer Immunol Immunother, 2013. **62**(10): p. 1563-73.
231. Mock, U., et al., *Automated manufacturing of chimeric antigen receptor T cells for adoptive immunotherapy using CliniMACS prodigy.* Cytotherapy, 2016. **18**(8): p. 1002-1011.
232. Arcangeli, S., et al., *Next-Generation Manufacturing Protocols Enriching TSCM CAR T Cells Can Overcome Disease-Specific T Cell Defects in Cancer Patients.* Front Immunol, 2020. **11**: p. 1217.
233. Zhang, W., et al., *Characterization of clinical grade CD19 chimeric antigen receptor T cells produced using automated CliniMACS Prodigy system.* Drug Des Devel Ther, 2018. **12**: p. 3343-3356.
234. Zhu, F., et al., *Closed-system manufacturing of CD19 and dual-targeted CD20/19 chimeric antigen receptor T cells using the CliniMACS Prodigy device at an academic medical center.* Cytotherapy, 2018. **20**(3): p. 394-406.
235. Hollie J. Pegram, V.L.R., Renier J. Brentjens, *Expansion and Anti-Tumor Efficacy of CD19 Targeted Cord Blood T Cells.* Molecular Therapy, 2011. **19**.
236. Singh, N. and D.M. Barrett, *Donor-derived CD19 chimeric antigen receptor T cells.* Curr Opin Hematol, 2015. **22**(6): p. 503-8.
237. D'Arena, G., et al., *Flow cytometric characterization of human umbilical cord blood lymphocytes: immunophenotypic features.* Haematologica, 1998. **83**(3): p. 197-203.
238. Harris, D.T., et al., *Phenotypic and functional immaturity of human umbilical cord blood T lymphocytes.* Proc Natl Acad Sci U S A, 1992. **89**(21): p. 10006-10.
239. Klebanoff, C.A., et al., *Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells.* Proc Natl Acad Sci U S A, 2005. **102**(27): p. 9571-6.
240. Hinrichs, C.S., et al., *Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity.* Proc Natl Acad Sci U S A, 2009. **106**(41): p. 17469-74.

241. Hinrichs, C.S., et al., *Human effector CD8+ T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy.* Blood, 2011. **117**(3): p. 808-14.
242. Garfall, A.L., et al., *T-cell phenotypes associated with effective CAR T-cell therapy in postinduction vs relapsed multiple myeloma.* Blood Adv, 2019. **3**(19): p. 2812-2815.

Appendix A: Sidra Medicine and Qatar University IRB approval

Tel: +974-4003-7747
Email: irb@sidra.org

Sidra IRB MOPH Assurance: MOPH-A-Sidra-00100
Sidra IRB MOPH Registration: MOPH -Sidra-IRB-099
Sidra IRB DHHS Assurance: FWA00022378
Sidra IRB DHHS Registration: IRB00009930

June 8, 2020

Approval

Dear Dr. Maccalli,

On 08 June 2020, the IRB approved the following through 07 June 2021 inclusive.

Type of review:	Continuing Review
Protocol Title:	Collection of Umbilical Cord Blood as source of T cell and CD34+ cells for the development and validation of either gene modified immune cells to target cancer or to correct genetic diseases
Principal Investigator:	Cristina Maccalli, PhD
IRB Number:	1500788
Sponsor/ Funding Agency:	PI Fund
Grant title and ID, if any:	N/A
Documents reviewed:	• IRB-400 Informed Consent Form English_v1.1/Oct2018 (UPDATED: 06/7/2020) • IRB-401 Informed Consent Form Arabic_v1.6/Nov 2019 (UPDATED: 05/19/2020) • Leafleft_CB collection_English (UPDATED: 06/7/2020) • Leafleft_CB collection-Arabic (UPDATED: 06/7/2020) • Sidra-IRB Research Proposal (UPDATED: 04/27/2020) • IRB-408 Modification Application Form-A -V2- 7-10-2019 (UPDATED: 04/05/2020) • IRB-407 Continuing Review Application_v2/7Oct2019 (UPDATED: 03/23/2020) • Sidra - IRB Application Form (UPDATED: 04/01/2020) • Training and credentials
Level of review:	Expedited
Expedited Categories:	3

Before *07 May 2021*, you are to submit a continuing review to request continuing approval or closure. If the IRB does not grant continuing review, approval of this protocol ends after 07 June 2021 .

Copies of approved consent documents are attached.

In conducting this study, you are required to follow Sidra's Policies and Procedures pertaining to Human Research Protection.

If you have questions or concerns, please call the IRB office at 4003-7747 or send an email to irb@sidra.org.

Sincerely yours,

Eileen McBride

Eileen McBride, MD

Vice Chair
Institutional review Board
t. +974 40032957
emcbride@sidra.org

October 4th , 2020

Dr. Layla Kamareddine
College of Health Sciences
Qatar University
Phone: +974 4403 6015
Email: lkamareddine@qu.edu.qa

Dear Dr. Layla Kamareddine,

Sub.: Research Ethics Review Exemption
Ref.: Student, Asma Mohammed Al-Sulaiti/ e-mail: aa1002755@student.qu.edu.qa
Project Title: "Generation and characterization of "off-the-shelf" CAR-T cells to target cancer patients with hematological malignancies"

We would like to inform you that your application along with the supporting documents provided for the above project, has been reviewed by the QU-IRB, and having met all the requirements, has been granted research ethics **Expedited Approval** based on the following category(ies) listed in the Policies, Regulations and Guidelines provided by MOPH for Research Involving Human Subjects. Your approval is for one year effective from October 4th, 2020 till October 3rd, 2021.

1) Present no more than minimal risk to human subject, and
2) Involve only procedures listed in the following category(ies).
Category 3: Prospective collection of biological specimens for research purposes by noninvasive means.

Documents Reviewed: QU IRB approval application, QU-IRB Application Material Check List, Research Proposal form, Sidra IRB approval letter, Informed Consent Form English, Informed Consent Form Arabic, QU-IRB Review Forms, responses to IRB queries and updated documents.

Please note that expedited approvals are valid for a period of **one year** and renewal should be sought one month prior to the expiry date to ensure timely processing and continuity. Moreover, any changes/modifications to the original submitted protocol should be reported to the committee to seek approval prior to continuation.

Your Research Ethics Expedited Approval Number is: **QU-IRB 1379-EA/20**. Kindly state this number in all your future correspondence to us pertaining to this project. In addition, please submit a closure report to the QU-IRB upon completion of the project.

Best wishes,
Dr. Noora Lari

Vice Chair, QU-IRB

Institutional Review Board
(IRB)
Office Of Academic Research