CANCER IMMUNOTHERAPY 
WITH VEGFR-3 TARGETED 
CAR T-CELLS 

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1. INTRODUCTION

1.1 Cancer and its vasculature

Tumorigenesis is the generation and proliferation of cells that have undergone genotypical and phenotypical alterations that disrupt the physiological balance between proliferation and death. In humans, evidence shows that tumorigenesis is a multistep process, where each step is a genetic mutational event that contributes to the cell tumorigenic traits. Douglas Hanahan highlighted in his highly cited “Hallmarks of Cancer” (2000) that mutated cells display six potentials that are shared by most if not all cancer types (Fig 1). Those characteristics, which dictate cancer expansion, include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (known as apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

Figure 1. The six hallmarks of cancer described by Douglas Hanahan and Robert A Weinberg (from ‘The Hallmarks of Cancer’, Cell, 100.1 (2000), 57–70).

Vessel Formation in Normal Tissues

The first step of vessel formation in normal tissues is called vasculogenensis which starts at the embryonic phase. It involves the differentiation of mesenchymal cells into hemangioblasts that form circular clusters called blood islands. The hemangioblasts on the periphery of those clusters will differentiate into endothelial cells that will congregate to form the wall of the vessel. Hemangioblasts in the center of the island will develop into hematopoietic cells, which represent the circulating blood components of the vessel’s lumen. The second step is called angiogenesis, during which secondary vessels emerge, either out of the pre-existing network by a mechanism called sprouting (Fig 2a), or by forming interstitial columns that assemble directly in the lumen of primary vessels in a process called intussusception (Fig2b). Both vasculogenesis and angiogenesis are regulated by a variety of molecules including tyrosine kinase receptors such as VEGFR1, VEGFR2 (vascular endothelial growth factors 1 and 2), Tie-1, Tie-2 (angiopoietin receptor 1 and 2).
Figure 2. Angiogenesis by blood vessel growth (a) or splitting by intussusception (b). (From Ralf H. Adams & Kari Alitalo, Nature Reviews Molecular Cell Biology 8, 464-478, June 2007.)

Angiogenesis and Tumors

At the start of the last century angiogenesis was described as a process occurring around tumors and then in the early 1970’s Folkman and colleagues demonstrated that tumor metastasis is an angiogenesis-dependent process in vivo. Finally, in the 1990’s, studies shed light on the molecular level of Folkman’s observations by demonstrating that anti-VEGF antibodies can prevent neovascularization and subcutaneous tumor growth in mice. It is now known that most solid tumors and some hematologic malignancies are characterized by an angiogenic phenotype that is an absolute requirement for tumor survival, progression, and metastasis. Tumors promote angiogenesis by disrupting the balance between pro-angiogenic (VEGFR, Tie, FGF) and anti-angiogenic (Thrombospondin-1, IFN-β) factors, a phenomenon called the angiogenic switch. The root causes of the angiogenic switch are mostly gene transcription alterations, such as the loss of p53, that occur during the transformation of malignant cells. P53 is a tumor suppressor gene that is altered in most human cancers and has the capability of up-regulating thrombospondin-1, a potent inhibitor of endothelial migration and angiogenesis. Ultimately, the loss of p53 function leads to thrombospondin-1 down-regulation, liberating endothelial cells from its inhibition, thus triggering the angiogenic switch. Along with enabling nutrient delivery and waste removal, more recently it has emerged that the tumor vasculature constitutes an important barrier to T cells, the so-called tumor endothelial barrier. It has been demonstrated that endothelial cells lining the vessels can (i) block T cells them from gaining entry into the tumor through the deregulation of adhesion molecules, (ii) suppress T cell activity via inhibitory molecules and receptors, and (iii) even target them for destruction via FasL/Fas interactions.
1.2 Rationale for the utilization of the immune system in cancer therapy

The interest in immune contexture and its influence on cancer has been increasing since the early 1900’s, when the first debates regarding immunological activity and tumor control emerged. Every nucleated cell among the vertebrates expresses a Major Histocompatibility Complex type I or MHC I (also called Human Leukocyte Antigen in humans; HLA). This receptor is anchored on the cell membrane and presents an intracellular processed peptide of 8-10 amino-acids, which plays the role of the cell’s «identity card». Those peptides are openly displayed to patrolling CD8+ T Lymphocytes (also called Cytotoxic T Lymphocytes; CTL’s), thereby allowing an immune reaction mediated by their T Cell Receptor (TCR) in case of foreign peptide recognition. MHC type II (MHC II), whereas, is only expressed by a subset of immune cells such as monocytes, macrophages, dendritic cells and B lymphocytes, and is responsible for the interaction with TCR’s from CD4+ T Lymphocytes (also called T Helpers).

Tumor cells also express MHC I which can display peptides from tumor associated antigens (TAA) as well as peptides from mutated proteins (also known as neoantigens) that can be recognized by CTLs. The tumor cells can also be neutralized by phagocytes, followed by an MHC II-mediated T helper cell recognition. Thus, natural immune responses involving both cellular and humoral arms can be triggered against tumor antigens. In fact, immunocompetency has shown to be a preventive factor of sarcoma formation in methylcholanthrene carcinogen-infected mice (Fig 3). Another study demonstrated a direct link between cytotoxic CD8+ T lymphocyte activity and lymphoma control, which accredited the Immunosurveillance Hypothesis of Burnet and Thomas in the 1950’s.

**Figure 3.** Tumors growth is faster in RAG2-/- immunocompromised mice. 15 wild-type mice and 15 immunodeficient mice with disruption of the recombination-activating gene-2 (RAG2), a gene responsible for genetical recombination and maturation of B and T lymphocytes, were subcutaneously injected with carcinogen methylcholanthrene (MCA) and their evolution was followed for 200 days. Tumors developed earlier in RAG2-/- mice and with greater frequency (P < 0.01). After 160 days, 2/15 wild-type mice formed carcinogen-induced tumors, whereas the proportion in RAG2-/- mice was 9/15. (From Vijay Shankaran and others, ‘IFNγ and Lymphocytes Prevent Primary Tumour Development and Shape Tumour Immunogenicity’, Nature, 410.6832 (2001), 1107–11)

The immunoediting hypothesis

The immune system plays a dual role in the development and progression of cancer. It protects the organism from tumor establishment by recognizing and destroying transformed cells, and conversely it can induce tumor initiation by promoting chronic inflammation. From this has emerged the concept of Immunoediting which postulates that after cellular transformation, tumor cells follow a standard pattern of proliferation and decline. This pattern is dictated by the dual protective/promoting behaviour of the immune system, as well as the tumor’s escape mechanisms. Immunoediting comprises three steps (Fig 4). The first step, termed elimination phase, involves the clearance of transformed cells before the tumor is clinically observable, and is mediated by both innate (natural killers, macrophages) and adaptive (CD4+, CD8+, dendritic cells) immunity which collaborate to promote malignant cell death. In case of elimination of all abnormal cells the host remains free of cancer. In the opposite scenario, the tumor enters an equilibrium. In the equilibrium phase the cancer is
maintained in a dormant state mediated by T lymphocytes and cytokines such as IL-12 and INF-γ. Equilibrium has also the capacity of shaping the immunogenicity of tumor cells, which represents their potential of activating the adaptive immune system allowing a qualitative control of the tumor. In the escape phase the malignant cells can escape immune recognition and death through different mutational mechanisms, discussed in detail below. Eventually, tumor escape results in clinically apparent cancer.

Although the immunoediting process has been well described in mice, its occurrence in humans is debated. Nevertheless, it has been reported that the presence of Tumor Infiltrating Lymphocytes (TIL’s) is associated with better prognosis in human patients with primary cutaneous melanoma. Furthermore, immunosuppressed patients who benefited from organ transplants show a higher incidence of malignancies, mostly due to viral-related cancers such as Kaposi’s sarcoma caused by Human Papilloma Virus (HPV). However, the incidence of noninfectious cancers also pointed some noticeable increase in transplant recipients (colon, pancreas, lung, endocrine system). Consequently, the human model, while more complex than mice, is constantly attracting more interest in the validation of the immunoediting hypothesis.

Tumor escape mechanisms

Tumors employ several mechanisms in order to escape immune surveillance and destruction. Immune escape was in fact recognized as an “emerging hallmark of cancer” in 2011 by Douglas Hanahan’s in his second manifesto of “Hallmarks of Cancer: The Next Generation”. Escape mechanisms can be divided in three categories, represented as A, B, C in Fig 5. One mechanism implicates the decrease of immune recognition and stimulation by downregulating tumoral antigens (TAA’s, TSMA’s), co-stimulatory proteins (CD80, CD40) or the MHCI machinery itself. Another way is the establishment of resistances towards death threats such as apoptosis via the increased expression of pro-survival growth factors such as Bel-2. Moreover, reluctant malignant cells work together in the promotion of an immunosuppressive microenvironment. Several mechanisms are employed such as the production of immunoregulating cytokines (TGF-β, VEGF), metabolic factors (PGE-2), and the expression of surface receptors that target ligands on effector cells from the adaptive immunity and thereby inhibit them (CTLA-4, PD-1). Apart from the induction of immunosuppressive networks, tumors often exhibit epigenetic silencing of TH1-type chemokines such as CXCL9 and CXCL10 which can prevent the trafficking of immune cells into the tumor microenvironment, and the secretion of chemokines such as CCL22 which chemoattracts CD4+CD25+ Tregs.
Figure 5. Tumor cells in the immune escape phase have three major ways of surviving (A,B,C).
(From Michele W.L. Teng et al, ‘From Mice to Humans: Developments in Cancer Immunoediting’, Journal of Clinical Investigation, 125.9 (2015), 3338–46.)

T cell distribution and clinical outcome

Over the past decade, research in the field of immuno-oncology has aimed to correlate sub-populations of T lymphocytes in the tumor site and clinical outcome. Fig 6 summarizes data gathered from 124 published articles and demonstrates that the presence CTL’s and CD45RO+ memory T cells are the most reliable markers of good prognosis, whereas Treg’s reflect, in most published papers, poor clinical outcome. This can be explained by their inhibiting effect on effector T cells. Other subtypes such as T Helpers1 and T Helpers17 show contradictory data and their effect on prognosis depends on the cancer phenotype.

Figure 6. Clinical outcome of cancer patients in relation to the presence of different T cell subtypes. Favorable prognosis is represented as “Good” (blue), bad prognosis is represented by “Poor” (red) and for no link between T cell sub-populations and prognosis as “None” (violet). Twenty different types of cancer were analyzed in the making of this graph. (From Wolf Herman Fridman and others, ‘The Immune Contexture in Human Tumours: Impact on Clinical Outcome’, Nature Reviews Cancer, 12.4 (2012), 298–306.)
1.3 Chimeric Antigen Receptor T Cell Therapy

Adoptive cellular immunotherapies (ACIs) involves the isolation and ex vivo expansion of tumor specific T cells followed by their infusion into cancer patients with the goal of recognizing, targeting, and destroying tumor cells. In recent years it has been shown that adoptive cell therapy (ACT) with autologous tumor infiltrating lymphocytes (TIL) in combination with recombinant interleukin-2 (IL-2) and nonmyeloablative lympho-depleting chemotherapy (NMA) is a powerful form of immunotherapy in patients with refractory melanoma, leading to objective clinical responses of 72%. Cancer therapy using CAR-expressing T CD8+ and/or CD4+ cells as a new type of ACI emerged in the mid-1980’s. The cells can be genetically modified using lentiviral or retroviral vectors, expanded ex vivo and then administered, into patients who might also receive non-myeloablative lymphodepleting chemotherapy in an effort to boost the persistence and function of transferred T cell in vivo. CARs are recombinant membrane proteins, which upon expression on T cells can redirect their specificity towards predefined tumor antigens. Due to the fact that their antigen-recognition domain is antibody-derived, CAR’s can target antigens in an MHC-independent fashion and therefore are able to bypass issues of tumoral MHC downregulation or dysfunction of the machinery involved in the generation of MHC-binding peptides.

CAR Design

Initially, in the 1980’s, CARs comprised an antibody-derived variable fragment and the α and β chains of TCR’s constant fragment. In the following decade, the single-chain variable fragment replaced the old antigen-binding part, a transmembrane domain was added and only the ζ endodomain of the TCR CD3 complex was kept. Nowadays CARs consist of 4 main components, the ectodomain, the hinge region, the transmembrane™ domain and the endodomain, which together allow recognition of antigen, mechanical flexibility for antigen access and signal transduction.

**Ectodomain:** this domain defines the tumor antigen specificity of the CAR and in most cases is comprised of a single-chain variable fragment (scFv) derived from the variable-heavy (V_H) and variable-light (V_L) regions of a monoclonal antibody, or isolated from yeast or phage scFv display libraries. As previously mentioned, because antigen recognition does not require the MHC machinery, CAR-T cell therapy can overcome this tumor escape mechanism. Moreover, in addition to protein epitopes, the scFv is also able to recognize lipid structures and carbohydrates allowing recognition of a wider range of cancer-related antigens. A limitation, however, of CARs not relying on antigen processing is that only surface expressed antigens can be targeted by CARs. In this project, the scFv used for the CAR T cell development is directed against VEGFR-3 which is overexpressed on both blood and lymphatic vasculature of tumors.

**Hinge region:** this extracellular component is most often derived from the constant fragment (Fc) of a monoclonal antibody, particularly CH2 and/or CH3 of IgG. CD8 can also be used. The hinge region plays an important mechanical role and must provide optimal length and flexibility to the CAR for reaching the target epitope. The importance of the hinge region is illustrated by studies which demonstrated that for the same targeting construct, optimal T cell activation depends on the distance of the epitope from the target cell membrane and the relative length of this spacer region. Hence the hinge region can critically impact tumor recognition, T-cell cytokine production and proliferation and synapse formation between the T-cell and target cell.
**TM domain:** this domain can be derived from T-cell signaling molecules such as CD3ζ, CD4, CD8, or CD28 and is anchored in the cell membrane. Depending on the type of scFv used, the choice of which transmembrane protein to use is crucial for the CAR’s stability and its expression on the cell surface.

**Endodomain:** this region, which is of crucial importance for CAR design, is an intracellular extension of the transmembrane domain and comprises intracellular signaling modules derived from lymphocyte signal-initiating molecules (i.e., CD3ζ, CD28 etc). First generation CARs comprise CD3ζ of the TCR/CD3 complex which provides signal 1 of T cell activation via the phosphorylation of its intracytoplasmic immunoreceptor tyrosine-based activation motifs (ITAM’s). Second and third generation CAR’s are developed by also adding one or more co-stimulatory endodomains, respectively, which can greatly improve T cell responses against tumor targets. Most second generation CARs used in the clinic comprise the endodomain of CD28 or 41BB.

![Figure 7. Figure of a CAR binding a cell-surface expressed antigen. (From Gianpietro Dotti and others, ‘Design and Development of Therapies Using Chimeric Antigen Receptor-Expressing T Cells’, Immunological Reviews, 257.1, 2014.)](image)

**Figure 8.** Components of (A), and design of (B) first, second and third generation CARs. (From Gianpetro Dotti and others, ‘Design and Development of Therapies Using Chimeric Antigen Receptor-Expressing T Cells’, Immunological Reviews, 257.1, 2014).
Co-stimulation and next generation CARs

Co-stimulation is critical for triggering optimal T cell responses. Most tumors, however, do not express costimulatory ligands leading to suboptimal stimulation of T cells and ultimately anergy highlighted by poor cytokine secretion and proliferation, and cell death.\(^5\) The most well studied T cell costimulatory receptor is CD28 which interacts with the B7 family molecules, B7.1 and B7.2. To overcome obstacles associated with the absence of costimulation, second generation CARs were developed which, along with CD3\(\zeta\), also incorporate a co-stimulatory endodomain derived from a variety of molecules such as CD28, 4-1BB (CD137), OX40 (CD134), CD27 and ICOS (inducible costimulatory). These costimulatory endodomains are fused with CD3\(\zeta\) (Fig 9).\(^6\) In comparison with CARs expressing only the CD3-\(\zeta\) subunit (i.e., first generation), studies have demonstrated that second generation CARs promote a higher rate of cytokine production and enhance T cell proliferation and survival \textit{in vivo}.\(^61,62\) Third generation CARs include two or more costimulatory signals endodomain, for example CD28 and CD137 as illustrated in Fig 9. Although signaling by third generation CARs has not been fully characterized they are believed to trigger different signaling cascades including ZAP70, TRAF1, PI3K, and GRB2 (Fig 3).\(^63\) In addition fourth-generation CAR T-cells redirected for universal cytokine killing (TRUCKs) have been described, where the vector encoding the CAR construct also encodes a cytokine expression cassette. Usually a pro-inflammatory cytokine is secreted, may be constitutively produced or induced once the T-cell is activated by the CAR in the target tumor. This locally produced pro-inflammatory cytokine can in turn attract an innate immune cell response against the cancer, including tumor cells that are invisible to CAR T-cells.\(^64\)

\textbf{Figure 9.} The three generations of CARs are represented as well as the different signaling pathways triggered by the third generation CAR archetype. (From Michael H. Kershaw, Jennifer A. Westwood and Phillip K. Darcy, ‘Gene-Engineered T Cells for Cancer Therapy’, Nature Reviews Cancer, 13.8 (2013), 525–41.)

\textbf{CARs in the clinic}

ACI using modified CAR T cells have proven to be particularly effective in refractory populations bearing haematological malignancies. A striking example is the development of CAR T cells targeting CD19, which is a B-lineage antigen that can only be found on normal mature B cells, malignant B cells, B cell precursors and plasmocytes.\(^65\) The anti-CD19 CAR therapy showed encouraging results in a patient bearing follicular lymphoma as the elimination and absence of B-cell precursors and B-cells were observed for at least 39 weeks
after modified T cell infusion.\textsuperscript{60} Another study showed long term persistence of anti-CD19 CAR T cells in 2 out of 3 patients bearing chronic lymphoid leukemia (CLL) highlighting that the prolonged presence of this receptor leads to the establishment of a population of memory T cells targeting the CD19.\textsuperscript{67} Regarding acute lymphoid leukemia (ALL), 3 studies exhibit the best response rates with complete remission observed in 70-90% of the patients treated.\textsuperscript{68,69,70} In general, persistence of remission is more present in CLL patients, whereas ALL patients show a higher response rates.\textsuperscript{71}

Important research efforts are also being undertaken to develop CAR T cells against solid tumors, targeting TAA’s such as CEA (carcinoembryonic antigen for colon cancer), GD2 (diganglioside for neuroectodermal tumors), HER2 (human epidermal growth factor receptor 2 for breast cancer) or EGFRvIII (for glioblastoma).\textsuperscript{72,73} Although clinical responses thus far have been rather disappointing, it has been recently demonstrated that treatment of a patient with recurrent multifocal glioblastoma with CAR–engineered T cells targeting the tumor-associated antigen interleukin-13 receptor alpha 2 (IL13Ra2) led to regression of all intracranial and spinal tumors accompanied with corresponding increases in levels of cytokines and immune cells in the cerebrospinal fluid.\textsuperscript{74} It is likely that obstacles presented by solid tumors, which are absent or neglectable in hematological tumors, must be overcome to improve CAR therapy against them. As described above, the trafficking of CAR T cell into the tumor sites and penetration into the tumor microenvironment can impaired due to the deregulation of chemokine networks and the formation of an inhibitory tumor endothelial barrier. Furthermore antigen loss is more likely to occur in solid tumors.\textsuperscript{75} In addition, T cells in solid tumors express immunosuppressive ligands (i.e PDL-1 and PDL-2), secrete immunosuppressive cytokines (i.e IL-10 and TGF-b), are more exposed to oxidative stress and nutritional depletion, and often contain T cell suppressive immune elements such as T regulatory cells (Tregs), myeloid derived suppressor cells (MDSCs) and tumor-associate macrophages (TAMs).\textsuperscript{76} Another limitation of CAR T cell therapy is the identification of tumor-restricted antigens that are completely absent in normal tissues, as well as of tumor antigens that are broadly expressed by different cancer-types.

**Toxicities associated with CAR transgene immunogenicity and recognition of antigens expressed on normal tissues**

The major adverse effect of CAR T cell infusion is cytokine release syndrome (CRS). This process is directly related to exponential T cell proliferation and the subsequent increase of inflammatory cytokines such as IFN-\(\gamma\), IL-10 and IL6.\textsuperscript{77} The main symptom is a mild flu-like state, but severe CRS can cause shock and even organ failure.\textsuperscript{78} The treatment is systemic corticosteroids, which have been shown to reverse the symptomatology of CRS without any drawback on the initial anti-tumor response.\textsuperscript{79} Occurrence and severity of the syndrome are not predictors of clinical outcome, however it has been observed in liquid tumors that the majority of responders to CAR T cell therapy develop a mild CRS.\textsuperscript{80} Probably also related to cytokine increase, neurological toxicities including confusion, delirium, seizures, aphasia have been observed, particularly with anti-CD19 T cell treatment.\textsuperscript{81}

In addition to CRS, anaphylaxis has been observed in cancer patients treated with CAR-T cells. Maus et al. (2013)\textsuperscript{82} reported the safety observed in four patients treated with mRNA electroporated murine anti-human mesothelin CAR T cells. One subject developed anaphylaxis and cardiac arrest within minutes of completing the third infusion, most likely because it induced an IgE antibody specific for the murine-based antibody sequences present in the CAR-modified T-cell product. These results indicate that the potential immunogenicity of CARs derived from murine antibodies may be a safety issue for CARs because of the continuous, persistent exposure to the product.\textsuperscript{83} Therefore CAR constructs based on fully humanized scFvs have been developed and are expected to have minimal antigenic potential as allergens.\textsuperscript{84} Another cause of toxicity is due to the fact that some antigens targeted by ACI are not specific to tumors (i.e., stroma-specific antigens) and have shared expression on essential healthy tissues. Several examples of this mechanism of
toxicity have been reported in the literature. “On-target” toxicity was first reported for the CAIX CAR which was used to treat patients with metastatic renal cell cancer and consisted of limiting the liver enzyme elevations that were most likely caused by the CAR T cells that recognized the CAIX antigen expressed at low levels on the bile duct epithelial cells. Moreover, patients bearing haematological malignancies and treated with anti-B lineage CAR T cells face B cell aplasia, thus requiring long term immunoglobulin replacement. Finally, a patient with metastatic colorectal cancer who received an infusion of autologous CAR T cells directed against the antigen ERBB2 (Her-2/neu) experienced acute respiratory distress and pulmonary edema and subsequently died, likely a result of CAR reactivity against ERBB2 expression on normal lung tissue. Hence detrimental “on-target off-tumor” effects are a serious issue that is currently being studied in depth. Strategies to minimize normal tissue targeting have been developed and include the introduction of suicide genes in the engineered T cells targeting of dual tumor antigen, transient expression of CARs via RNA electroporation, development of antigen-specific inhibitory chimeric antigen receptors (iCARs) and inclusion of heterodimerizing small molecule that control assembly of antigen-binding and intracellular signaling components.

Anti-VEGFR-2 CAR T cell as a model for CAR vascular disruption therapy

Several immunotherapeutic approaches targeting VEGFR-2 on tumor endothelial cells have been used in the past including immunization against VEGFR-2, neutralization of VEGFR-2, disruption of VEGFR genes, and coupling of VEGF to toxins to target and destroy VEGFR-2–expressing cells. However results in murine models targeting VEGFR-2 have been modest, and few evaluations have been performed in humans such as the administration of an anti-VEGFR-2 antibody to patients with cancer where partial responses were seen in 15% of patients. Therefore genetically engineered CAR T cells have been explored as alternative strategy for the destruction of tumor endothelial cells.

Since VEGFR-2 and VEGFR-3 are much alike in terms of structure and function, it is worth mentioning the preclinical studies covering anti-VEGFR-2 CAR T cells. A 2010 study assessed the activity of a single dose of 2x10⁷ mouse T cells bearing a VEGFR-2-specific CAR and demonstrated its effectiveness in vivo on 5 vascularized tumors of different histological types such as melanoma, colon adenocarcinoma, colon carcinoma, renal cell carcinoma and sarcoma. Combinatorial therapy of anti-VEGFR2 CAR T cells with high doses of administrated IL-2 appeared to delay the progression of all tumors and improved overall survival of the mice. Nevertheless, cures were few and relapses of tumor progression occurred 2-3 weeks after treatment. This study also tested a fully human VEGFR-2-specific scFv against a human VEGFR-2 antigen and demonstrated its capability of generating specific immune response against human VEGFR-2-expressing endothelial cells in vitro. A later study tested the therapeutic efficacy of murine VEGFR-2-specific CAR T cells co-transduced to express IL-12. IL-12 is a pleiotropic cytokine produced by macrophages, dendritic cells and B cells, and plays an important role in NK and T cell activation. IL-12 has also been shown to have antitumor, antiangiogenic and antimitostatic properties. Treatment of mice bearing five different established subcutaneous tumors (melanoma, 2 types of sarcoma, colon carcinoma, colon adenocarcinoma) with T cells co-transduced with an anti-VEGFR-2 CAR and an inducible IL-12 gene led to a robust tumor regression and survival without the need for IL-2 administration. Furthermore, more recently it has been shown that simultaneous targeting of tumor vasculature using VEGFR-2-specific CAR T cells and tumor cells using T cells specific for tumor antigens such as gp100 can synergistically induce rapid regression of established tumors in mice. Together these results opened the door for the utilization of VEGFR-2 CAR-transduced cells as a cancer therapeutic strategy and a clinical trial has been recently completed (NCT01218867); publication of its results is pending.)
Vascular endothelial growth factor 3 is a tyrosine kinase receptor and its signaling requires the Notch pathway.\textsuperscript{106} Compared with VEGFR-1 and 2, VEGFR-3 is not only present on the endothelium of tumor blood vessels, but is also highly expressed on tumor lymphatic endothelial cells. Hence, VEGFR-3 plays an important role in both angiogenesis and lymphangiogenesis.\textsuperscript{107,108} The vascular endothelial growth factors C and D (VEGF-C, VEGF-D) bind and activate VEGFR-3 by triggering receptor phosphorylation.\textsuperscript{109} VEGFR-3 is widely present on blood endothelium and indispensable for angiogenesis during the embryonic period, but its expression lowers during development to become eventually become lymphatic-restricted.\textsuperscript{110} Nevertheless, it appears that VEGFR-3 is upregulated in tumor vasculature, especially in endothelial tip cells, which are the cells leading the angiogenic sprouts.\textsuperscript{111} Several studies have shown that VEGF-C and VEGFR-3 are expressed by malignant tumor cells including breast, papillary thyroid carcinoma, prostate, Kaposi sarcoma, gastric carcinomas and colorectal cancer.\textsuperscript{112,113,114,115,116} Furthermore, studies demonstrated that the blocking of VEGFR-3 can delay lymphangiogenesis and retard tumor growth and metastasis. For example, a rat monoclonal antibody with specificity for mVEGFR-3 and capability of antagonizing the binding of VEGF-C to VEGFR-3 prevented both physiologically normal and tumor VEGF-C enhanced lymph-angiogenesis.\textsuperscript{117} In another study administration of soluble VEGFR-3-Ig fusion protein via adenoviral or adeno-associated viral vectors led to inhibition of VEGFR-3 signaling and blockade of lymph-angiogenesis and lymph node metastasis. Of note it has been also demonstrated that combination of antibodies against VEGFR-3 and VEGFR-2 can result in additive inhibition of angiogenesis and tumour growth highlighting that VEGFR-3 may provide additional efficacy for anti-angiogenic therapies, especially towards vessels that are resistant to VEGF or VEGFR-2 inhibitors.\textsuperscript{118} These findings suggest that VEGFR-3 represents a promising therapeutic target for the development of novel anti-angiogenic approaches.
2. MATERIALS AND METHODS

2.1 Human modified CAR T cell development and functional assessment

Cell lines. Mouse endothelial cell lines used in immune-based assays include MS1, bEnd3 and H5V. 293T cells and tumor cell lines were maintained in high glucose DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mmol/l L-glutamine, and 100 µg/ml penicillin, and 100 U/ml streptomycin. The 293T human embryonic kidney cells, the Phoenix Ecotropic cells and the human ovarian cancer cell line A2780 were cultured in RPMI-1640 (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mmol/l L-glutamine 100µg/ml penicillin, and 100 U/ml streptomycin. The Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in Endothelial cell growth media 2 (ready-to-use) from Promocell. All cell lines were routinely tested for mycoplasma contamination.

Anti-hVEGFR3 CAR construction. A plasmid containing the anti-VEGFR3 scFv was used as a template for PCR amplification of the scFv fragment using the following primers: forward 5'-CAGTCTAGATCTGAGGTGACGCTGGGTGAGTCTGG-3' (BglII in bold) and reverse 5'-ACGTTGACCTAGCCGGCTAGGACGGTCAGGCTGG -3' (NheI in bold). The resulting PCR product containing a BglII site and a NheI site. Second generation expression vectors pRRL were digested with BamHI and NheI to create compatible cohesive ends and gel purified. The digested PCR products were then ligated, the resulting construct containing the scVEGFR3, CD8 Hinge transmembrane protein, CD28-CD3ζ T cell signaling domains and a WPRE post transcriptional regulatory element; transgene expression was allowed by hPGK promoter. The construct was then digested with BglIII and NheI for verification on an analytic gel. Finally, the resulting construct was sequenced and DNA concentration was measured. The resulting construct was designated pRRL-scVEGFR3-28z.

Recombinant lentivirus production. High-titer replication-defective lentiviral vectors were produced from 293T human embryonic kidney cells and concentrated. For the transfection protocol 293T cells were seeded at 10 × 10^6 per T-150 tissue culture flask 24 hours before the procedure. All plasmid DNA were purified using the QIAGEN Endo-free Maxi prep kit (Qiagen, Valencia, CA). Cells were transfected with 7 µg pVSV-G (VSV glycoprotein expression plasmid), 18 µg of pRSV.REV (Rev expression plasmid), 18 µg of pMDLg/p.RRE (Gag/Pol expression plasmid) and 15 µg of pPRLL transfer plasmid using Turbofect. The viral supernatant was harvested at 24 and 48 hours post-transfection. Viral particles were concentrated and resuspended in 0.4 ml by ultracentrifugation for 2 hours at 24,000 rpm with a Beckman SW28 rotor (Beckman Coulter, Fullerton, CA).

Human T cell transduction. Primary human T cells, were isolated from buffy coats of healthy donors. T cells were cultured in complete media (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 10-mmol/l HEPES) and stimulated with anti-CD3 and anti-CD28 mAbs coated beads (Invitrogen, Carlsbad, CA) at a bead:cell ratio of 3:1. Recombinant human interleukin-2 (IL-2) was added at a concentration of (50 IU/ml). Twenty-four hours after activation, T cells were transduced with lentivirus. Human recombinant IL-2 was added every other day to a 50 IU/ml final concentration. Cell density of 0.5–1 × 10^6 cells/ml was maintained. Rested engineered T cells were adjusted for identical transgene expression before functional assays.

Flow cytometry. Endothelial cell surface expression of mouse and human VEGFR3 was performed using APC-conjugated anti-mouse (AFL4) or anti-human VEGFR3 (clone 9D9F9) from Biolegend. VEGFR3 CAR expression was evaluated using human IgG Fc-tagged recombinant mouse or human VEGFR3 VEGF (from R&D Systems) followed by PE-conjugated anti-human IgG Fc Antibody (clone HP6017 from Biolegend). Acquisition and analysis was performed using a BD FACS LSR II flow cytometer and DIVA software (BD Biosciences).
2.2 Mouse modified CAR T cell development and functional assessment

**Anti-mVEGFR3 CAR construction.** A plasmid containing the anti-VEGFR scFv was used as a template for PCR amplification of the scFv fragment using the following primers: forward 5’-ATATGAATTCCAGGTGCAGCTGGATGTGGCTTG-3’ (EcoR1 in bold) and reverse 5’-AATGCCGGGCCGCGCTTCTGGATGTGGCTTG-3’ (Not1 in bold). The resulting PCR product containing a EcoR1 site and a Not1 site. pMSGV vectors were used, containing a VEGFR2 single chain sequence limited by EcoR1 and Not1 restriction sites. Those vectors were digested to create compatible cohesive ends and gel was purified. The digested PCR products were then ligated, the resulting construct containing a retroviral PSI packaging sequence, a signal peptide, the scVEGFR3, CD8 Hinge transmembrane protein and CD28-CD3z T cell signaling domains; transgene expression and integration was allowed by retroviral LTR sequences. The construct was then digested with EcoR1 and Not1 for verification on an analytical gel. Finally, the resulting construct was sequenced and DNA concentration was measured. It was designated pMSGV-scVEGFR3-28z.

**Recombinant retrovirus production.** High-titer replication-defective retroviral vectors were produced upon transfection of Phoenix Ecotropic cells and concentrated. Phoenix Ecotropic cells were seeded at 10 × 10^6 per T-150 tissue culture flask 24 hours prior transfection. Cells were transfected with 14.4μg ecotropic envelope protein plasmid and 21.4 μg of pMSGV transfer plasmid using Turbofect. The viral supernatant was harvested at 24, 48 and 72 hours post-transfection. Viral particles were concentrated and resuspended in 0.4 ml by ultracentrifugation for 2 hours at 24,000 rpm with a Beckman SW28 rotor (Beckman Coulter, Fullerton, CA).

**Mouse T cell transduction.** Primary mouse T cells, were isolated from the spleens of wild-type C57BL/6 using magnetic cell sorting (EasySep™ Mouse T Cell Isolation Kit). T cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 1x Glutamax, 1mM Pyruvate, 50μM BME and 1x non-essential Amino acids. Mouse T cells were stimulated with anti-CD3 and anti-CD28 mAbs coated beads (Invitrogen, Carlsbad, CA) at a bead:cell ratio of 3:1. Recombinant human IL-2 was added at 50IU/ml. Twelve to twenty-four hours after activation, T cells were transduced with retrovirus. Retrovirus was first added to a well of a RetroNectin-coated 24-well plate and centrifugation was performed at 3000 rpm for 1.5 hours at 320C. Then retroviral supernatant was then removed and the mouse T cells were added and centrifuged for 10 minutes at 1200 rpm. A second round of retroviral transduction was performed 24 hours post 1st T cell transduction. Human recombinant interleukin-15 (IL-15) and interleukin-7 (IL-7) were added every other day (starting from day 3) to a 10 ng/ml final concentration. Cell density of 0.5–1 × 10^6 cells/ml was maintained. Rested engineered T cells were adjusted for identical transgene expression before functional assays.

**Generation of mVGFR3 overexpressing cell line.** Retroviral particles bearing the MSGV-mVEGFR3 recombinant plasmid were produced using Phoenix Ecotropic cells and PCL-Eco packaging envelope plasmid as described above. The mouse endothelial cell line MS1 was transduced with MSGV-mVEGFR3 to overexpress VEGFR3. Retrovirus was added to MS1 cultured cells with confluency of 30% followed by addition of protamine sulfate (10ug/ml final concentration) and centrifugation at 2000rpm for 15 minutes. The medium was changed the following day and VEGFR3 expression was assessed by flow cytometry day days later as described above.

**ELISA for IFN-γ.** The assay was performed using supernatants derived from 24h co-cultures of 1x10^5 mouse CAR T cells with 1x10^5 mouse endothelial cells (MS1, MS1-MVEGFR3, bEnd3, H5V), human endothelial cells (HUVEC) or mouse ovarian cancer cells (A2780). The co-culture were performed in duplicate wells in a final volume of 200 μl of T cell media. IFN-γ was also measured in the supernatants of T cells exposed to immobilized recombinant human or mouse VEGFR3. IFN-γ concentration was determined using an ELISA Kit, according to manufacturer's instructions (Biolegend, San Diego, CA)
3. RESULTS

The anti-VEGFR3 scFv utilized in our study was previously isolated from a human phage display library and demonstrated specific cross-reactive binding to both human and mouse recombinant VEGFR-3. To generate a second generation CAR specific for the VEGFR3 we cloned the anti-VEGFR3 scFv into a retroviral vector bearing the CD3ζ T cell signaling domain fused to the CD28 costimulatory moiety. The final construct (termed pMSGV-scVEGFR3-28z) comprises the anti-VEGFR3 scFv linked to a CD8α hinge and transmembrane region, followed by CD28 and CD3ζ signaling domains (Fig 1). Primary mouse T cells were efficiently transduced with CAR retroviral viruses with transduction efficiencies reproducibly in 72% for mouse VEGFR3 soluble antigen and 73% for human antigen (Fig 2). A negative control was performed using T cells that were not exposed to retrovirus (untransduced T cells). Figure 2 illustrates the frequency of primary mouse T cells that were successfully transduced (dots in the smaller squares) and exhibited strong and specific binding to both mouse and human soluble antigens (mVEGFR3 and hVEGFR3).

Figure 1. Map of the pMSGV vector encoding a second generation anti-VEGF3 CAR.
Figure 2. Retrovirus transduction efficiencies of primary murine T cells with the VEGF3 CAR as measured by binding to recombinant murine VEGFR3-Fc (top right plot) and human VEGFR3-Fc (bottom right plot) as detected by flow cytometric analysis. No binding was observed for untransduced T cells (top and bottom left plots) to either of the recombinant proteins.

Similar results were obtained upon lentiviral transduction of primary human T cells using the pRRL-scVEGFR3-28z transfer plasmid (Figure 3). In brief, anti-VEGFR3 CAR expression was detected on genetically engineered human T cells via recombinant mouse or human protein staining upon lentiviral transduction. Again, untransduced T cells were utilized as negative control for the CAR expression analysis. Figure 4 demonstrates that human T cells were efficiently transduced with lentiviral vectors and were capable of binding mouse VEGFR3 at a frequency of 75 % and human VEGFR3 at a frequency of 48%.

Figure 3. Map of the pRRL lentiviral vector encoding a second generation anti-VEGF3 CAR.
Figure 4. Lentivirus transduction efficiencies for primary human T cells with the VEGF3 CAR as measured by binding to recombinant murine VEGFR3-Fc (top right plot) and human VEGFR3-Fc (bottom right plot) detected by flow cytometric analysis using detection by fluorescenated anti-Fc Ab. No binding was observed for untransduced T cells (top and bottom left plots) to either of the recombinant proteins.

To assess the functional capacity of the CAR-transduced T cells we first tested whether immobilized mouse or human VEGFR3 protein stimulation induces VEGFR3 CAR-transduced T cell activation in vitro. For this purpose, VEGFR3-specific CAR T cells were incubated with different protein concentrations of plate-bound mouse or human VEGFR3. The ELISA assays demonstrated that transduced T cells exert a robust activation in response to both recombinant mouse and human VEGFR3 and secreted high levels of mouse IFN-γ in a dose-dependent manner (Fig 5). IFN-γ was absent in mouse T cells that were culture in medium without immobilized protein highlighting the specificity of anti-VEGFR3 CAR T cells against their cognate antigen.

Figure 5. IFN-γ production of murine anti-VEGR3 CAR T-cells activated with decreased concentration of recombinant murine VEGFR3 (in blue) and recombinant human VEGFR3 (red).
In order to evaluate the ability of scVEGFR3-28z mouse CAR T cells to respond to antigen expressed on the surface of endothelial cells, the expression of mouse VEGFR3 was assessed for a panel of mouse endothelial cell lines by flow cytometry. We observed that the H5V (an endothelial polyoma middle T antigen transformed cell line) and bEnd3, had the highest expression levels, while a low amount of VEGFR3 was detected on MS1 cells (a mouse neuroendocrine skin carcinoma cell line) (Fig 6). 2H11, an endothelial SV40 transformed cell line, had almost no VEGFR3 expression.

**Figure 6.** Evaluation of VEGFR3 expression by MS1, 2H11 bEnd3 and H5V cells as measured by flow cytometric analysis followed by staining with mVEGFR3-Fc + fluorescenated anti-Fc Ab.

ELISA was performed to assess the functionality of anti-VEGFR3 CAR T cells in response to mouse or human cell surface-expressed VEGFR3. In parallel co-cultures of CAR T cells with immobilized mouse or human VEGFR3 proteins were set-up (similar as above) and served as a positive control for our functional assays. We again observed a strong and specific secretion of IFN-γ upon stimulation of CAR T cells with plate-bound protein (Fig 7). However, no IFN-γ secretion was observed upon stimulation with mouse endothelial cells expressing high levels of VEGFR3 (H5V, bEnd3) or low levels of VEGFR2 (MS1). We further gene-engineered MS1 cells to express high levels of VEGFR3 (the retroviral vector pQGXIP encoding the protein is shown in Fig 8). No IFN-γ was observed against human endothelial cells (HUVEC) or human ovarian cancer cells (A2780) known to express VEGFR3 but approximately 1000 pg/ml of IFN-γ was secreted by CAR T cells upon co-culture with the genetically engineered VEGFR3-MS1 cells (Fig 7). In summary, Figure 7 demonstrates that VEGFR3-specific T cells exert high reactivity against immobilized protein, low reactivity against genetically engineered endothelial cells expressing mouse VEGFR3, and no reactivity against mouse or human endothelial cells naturally expressing mouse or human VEGFR3
Figure 7. IFN-γ is produced for VEGFR3 CAR T cells stimulated with recombinant human and mouse VEGFR3 (left). No IFN-γ could be detected for VEGFR3 CAR T cells co-cultured with cell lines naturally expressing VEGFR3 (right), only in the case of MS1 endothelial cells that had been gene-engineered to overexpress VEGFR3.

Figure 8. Retroviral vector pQGXIP encoding recombinant VEGFR3 and used to gene-engineer MS1 cells.

The low level of binding of our CAR T cells with endothelial cells naturally expressing VEGFR3 could be due to the poor display or accessibility of the antigen’s epitope on the targeted cells. To test our hypothesis, three supplementary third generation CAR’s destined to lentiviral transduction were constructed. Three IgG4 hinge-based domains of different lengths were designed in order to increase the total length and flexibility of the extracellular subunit. The first construct contains a simple Ig4 hinge unit and was designated scVEGFR3-IgG4-TM-28BBz (Fig 9A). The second CAR’s hinge domain contains the Ig4 hinge associated with an antibody-derived CH3 subunit. This construct was designated scVEGFR-IgG4-CH3-TM-28BBz (Fig 9B). The third construct designated scVEGFR3-IgG4-CH2/3-TM-28BBz is similar to the second but contains an additional antigen-derived CH2 subunit (Fig 9C). All three CAR’s were successfully generated using molecular cloning. Lentivirus production and T cell transduction has been completed. Functional immuno-assays are underway.
Figure 9. Lentiviral vectors encoding the VEGFR3 CAR varying in linker composition. The first CAR comprises a short IgG4 hinge only (A), the second one is medium in length comprising the IgG4 hinge linked to the Ig CH3 domain (B), and the final one is a long linker made up of the IgG4 hinge fused to CH2 and CH3 (C).
4. DISCUSSION

There is strong evidence that tumor infiltration by T lymphocytes is associated with good patient prognosis in several types of cancer including colorectal, bladder, ovarian, breast and melanoma\textsuperscript{119} thus indicating great potential for ACI in the treatment of cancer patients. CAR T cell therapy is a particularly interesting form of ACI because of its high specificity, the possibility of targeting antigens of various natures, and its ability to bypass some mechanisms that tumors employ to escape T cell attack such as of MHC downregulation or deregulation of the antigen processing machinery.\textsuperscript{120,121} Furthermore, co-engineered CAR T cells may offer potential benefits such as active trafficking into tumor sites, \textit{in vivo} expansion and long-term persistence.\textsuperscript{122} Because blood supply is essential for tumor growth, survival, metastasis,\textsuperscript{123} and since pro-angiogenic elements are over-expressed in tumor vasculature,\textsuperscript{124} there is great interest in developing vascular-specific CAR T cell therapy approaches.

The aim of this project was to design and develop CAR T cells specifically targeting VEGFR3, a pro-angiogenic receptor that can be found mostly on lymphatic endothelium, but whose expression is largely induced on vascular endothelium in the context of tumorigenesis.\textsuperscript{125} We were able to build plasmid constructs containing 2nd generation VEGFR3-specific CAR transgenes and produce viruses with them (plasmid pRRL-scVEGFR3-28z for lentiviral transduction in human T cells, pMSGV-scVEGFR3-28z for retroviral transduction in mouse T cells). Then we successfully transduced primary human and mouse T cells with the lentiviral and retroviral vectors respectively; this was demonstrated by a strong binding of soluble VEGFR3 antigen on both human and mouse transduced CAR T cells. ELISA assays showed robust recognition of immobilized VEGFR3 antigen and massive IFN-$\gamma$ production by transduced human and mouse T cells in a dose dependent manner. We further screened a panel of endothelial cells for the expression of VEGFR3 and identified endothelial lines that express differential levels of VEGFR3 and endothelial lines that lack VEGFR3 and can be utilized as negative controls in our functional assays. However, no reactivity of transduced T cells was observed towards mouse or human endothelial cells naturally expressing the VEGFR3, whereas low reactivity was observed towards genetically-engineered endothelial cells artificially overexpressing the VEGFR3. To hopefully overcome this issue, we developed three new plasmid vectors containing hinge domains of various lengths (short, medium and long), with the hypothesis that modest epitope access, and insufficient length and lack of flexibility of the CAR are responsible the absence of reactivity. These constructs have been built, virus produce and primary human T cells successfully engineered. Immuno-assays are planned. According to those results, we will follow the same framework for engineering mouse T cells.

There are several advantages in targeting the vasculature of tumors. Unlike specific tumor antigens, vascular antigens like the VEGFR family are over-expressed in numerous types of cancer, which offers the possibility of targeting a broad range of solid tumors regardless of their histology and immunogenicity.\textsuperscript{126,127} It is also interesting to note that the endothelium which is targeted by vascular-specific CAR T cells consists of genetically stable non-malignant cells. Therefore, unlike tumor cells, tumor endothelial cells are less likely to mutate into drug-resistant or antigen-loss variants.\textsuperscript{128} This encourages to expect more durable responses from this vascular approach, compared to tumor-targeting CAR T cell therapies. Moreover, targeting the endothelium of tumor vessels facilitates the access of effector T cells to the area of interest, avoiding difficulties related to deep infiltration into the tumor bed.\textsuperscript{129} Finally, angiogenic markers are either not expressed or expressed at low levels in healthy vessels, and therefore vascular disruption is anticipated to have minimal levels of "on-target/off-tumor" toxicities.\textsuperscript{130,131}

Although our transduced VEGFR3-specific CAR T cells showed strong binding to either soluble or immobilized VEGFR3, they demonstrated absence or poor reactivity to the antigen once expressed on the surface of endothelial cells. This implies that despite the fact that the anti-VEGFR3 scFv exhibits strong binding to the human or mouse recombinant VEGFR3, modified anti-VEGFR3 CAR T cells are perhaps unable
to reach its epitope correctly in the context of cell-to-cell interaction. There is evidence that optimal T cell activation depends on the length of the spacer (hinge domain) and the distance of the epitope from the target cell membrane.\textsuperscript{132} Studies have been conducted in order to better understand the criteria that determine suitable hinge candidates. For example, \textit{Guest and colleagues} (2005)\textsuperscript{133} compared the function of CAR T cells expressing scFVs specific to CEA (carcinoembryonic antigen), NCAM (neural small adhesion molecule), 5TA and CD19, on which an IgG1 derived CH2-CH3 Hinge was added. Whereas 5TA- and CD19-specific CAR T cells showed a better effector function with the CH2-CH3 Hinge subunit, CEA- and NCAM-specific CAR T cells had optimal activity without the spacer. Research on length or presence of the hinge demonstrates conflicting data so far,\textsuperscript{134} however it clearly highlights the importance of the hinge region in defining the effector activity of CAR T cells. It is therefore crucial to test different hinge/scFv combinations in an empirical manner.\textsuperscript{135} Consequently in this project, we have already started to work in this direction.

The goal of T cell transduction is to obtain constitutive expression of the CAR on the cell’s surface. To do so, the most commonly used vectors are \(\gamma\)-retroviruses or lentiviruses, which are both members of the \textit{Retroviridae} family.\textsuperscript{136} Those vectors are lacking key genes for replication (\textit{gag}, \textit{pol}, \textit{env}), meaning that despite being able to penetrate host cells, integrate their genome and use the cell’s nuclear machinery for transgene expression, they do not have the genetic material to generate new viral entities after integration.\textsuperscript{137} Practically, \(\gamma\)-retroviruses appear to be more potent transducers for murine T cells, as described in \textit{Kerkar and colleagues’} (2011)\textsuperscript{138} study, in which MSGV retroviral transduction of murine naïve/memory-stem cells and central memory CD8\(^+\) cells was shown more efficient compared to lentiviral transduction. However, \(\gamma\)-retroviruses have a consequent drawback since they cannot ensure proper integration of their transgene into the genome of a host cell that is non-proliferating.\textsuperscript{139,140} In fact, \(\gamma\)-retroviruses get internalized into the cell’s cytoplasm where they operate reverse transcription. Then the nuclear membrane of the cell needs to be broken during mitosis for the preintegration complex to access the genome.\textsuperscript{141} Target cells such as blood lymphocytes are not actively cycling, thus they need to be activated in vitro prior to \(\gamma\)-retroviral transduction.\textsuperscript{142} On the contrary, HIV1-derived lentiviral vectors have shown the capability of transducing human T cells in a mitosis-independent manner.\textsuperscript{143,144} Moreover, lentiviruses demonstrate less propensity to integrate into promoter regions, which lowers the risk of insertional oncogenesis.\textsuperscript{145} Lentiviruses have empirically shown robust transgene delivery in human CD4\(^+\) and CD8\(^+\)\textsuperscript{146,147} whereas poor gene transfer has been observed in murine T cells.\textsuperscript{148} In sum, despite the lack of causal explanations, lentiviruses are generally utilized for human T cell transduction considering all their practical advantages, whereas \(\gamma\)-retroviruses remain the most reliable tool for murine T cell transduction.

VEGFR3 has a unique characteristic compared to VEGFR1 and 2 due to the fact that it is widely expressed on lymphatic vessels.\textsuperscript{149} It is well documented that tumors over-expressing VEGFR3 have enhanced lymphatic sprouting and are thus more likely to develop regional lymph node metastasis.\textsuperscript{150,151} This offers a potential new approach in the treatment of tumors, by targeting their lymphatic network. Some investigators have already started to explore this direction. For example, \textit{Lin and colleagues} (2005)\textsuperscript{152} were able to inhibit tumor-derived VEGFR-C (a ligand of VEGFR3) with an antibody-derived VEGFR3 decoy receptor, which blocked tumor lymphangiogenesis and metastasis in mice bearing human melanoma and prostate cancer. \textit{Laakonen and colleagues} (2007)\textsuperscript{153} demonstrated potent inhibition of tumor growth in mice bearing human large-cell lung carcinoma with the use of a VEGFR3-specific monoclonal antibody that blocked the formation of tumor-associated lymphatic vessels (without affecting pre-existing lymphatic and blood vessels). Furthermore, the study demonstrated that the anti-VEGFR3-treated tumors contained significantly less blood vessels than the control antibody-treated tumors. Since VEGFR3 is also over-expressed in tumor blood vessels\textsuperscript{154,155} development and evaluation of VEGFR3-specific CAR T cell therapy is very promising because it can counteract both tumor lymphangiogenesis and angiogenesis. This double benefit could not be so firmly expected from the other VEGFR approaches. Besides, it is interesting to note that the lymph-restricted
expression of VEGFR3 could be a valuable protective factor against toxicities on normal blood vessels, although the expression of the other VEGFRs also appears to be lower in healthy blood vessels.

In conclusion, although there is no data yet published related to VEGFR3-specific CAR T cell therapy, the encouraging preclinical results with the VEGFR2 approach\textsuperscript{156,157} generated high enthusiasm for our project. We have achieved proper human and mouse T cell transduction, and robust immobilized antigen recognition and T cell activation. Future goals aim to transduce T cells with optimal CAR constructs which will render the engineer T cells capable of recognizing and reacting against endothelial cell line naturally expressing VEGFR-3. To achieve the latter, we will assess human transduced-T cell reactivity with the use of three new CAR constructs that we managed to build already, and which contain antibody-derived hinge spacers of different lengths. Depending on the outcome, we will use the same linkers for mouse T cell engineering as well. Hopefully we will be able to select one of the resulting CARs as the best fitted for the next step which is the assessment of functional activity of CAR T cells \textit{in vivo}. We could further design and develop dual CAR-transduced T cells containing both anti-VEGFR2 and anti-VEGFR3 CARs. This future aim is based on Tammela and colleagues (2008)\textsuperscript{158} study which postulates that the combined blockade of VEGFR2 and VEGFR3 using monoclonal antibodies has additive anti-angiogenic and anti-tumor effects on tumor-bearing mice. On the other hand, in case we observe unexpected "on-target off-tumor toxicity" in our mouse preclinical models we can employ a trans-signaling CAR strategy,\textsuperscript{159} whereby T-cell activation signal 1 (CD3\zeta) can be physically dissociated from costimulatory signal 2 (CD28) in two CARs of differing endothelial antigen specificity: VEGFR-3 and VEGFR-2. Ultimately, the field of vascular-specific CAR T cell therapy is still wide open for research and discoveries.
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