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Development of innovative CAR molecules to be transduced in Cytokine Induced Killer cells for the treatment of different neoplasia

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CHAPTER 1:

GENERAL INTRODUCTION

Immunotherapies for hematological malignancies

Over the past decade, the advent of a range of novel pharmacologic and cellular strategies with the capacity to deliver specific immunologic antitumor responses has transformed the management of hematologic malignancies. Immune-based therapies for hematological malignancies aim at generating new agents, such as monoclonal antibodies, engineered monoclonal antibodies called bispecific T-cell engagers, cell therapies involving cells of the innate and adoptive immune system, adoptive cell transfer therapy with T cells engineered to express chimeric antigen receptors or T cell receptors, immune cell redirection strategies, vaccines and checkpoint inhibitors. However, immunotherapy is an emerging field and on-going research will have to address several issues to improve safety and efficacy of this novel treatment modality. There is also the need to extend the application to other malignancies.

Monoclonal and bispecific Antibodies

Monoclonal antibodies (MAbs) are produced by B cells and specifically target antigens. The hybridoma technique introduced by Köhler and Milstein in 1975 has made it possible to obtain pure MAbs in large amounts, greatly enhancing the basic research and potential for their clinical use.[1] Most anti-cancer MAbs approved to date have been shown to work, at least in part, through activation of innate immunity by the Fc region of the antibody. In particular, they mediate antibody-dependent cellular cytotoxicity (ADCC) by Natural Killer (NK) cells, as well as antibody-dependent cellular phagocytosis (ADCP) by macrophages. Many anti-tumor MAbs that have been

tested in preclinical studies or early phase clinical studies have shown poor efficacy and/or toxicity, mainly due to low expression on tumor tissue or poor relative expression on tumor versus normal tissues.[2] This has led to the design of alternative formats, such as MAbs conjugated to radioactive payloads or to potent chemotherapeutic drugs (antibody-drug conjugates, ADC) and Bispecific Antibodies (BsAbs).

BsAbs are second generation antibodies for therapeutic application in immunotherapy. An important class of BsAb is the “bi-specific T cell engagers (BiTE)” and consists in the recruitment of immune effector cells by combining an anti-CD3 or anti-Fc-gamma receptor (FcγR) binding domain with an anti-tumor binding domain. These BsAbs activate T or other immune cells, respectively, after tumor target recognition, and bring these effector cells close to the tumor target so that the latter are efficiently killed.

There are two main formats of BsAbs, the immunoglobulin G (IgG)-like molecules and the non-IgG-like molecules (Figure 1). IgG-like BsAbs carry a functional or mutated Fc linked to variable fragment antigen binding (Fab) or single chain fragment variable (scFv) domains carrying the two different specificities. This class of molecules offers Fc mediated effector functions, such as ADCC, complement-dependent cytotoxicity (CDC), and ADCP, and retain physical properties associated with the Fc, including serum stability. Non IgG-like molecules are based on the sole scFv or Fab portions of two antibodies linked together, usually by peptide sequences, and lack Fc. The advantage of non-IgG-like BsAbs is their smaller size which enables enhanced tissue penetration and reduced nonspecific

activation of innate immune cells due to lack of Fc region; however, short half-life is an issue owing to their smaller size and the potentially greater immunogenicity given by non-natural peptide linker sequences.[3,4] Approved BiTEs include the tandem single chain variable fragment BiTE antibody blinatumomab (CD3×CD19) for refractory Philadelphia chromosome-negative B cells acute lymphoblastic leukemia (B-ALL).[5,6]

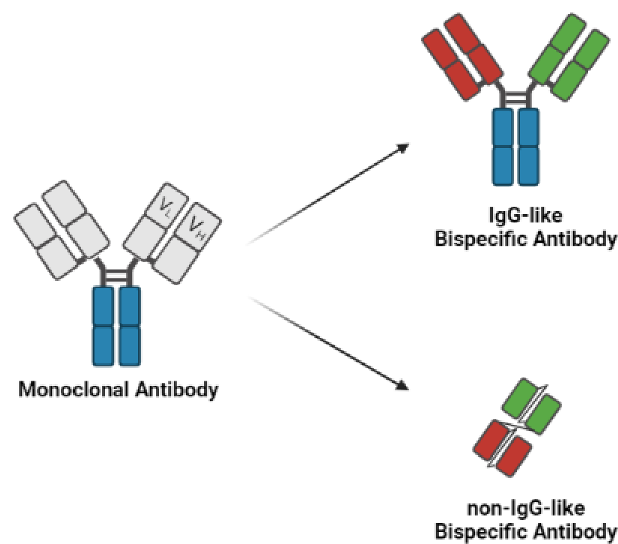

Figure 1 –MAb and BsAbs structures. Examples of one IgG-like, containing the Fc region, and one non-IgG like, lacking the Fc region, bispecific antibodies structure. (Image created with Biorender)

The drug blinatumomab (Amgen) is the first representative of BiTEs authorized for use in the US. The BsAb blinatumomab molecule is constituted by two scFv, the first antigen-binding site is directed against the CD19 protein on the surface of B lymphocytes, the second against the CD3 receptor on the surface of cytotoxic T lymphocytes. Once blinatumomab binds to its targets a cytolytic synapse is formed and cytotoxicity is induced by the release of perforin and granzymes from granules in the cytotoxic T cell, inducing apoptosis and lysis of

the malignant B cell. Cross-linking CD19$^+$ tumor cells and CD3$^+$ T cells by blinatumomab also promotes T cell proliferation and increases the secretion of inflammatory cytokines, like interleukin (IL)-6, IL-10, interferon γ (IFNγ). Blinatumomab is administered intravenously as a continuous infusion, since it has a mean half-life of about 2 hours *in vivo*.[7,8]

Blinatumomab therapy in a Phase II clinical trial for adult patients with recurrent B-ALL leads to complete remission in 69% of patients. Since CD19$^+$ normal B cells are also affected, lymphopenia is the most frequent severe adverse event. Nonetheless , since a significant portion of patients with CD19$^+$ neoplasms do not respond or relapse early after Blinatumomab treatment, combination strategies are also being extensively investigated, including combination with other biological therapies or standard drugs.[9,10]

Cell therapies

- ***Chimeric Antigen Receptor T cells (CAR-T)***

Chimeric antigen receptor-modified T cell (CAR-T) are genetically engineered T cells that express a recombinant receptor conferring major histocompatibility complex (MHC)-independent specificity against a target antigen on the tumor cell surface. CAR-T cells are therefore insensitive to tumor escape mechanisms related to MHC loss. The CAR is introduced into the T cells through different possible mechanisms, such as viral vectors (gamma-retroviral and lentiviral vectors) or non-viral vectors (e.g. transposase-based system, such as Sleeping Beauty and PiggyBac).[11,12] The production of CAR-T cells typically involves cell collection from the patient or from the

matching donor peripheral blood, respectively for autologous or allogenic production, followed by transgene delivery in T lymphocyte and *ex vivo* expansion.

The essential components of these synthetic receptors are an extracellular antigen-targeting moiety, such as a scFv, a hinge and transmembrane domain that anchors the receptor on the cell surface and projects the scFv out to the extracellular space, and intracellular signaling domains that are triggered on antigen engagement. The scFv confers the antigen specificity of a CAR, it contains only the MAb variable regions light and heavy chain fused via a flexible linker. The flexible hinge domain is a short peptide fragment that provides conformational freedom to facilitate binding to the target antigen on the tumor cell. It may be used alone or in conjunction with a spacer domain that projects the scFv away from the T-cell surface. Based on the characteristics of the intracellular moiety, four different generations of CARs can be distinguished. First-generation CARs relied solely on the intracellular domain of CD3ζ for signaling, but they had limited efficacy in clinical trials. Incorporation of one or two co-stimulatory domains, respectively second and third generations CARs, to provide additional activating signals has resulted in enhanced clinical responses and persistence. Fourth generation CARs contain additional elements including cytokines or chemokine to increase immune activation, a second CAR to enhance specificity and efficacy, or suicide genes to ablate CAR-T cells after once they are not anymore needed (Figure 2). [13,14]

Recent clinical trials have highlighted the need for developing control programs to modulate the activity of CARs in presence of toxicities,

such as the introduction of a suicide gene or combinatorial strategies requiring multiple Tumor Associated Antigens (TAAs) to be engaged for full T cell activation. [15]

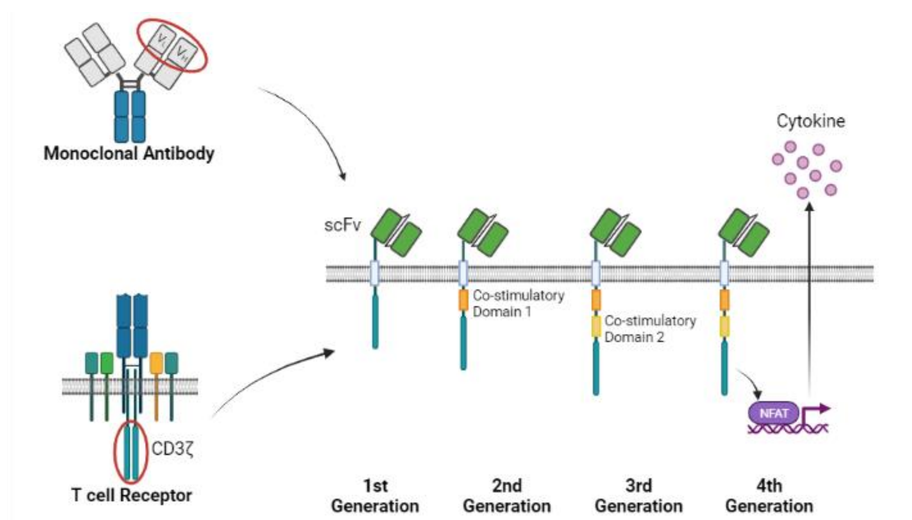

Figure 2 – CAR-T cells generations. Structure of a MAb and the T cell Receptor complex, the scFv and the CD3ζ together give rise to the first generation of CAR-T cells. The introduction of one or two co-stimulatory domains give rise to the second and third generation. Example of a fourth generation CAR that synthetizes and releases a specific cytokine upon antigen binding. (Image created with Biorender)

A great number of different CAR-T cell have been developed in different pathological contexts, like B cell malignancies, neuroblastoma and sarcoma. In the context of B cell malignancies CD19-directed CAR-T cell have given the most promising results. In this regard, CD19-directed CAR-T cell treatment of patients resulted in persistence of immunological memory, trafficking to the tumor sites, and anti-tumor activity, which led to tumor regression and, in most of the patients, complete remission. Since 2017, six CAR-T cell therapies have been approved by the Food and Drug Administration (FDA) and by European Medicines Agency (EMA), among which 4 anti-CD19 and 2 anti-BCMA CAR-T cells. All have been approved

for the treatment of hematologic malignancies, including relapsed or refractory B-ALL, B-cell Non Hodgkin Lymphoma (B-NHL), follicular lymphoma (FL), mantle cell lymphoma (MCL) and multiple myeloma (MM).[16–18]

Despite this early success, major challenges to the broad application of CAR-T cells as cancer therapies remain, including treatment-associated toxicities and cancer relapse. Although the safety levels of CAR-T therapy are generally acceptable, several fatal outcomes due to severe cytotoxicity have been reported in clinical trials of CAR-T therapies. Cytokine release syndrome (CRS) is the most common adverse event and is correlated to the expansion and activation of CAR-T cells, which leads to a massive over-production of cytokines that result in an elevated systemic inflammatory response.[19] Lack of response or early relapses are still a common problem of present day CAR-T therapy.

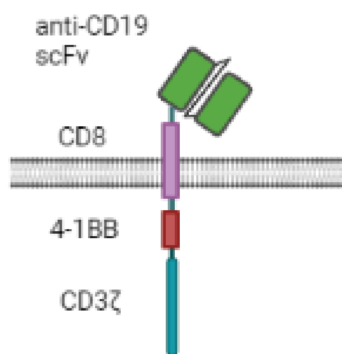

Figure 3 - Anti-CD19 CAR schematic structure. (Image created with Biorender)

The first FDA approved CAR molecule is Tisagenlecleucel, sponsored and designed by Novartis Pharmaceuticals.[20] This second-generation CAR is composed by the anti-CD19 scFv from clone FMC63, the spacer and the transmembrane domains are derived from CD8, and the intracellular moiety is constituted by 4-1BB (CD137) and CD3ζ (Figure 3). Tisagenlecleucel are generated from autologous cells, in which T cells are enriched and activated by addition of anti-CD3/CD28–coated paramagnetic beads, depleting

leukemic cells. T cells are transduced by the use of a lentiviral vector and the expansion lasts for around 10 days.[21]

A phase 2 global study of Tisagenlecleucel in pediatric and young adult patients with CD19^{+} relapsed or refractory B-ALL has been conducted and it demonstrated durable remission with long-term persistence after a single infusion of Tisagenlecleucel. The overall remission rate within 3 months was 81%, persistence of CAR-T cells in the blood was observed for as long as 20 months and modest to severe adverse events occurred in 73% of patients.[16] High response rates have also been observed among adult patients with relapsed or refractory diffuse large cell lymphoma (DLBCL) in an international, phase 2, pivotal study: 40% of the patients had complete responses and grade 3 or 4 adverse events included CRS in 22% of the patients.[17] CAR-T cells were infused also into patients with relapsed/refractory chronic lymphocytic leukemia (CLL), the overall response rate of the study was 57%, with CAR-T cells persistence beyond 4 years.[22]

- ***Cytokine Induced Killer (CIK) cells***

Cytokine Induced Killer cells (CIK) are CD3^{+}CD56^{+} double positive T cells that have acquired phenotypic markers of NK cells and show MHC-unrestricted cytotoxicity toward neoplastic but not normal targets.

The experimental protocol to expand and enhance the cytotoxicity of effector cells directed against a B-NHL cell line was proposed for the first time by Wolf et al..[23] CIK cells are therefore *ex vivo* activated lymphocytes that can be obtained from either human peripheral blood (PB), bone marrow (BM) or cord blood (CB) mononuclear cells in 15

to 21 days of culture by the sequential addition of IFN-γ, anti-CD3 antibody (OKT3), and high doses of recombinant human IL-2.[24,25] CIK cells represent a heterogeneous cell population, including a large majority of $CD3^+CD56^+$ cells and minor fractions of typical T cells ($CD3^+CD56^-$) and NK cells ($CD3^-CD56^+$). CIK cells are characterized by a dual function, acting both as $CD8^+$ specific effector T and NK-like cells. The T cell receptor (TCR) on CIK cell is polyclonal and with a similar proportion of αβ chains compared to γδ, like peripheral blood cells.[26] Except for NKG2D and CD56, which are non-specific NK-cell markers, CIK cells do not significantly express NK-cell markers, such as KIR receptors, CD16 and NKG2A. CIK cells only express NKp44 and NKp30, but to rather low levels (10-20%) compared to NK cells. Moreover, gene expression microarray analysis has revealed that CIK cells share a larger number of genes with T cells present at the end of culture rather than with NK cells. CIK cells can lyse a broad array of tumor targets, including acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), and CLL by a non–MHC-restricted, NK–like mechanism. Moreover, their dual effector function (TCR and non-TCR mediated) has been demonstrated by showing that anti-cytomegalovirus (CMV) specific CIK cells expand and are functional during standard CIK cultures. These mediate both specific MHC-restricted recognition of CMV antigens and TCR-independent NK-like cytolytic activity against leukemic cell lines or fresh leukemic blasts.[27,28] These data suggest that CIK cells, when used in a clinical setting, may control both neoplastic relapses and viral infections, two frequently associated complications in patients who received a transplant.

The analysis of different *in vivo* models has demonstrated that CIK cells have an important anti-tumor activity. Their activity has been confirmed against different hematologic tumor models, among which the B lymphoma SU-DHL-4 cell line, autologous CML $CD34^+$ blasts and the Bcl-1 leukemia cell line.[29,30] At the same time, it was evident that CIK cells had minimal cytolytic activity against normal syngeneic BM, while, anti-tumoral activity have been described also for other tumors, such as hepatocellular carcinomas, undifferentiated stomach adenocarcinoma and melanomas.[31] These experiments have also demonstrated the tumor-specific homing *in vivo*. Most interestingly, since from early studies, CIK cells appeared to be endowed with a reduced alloreactive potential. In *in vivo* models very little, if any, graft versus host disease (GvHD) was induced by CIK cells. Indeed, specific *in vivo* studies, using murine GvHD models, convincingly evidenced the lack of GvHD activity of CIK cells, in spite of their intense proliferating capacity.[32]

These data have been confirmed in different clinical trials evaluating the safety and activity of CIK cells infusions for different hematological neoplasms and solid tumors. CIK-based clinical studies indicate that CIK cells, whether used in autologous or allogeneic combinations, show a modest but reproducible anti tumoral activity against most types of cancer, with particular emphasis for the hematologic neoplasia.[33–35] Moreover, it is also clear that, at the doses and schedules that have been studied so far, CIK cells have shown very minor toxicity and, even in the extreme genetic disparity as the haploidentical donor, have almost never shown severe GvHD related

toxicity exceeding grade II and a standard treatment by steroids has resulted in the disappearance of such symptomatology.[36,37]

- ***How to increase CIK cells specificity and efficacy***

CIK cells have been used in several clinical trials to treat patients with cancer and have shown therapeutic activity with limited toxicity. Nonetheless the wider use of CIK cells as a therapeutic agent in cancer treatment still needs to overcome several hurdles, in particular CIK cells alone have shown so far therapeutic activity mostly in a minimal residual disease or low tumor burden context, so that strategies feasible in the clinic to increase the therapeutic efficacy of CIK cells are still required.

  - ***CIK cells combined with BiTEs***

Golay et al. and other groups have developed the idea that BsAbs could be used to redirect CIK effector cells toward tumor targets, rendering these cells more effective and specific *in vitro* and *in vivo* in animal models.[38–41] In *in vivo* models CIK cells could control the growth of the aggressive patient derived (PDX) model of Philadelphia-positive B-ALL in NOD-SCID mice when redirected to the tumor by the BsAb Blinatumomab. Interestingly, no GvHD could be noted in this model, as expected also from the literature, confirming that little GvHD is induced by allogeneic CIK cells.[42]

  - ***CIK cells modified with CAR***

The Papa Giovanni XXIII hospital in Bergamo has participated to a single arm, open-label, multi-center, dose escalation phase I/IIa study (Protocol number FT01CARCIK; Eudract number 2017-000900-38; ClinicalTrials.gov Identifier NCT03389035) that used CAR-modified

CIK cells. The trial was sponsored by the Fondazione Matilde Tettamanti Menotti De Marchi Onlus, the principal investigators are prof. Andrea Biondi and prof. Alessandro Rambaldi. Cells were produced in two facilities, the “S. Verri” cell factory in Monza, and the “G. Lanzani” cell factory in Bergamo. The aim of this study was to determine the feasibility and safety of a single dose of allogeneic donor derived CIK cells transduced with a transposon CD19 CAR gene. Adult and pediatric patients with relapsed or refractory B-ALL after hematopoietic stem cell transplantation were enrolled. The primary end points of the trial were the determination of the maximum tolerated dose and/or the recommended Phase II dose and the safety of CARCIK-CD19. Early results of the phase I/II trial have reported complete remission 28 days after treatment in six out of 7 patients receiving the highest doses achieved. Toxicities reported were 2 grade I and 1 grade II CRS cases at the highest dose in the absence of GvHD, neurotoxicity, or dose-limiting toxicities. Robust expansion was achieved in the majority of the patients and CARCIK cells were detectable up to 10 months.[43] The success of the trial has led to the opening of the phase II trial that allows a second CARCIK-CD19 cells infusion, based on the disease status from one month after the first infusion, in adult and pediatric patients with B-ALL (Protocol number FT03CARCIK; Eudract number 2020-005025-85; ClinicalTrials.gov Identifier NCT05252403).

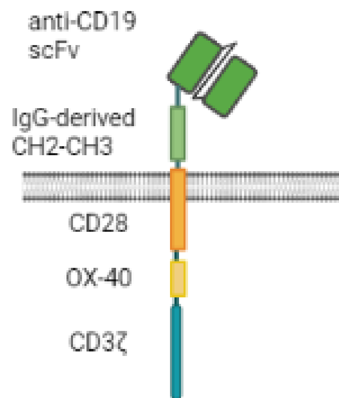

Figure 4 - Anti-CD19 CAR schematic structure. (Image created with Biorender)

Magnani et al. have developed this novel clinical-grade protocol to produce CIK cells transduced via Sleeping Beauty (SB) transposon system. CARCIK-CD19 is an adoptive immunotherapy product consisting of allogeneic T lymphocytes differentiated according to the CIK protocol and engineered with non-viral method to express a third generation CAR against CD19, an antigen present on B-ALL. The CAR is composed of the scFv from the anti-CD19 monoclonal antibody FMC63. The recognition domain is bound to the trans-membrane domain by a spacer carrying the hinge and the CH2-CH3 domains of human IgG1. The intracellular moiety is a third generation signaling tail constituted by CD28, OX40 and TCRζ signaling modules (Figure 4). Cells are transduced using the SB transposon system, which allows the insertion of a synthetic DNA sequence in the genomic DNA through the activity of the transposase enzyme. The vector contains a constitutive promoter upstream of CAR sequence and the whole sequence is flanked by the two Terminal Inverted Repeats (TIRs) allowing genomic insertion by the SB transposase. The second plasmid coding for the SB11 transposase is co-transfected with the CAR plasmid by electroporation. Cells are cultured according to CIK cell differentiation protocol for about 21 days. At the end of the culture cells are frozen and the product tested for viability,

immunophenotype, sterility and other safety parameters. If the product is compliant, it can be released and infused in the recipient.[44,45]

Immunotherapies for solid tumors

Surgical resection, radiation and cytotoxic therapies have long formed the basis of cancer care, but it is not enough to control the disease. A major limitation of cytotoxic chemotherapy drugs is that they generally lack specificity and attack both normal and tumor cells, causing potentially severe side effects. Radiation therapy is often used in combination with chemotherapy or surgery because the use of radiation alone cannot cure most forms of cancer.

The success of biological therapies (antibodies and CAR-T cells) for hematopoietic neoplasms has given an impetus to transfer these technologies also for the treatment of solid cancers. Therapeutically attractive targets are, for example, the checkpoint inhibitor molecules, which include CTLA-4, PD-1, and its ligand, PD-L1. Currently, the majority of the BsAbs in pre-clinical trial are BiTEs, but immunologically "cold" tumors, with a negligible lymphocyte presence, may pose significant challenges for this class of drug. Also CAR-T cells are in pre-clinical and clinical trials for solid tumors, although the major obstacle is, as for MAbs, the necessity to find a target antigen specific for or sufficiently overexpressed by the tumor.[46] CARs have been developed against a variety of solid tumor surface antigens including mesothelin[47,48], carcinoembryonic antigen (CEA)[49], disialoganglioside (GD2)[50], interleukin-13 receptor α2 (IL-13Rα2)[51], mucin-1 (MUC1)[52], ephrin type-A receptor 2 (EphA2)[53], human epidermal growth factor receptor 2 (HER2)[54] and other growth factor receptors. Currently there are several ongoing clinical trials assessing the safety and efficacy of CAR-T immunotherapy in various solid malignancies. Perhaps the greatest challenge facing the

successful application of CAR-T technology to solid tumors in human patients is the selection of acceptable antigen targets. Enhancing CAR-T cytotoxicity against epitopes not restricted to malignant cells can induce toxicity against healthy tissues. On-target off-tumor toxicity has been observed in different clinical trials.[55] However immunotherapeutic approaches have proved less effective against solid tumors than against blood cancers for different reasons: 1) the difficulty to identify target antigens that are either significantly overexpressed in tumor cells or specific for these cells compared to normal tissues; 2) solid tumors are often immunologically silent (cold tumors), i.e. they are poorly infiltrated by immune cells that are required for the efficacy of the immunotherapy; 3) the solid tumor microenvironment (TME) is often immunosuppressive, i.e. molecules or cells that repress immunity are over-represented; 4) immunotherapeutic agents may experience difficulty in penetrating solid tumors which are quite compact and may not easily reach the tumor core. Expanding the impact of immunotherapy to include more solid tumor types will therefore require further advances that overcome critical barriers related to immunosuppression and targeted delivery, among others.[56]

Targeting the Tumor Microenvironment

The TME is a highly heterogeneous environment that includes cellular components like fibroblasts, endothelial cells, adipocytes, immune and inflammatory cells, and a non-cellular component termed the extracellular matrix (ECM). Tumor markers expressed in the ECM of the TME exemplify attractive molecules for the targeted delivery of

therapeutics. ECM components are good candidate targets because they are easily accessible, due to their low shedding profile, and due to their abundancy and stability.

The ECM is a three-dimensional network of macromolecules that provides structural and biochemical support to surrounding cells. The ECM is constituted of collagens, laminins, elastin and elastic fibers, glycoproteins and proteoglycans. The ECM also contains matricellular proteins such as thrombospondins, secreted protein acidic and rich in cysteine (SPARC), cartilage oligomeric matrix protein (COMP), tenascin C (TNC) and osteopontin. Matricellular proteins interact with other matrix components and growth factors as well as with cell surface receptors contributing to tissue specific functions. The ECM has a structural function, forming a scaffold in which cells are embedded, and regulates cells biological processes, such as survival, proliferation, differentiation, and migration. Every tissue has a unique ECM composition, ECM components being produced by resident cells based on the needs of the tissue. Interactions of cells with the ECM are mediated by their surface receptors, such as integrins, syndecans and discoidin domain receptors (DDRs). In solid tumors the barrier function of the ECM can physically block the immune response. Besides, ECM molecules can suppress the immune responses via direct engagement of ECM receptors on immune cells.[57]

Fibroblasts are the major producers of ECM in normal physiology and in tissue repair. During tissue repair different signals, such as TGF-β, direct fibroblasts to a pro-fibrotic phenotype, switching to ECM protein synthesis, or to myofibroblasts which participate in wound contraction. In a tumor context, Cancer Associated Fibroblasts (CAFs)

driven by cancer cells are responsible for ECM synthesis and remodeling. CAFs are a heterogenous population of cells which are characterized by increased proliferative capacity, elevated production of growth factors and ECM proteins and increased metabolic activity. Not only CAFs, but also some tumor cells themselves can synthesize components of the ECM such as collagen.[58] Fibroblast activation protein (FAP) is a membrane protease that is highly expressed on CAFs and its over-expression is associated to poor prognosis in various cancers. Two clinical trials have been started to assess the safety and efficacy of CAR-T cells directed against FAP. First results have not reported severe toxicities, further studies will give more information on the efficacy.[59]

Furthermore, CAFs induce migration, invasion and metastasis of cancer cells by producing target-specific proteases such as matrix metalloproteinases (MMPs). Several signals from the tumor environment, such as growth factors, hypoxia, extracellular pH and metabolism induce the synthesis of specific MMP essential for the degradation of ECM components to permit cell invasion.[60]

In the next paragraphs some ECM proteins will be described and following some therapies targeting these proteins will be presented.

- ***Collagen***

Collagens are the major proteins of the ECM. Collagen is formed from three polypeptide α chains, organized into a triple helical structure and are typically homo- or heterotrimers. In vertebrate animal organisms, 46 chains have been identified that can organize into 28 different collagen types. Collagen assembles into a three-helix structure at the

C-terminus to form procollagen, this process is accompanied by chaperone proteins like the heat shock protein 47. Hydroxylation and glycosylation in the endoplasmic reticulum are the two main modifications that occur after translation. Still in the endoplasmic reticulum procollagen is hydrolyzed to form collagen and is secreted. The effects of collagens on cell–matrix interactions are mediated by receptors like integrins, DDR, Glycoprotein VI and Lair1.
The collagen fibers in tumors are often straightened, as well as quantitatively and qualitatively reorganized. Linearly aligned collagen fibers are thought to generate migration highways that allow cancer cell invasion and dissemination. It was demonstrated that ECM deposited by CAFs was remarkably aligned in a parallel pattern, and CAFs promoted the dissemination of malignant cells in vivo. DDR-collagen signaling plays an important role in cancer progression and metastasis, activating pathways such as Notch and AKT. DDR1 function is to realign collagen fibers into a dense structural barrier that can block T cell infiltration. Collagen interaction with the inhibitory receptor Lair1 on T cells resulted in $CD8^+$ T cell exhaustion, as evidenced by the expression of PD1 and TIM3. In breast cancer, it has been demonstrated that the metastasis formation is preceded by an increased collagen production due to hypoxia and TGF-β. In colorectal carcinoma (CRC) patients, the increased collagen gene expression level is directly correlated to a worst overall survival.[61–64]

- ***Fibronectin***

Fibronectin (FN) is a high-molecular-weight glycoprotein that consists of two subunits, covalently linked by a pair of disulfide bonds at the

C-terminal (Figure 5). There are 20 isoforms of human FN, which can be divided in two main groups: the soluble dimer, that can be found in plasma, or insoluble protein, that is part of the ECM. FN has been shown to interact with integrins, collagen, TNC, fibrillin, glycosaminoglycans as well as with growth factors. A single gene encodes FN, but alternative splicing of pre-mRNA as well as post-translational modifications allow the formation of multiple isoforms.

Figure 5 - Fibronectin structure. From the N-terminus is represented the Fibronectin type I domains (FN I, green ovals), the fibronectin type II domains (FN II, orange ovals) and the fibronectin type III domains (yellow rectangles). The V domain (blue) is subjected to alternative splicing. (Popova e Jucker, 2022)

In cancer, CAFs secrete and assemble FN as parallel fibers mediating directional cancer cell migration. The role of FN in promoting growth, survival and invasion of cancer cells is exploited through the inhibition of mitochondrial dysfunction and caspase activity. [65,66]

In breast cancer, FN can promote the epithelial-mesenchymal transition via ERK/MAP kinase pathways. In Pancreatic Ductal Adenocarcinoma (PDAC), a highly metastatic disease, FN is one of the major components of the stroma, it supports metastatic spread, chemoresistance, and angiogenesis.[67]

- ***Periostin***

Periostin is a multi-modular protein composed of a signal peptide, necessary for secretion, a small cysteine-rich module (EMI domain) probably involved in the formation of multimers, four fasciclin-like domains (FAS1) that interact with integrins, and a hydrophilic C-terminal region known to interact with other ECM proteins such as

collagens, fibronectin, TNC, or heparin. Alternative splicing of the C-terminal sequence gives rise to four isoforms. These isoforms are differentially expressed during embryogenesis and bone development. Periostin is a key player in the regulation of cell behavior and organization of the ECM. Periostin has been shown to bind integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ in osteoblasts and several types of normal and cancer cells where it elicits activation of FAK, PI3-Kinase, and AKT signaling pathways.

Periostin expression is deregulated in several pathologies such as inflammation, tissue repair and malignant transformation. The deregulation of periostin expression in many cancers suggests that it plays an important role in cancer development and progression. Also, periostin is an important component of the pre-metastatic niche. High periostin expression is associated with poor survival in breast cancer, CRC and PDAC. In breast cancer, periostin increase cancer stem cells survival and facilitate metastasis to the lung.[68]

- ***Tenascin C***

TNC is a large homohexameric extracellular matrix protein with disulfide-linked subunits. This protein contains at the N-terminus 3 cysteine-rich heptad repeats which form a coiled-coil region, followed by epidermal growth factor (EGF)-like domains, a string of fibronectin-type III (FNIII) repeats and a fibrinogen-like globe. Following translation, TNC assembles intracellularly into a 6-armed hexabrachion structure comprised of 6 monomers (Figure 6). This process is largely determined by the N-terminal heptad repeats.

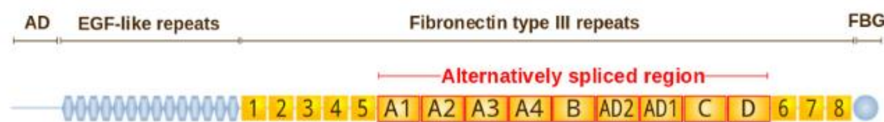

Figure 6 - Tenascin C structure. From the N-terminus is represented the coiled-coil domain (AD), the EGF-like repeats (light blue ovals), the fibronectin-like repeats (yellow rectangles) and the fibrinogen-like globe (FBG). (Popova e Jucker, 2022)

A striking feature of TNC is its tightly controlled gene expression pattern. Regulation of the TNC promoter is influenced by many transcription factors, it can be induced or repressed in response to different subsets of stimuli including pathogen-associated molecular patterns (PAMPs), cytokines like TGFβ and fibroblast growth factor (FGF), reactive oxygen species, growth factors and mechanical stress. Moreover, TNC FNIII domains can undergo alternative splicing resulting in small (220 kDa) or large (>300 kDa) isoforms. Pro-inflammatory cytokines can either up-regulate the expression of all TNC isoforms or induce the preferential expression of one isoform. The smallest TNC isoform, with no alternatively spliced FNIII included, is known to promote cell attachment and the formation of focal adhesions. This contrasts with larger TNC isoforms, which

prevent focal adhesion formation and drive cell migration. The TNC multimodular structure enables the interaction with a high number of highly diverse ligands, like other ECM components and this likely plays a structural role, but it can also modulate how ECM components signal to the cell.[69,70]

Wide distribution of TNC occurs in embryonic tissues, while in postnatal development its expression is much lower and its synthesis is tightly regulated. TNC expression has been observed in malignant tissues, spatially and temporally related to tumor neovascularization, it may exert anti-adhesive and immunosuppressive activities. In many cancers the strongest TNC expression has been associated with the invasive front. Within the stroma, activated fibroblasts (myofibroblasts) and angiogenic blood vessels are major producers of TNC. TNC expression levels represents a predictive value for local recurrence and metastatic dissemination for breast cancer, glioblastoma and other cancers, like melanoma.[71] TNC can inhibit the spreading on adhesive ECM substrates, as a result, intracellular signaling pathways that normally are triggered by cell contact with adhesive ECM substrates are altered. This stimulates cell motility and invasion. TNC has been shown to promote metastatic fitness of disseminated cancer cells and facilitate colonization of secondary organs by engaging the Notch and Wnt signaling pathways.[72] Many different tumors have been investigated for the expression of TNC, like B and T cell NHL, primary melanoma, brain tumors, breast and lung cancer as well as in their metastasis. [73–75]

- ***ECM Proteins as Targets for Anticancer Therapies***

  - **Collagen**

Collagen is basically present in various types of cancer and contributes to the cancer development by cells proliferation, migration, and metastasis. ECM components like collagen, that are highly expressed in areas of active tumor invasion, are promising targets. Collagen can be regulated by different types of inhibitors by interfering with collagen biosynthesis enzymes, disturbing cancer cell signaling pathways, or directly utilizing collagenases. Two examples of therapies directed against collagen are the administration of endostatin, a collagen fragment with specific anti-tumor properties, and the anti-DDR1 antibody, a neutralizing antibody acting on collagen receptor DDR1. Endostatin is a C-terminal fragment derived from collagen type XVIII that has shown to regulate multiple signaling pathways. Endostatin has shown anti-angiogenic properties and it regulates Tumor Associated Macrophages (TAM) polarization towards the pro-inflammatory M1 phenotype. Clinical studies have demonstrated the significant survival benefit of endostatin treatment for late stage non-small cell lung cancer (NSCLC), indeed in breast cancer the combination of endostatin and chemotherapy has shown an increased efficacy compared to chemotherapy alone.[76] DDR1, a collagen receptor with tyrosine kinase activity, promotes tumor progression and metastases by promoting collagen fiber alignment. DDR1 increased expression in breast cancer correlates with poor prognosis. Anti-DDR1 neutralizing antibodies disrupt collagen fiber alignment, mitigate immune exclusion and inhibit tumor growth in

immunocompetent hosts. Anti-DDR1 antibody has been tested in *in vivo* breast cancer mouse models and the resulting data suggest DDR1 as a good immunotherapeutic target for increasing immune accessibility through reconfiguration of the tumor ECM[77]

- **Fibronectin**

Since specific FN isoforms have been identified in tumors, they have been largely investigated as therapeutic targets. Examples include the development of inhibitory molecules that block the interaction of FN with integrins and other receptors on the cell surface, and the development of antibodies, which can be used for the selective delivery of therapeutic agents to the tumor environment. For example, Fibulin-5 (Fbln5) is a matricellular protein that competes with FN for integrin binding. The loss of the Fbln5-integrin interaction in mouse models of PDAC resulted in augmented fibronectin signaling, driving integrin-induced ROS production. These results suggests that Fbln5 is a promising therapeutic target for pancreatic cancer.[78] Also, the cytokine endothelial monocyte activating polypeptide II (EMAP II) blocks the fibronectin-integrin interaction. *In vivo* PDAC mouse models have demonstrated a reduction in tumor growth after EMAP II administration.[79] Schliemann's laboratory together with Philogen SpA have developed two antibodies called L9 and F8 that selectively recognize splicing isoforms of FN. The L19 radio-immunoconjugate (L19-^{131}I) has been shown to selectively localize on neoplastic lesions in Hodgkin lymphoma, leading to objective responses in heavily pre-treated patients. Furthermore, the immuno-cytokine L19-IL2 was shown to potentiate the therapeutic action of the anti-CD20 antibody Rituximab in three mouse models of human B-NHL.[80,81] Also CAR-T

cells have been developed against FN with promising results. EIIIB is an alternatively spliced domain of fibronectin strongly expressed in tumors and during angiogenesis, but not in most normal tissues. Anti-EIIIB fibronectin CAR T cell targeted tumor ECM and neovasculature, they inhibited the growth of the aggressive B16 melanoma in an immunocompetent mouse. These results shown that CAR-T cells targeting selectively to tumor ECM and neovasculature can be very effective in suppressing tumor growth.[82]

- **Periostin**

Increased expression of periostin has been associated with advanced carcinogenesis and cell proliferation, adhesion, and migration. Kim et al. have shown that overexpression of spliced periostin, which lacks the exon 17 region, suppresses lung metastasis in mouse models.[83] This study supported the hypothesis that exon 17 is essential to tumor progression and metastasis. Based on these findings a neutralizing antibody against the peptide encoded by exon 17 (PN1-Ab) has been developed. *In vivo* administration of PN1-Ab significantly inhibited the growth of primary as well as metastatic tumors, resulting in increased survival of mice. Further development of this antibody, such as the generation of a humanized antibody, may provide a new therapeutic agent.[84] Nucleic acid–based aptamers are an emerging class of targeted therapeutic molecules. Aptamers are single-stranded DNAs or RNAs that are designed to bind to proteins with similar or better affinity and specificity than antibodies. By directly binding to the target, aptamers can modulate the activities and functions of target molecules. PNDA-3 is a DNA aptamer capable of binding to periostin with high affinity and inhibiting its function. PNDA-3 selectively

bound to the FAS-1 domain of periostin disrupting the interaction with integrins. PNDA-3 markedly antagonized the periostin-induced adhesion, migration, and invasion of breast cancer cells and blocked the activation of the integrin signal transduction pathways. In *in vivo* models PNDA-3 administration significantly reduced primary tumor growth and distant metastasis.[85]

- **Tenascin C**

The tightly regulated expression and the presence of TNC within tumor infiltrated tissues made it an attractive candidate for immunotherapy. Several antibodies have been raised against TNC. In this context, radio-immunotherapy (RIT) approaches have been developed, pre-clinical and clinical trials in different settings have shown both feasibility and therapeutic efficacy of tenascin-targeting therapeutic approaches. One example of anti-TNC antibody is the IgG1 murine antibody BC2, specific to an epitope of the alternative spliced A4 domain of TNC. BC2 was radio-labelled with iodine-131 (^{131}I) and patients with glioma and malignant glioblastoma were treated with direct intralesional administration. Preliminary results have demonstrated the modest effectiveness of this method and the absence of systemic and cerebral toxicity.[86,87] Different antibodies have been developed against the TNC domain D, such as the IgG2 81C6 antibody that was investigated in clinical trials as a ^{131}I radio-conjugate for the treatment of B-NHL by intravenous injection. One complete and one partial remission were reported, but further attempts are needed to enhance tumor localization and decrease normal visceral uptake through the use of unlabeled antibody.[88,89] Also P12 and R6N antibodies have been developed against TNC domain D, but the first

reported intestinal accumulation in *in vivo* models, while the second has been proposed fused to the murine cytokine IL-12 (mIL12-R6N) with better results. mIL12-R6N exhibited potent antitumor activity in immunodeficient mice bearing renal cell carcinoma and glioma.[90,91]
Sigma Tau has developed an anti-TNC murine antibody ST2146 for radio-immunotherapy. They proposed a therapeutic strategy called Pretargeted Antibody-Guided RadioImmunoTherapy (PAGRIT), based on intravenous, sequential administration of the biotinylated antibody, avidin/streptavidin and yttrium-90 labelled biotin. ST2146, later called tenatumomab, is a murine IgG2b/k antibody recognizing an epitope within the EGF-like repeats of human tenascin, shared by both small and large tenascin isoforms.[92,93] *In vivo* biodistribution studies of biotinylated Tenatumomab were done in nude mice transplanted with human HT-29 colon carcinoma and human U-118MG glioblastoma cells characterized for low and high tenascin expression, respectively. Despite the antibody cross-reactivity with the tenascin expressed at low level in the normal mouse organs, good tumor/non-tumor uptake ratios confirmed the high tumor selectivity of Tenatumomab.[94] Another conjugate was investigated in order to provide an efficient PAGRIT application protecting biotin from enzymatic degradation; in this case biotin has been bound to the chelating agent DOTA and labelled with yttrium-90.[95] Nevertheless, PAGRIT therapeutic strategy was discontinued in favor of simpler Tenatumomab versions, because PAGRIT requires the simultaneous production of multiple drugs and so, a disadvantage for the development of a new treatment. Tenatumomab was investigated in a Phase 1 clinical trial labelled with ^{131}I, as a delivery agent for

radionuclides to neoplastic lesions (NCT02602067). Trial preliminary results suggested revising infusion modality pointing out the need of an imaging guided approach to personalize treatment. In fact, despite fine specificity, MAbs display inter-individual variability due to unpredictable factors like Fc receptor affinity, level of circulating antigen or presence of Anti-Drug Antibodies (ADA), thus requiring patient-specific information on the antibody distribution to personalize the radiotherapeutic treatment.[96]

Scope of the thesis

The aim of my PhD thesis project is to compare the functional activity of two strategies to improve CIK cells, bispecific antibodies and CAR genetic engineering, both directed against the CD19 antigen, and, based on these findings, to apply such “optimized” CIK cells to target a novel TME protein in the context of solid tumors.

The project has been developed as follows:

- The first part of the project has characterized and better defined the different options for enhanced and redirected anti-tumor CIK cells activity. This was done by comparing CIK combined with a BiTE, the CD19xCD3 blinatumomab, or CIK modified by 2 different CARs carrying different signaling modules, one being the anti-CD19 CAR used by our group in clinical trials (CARCIK-CD19) and the other recapitulating the Tisagenlecleucel CAR structure. In all cases, the target antigen was CD19 and the BiTE or CARs carried an anti-CD19 scFv, respectively MT-103 or FMC63. The two anti-CD19 CARs were in both cases stably transfected into CIK cells through Sleeping Beauty transposon system. These different CIK redirecting approaches were analyzed in terms of proliferation, cytotoxicity, cytokine release and intracellular signaling, in response to $CD19^{+}$ tumor targets *in vitro*.
- The second part of the project developed a novel CAR directed against a specific TME protein, which identity will not be revealed due to patenting reasons and we have called it TMA (Tumor Microenvironment Antigen), to be introduced into CIK cells. Also in this case, 2 different anti-TMA CARs have

been generated with structures similar to the anti-CD19 CARs of Part 1, and their functionality *in vitro* against different TMA positive cells was investigated. The expression of the TMA target was analyzed in different solid and hematopoietic tumors and normal cells.

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CHAPTER 2

Functional activity of CARCIK-CD19 cells compared to unmodified CIK cells combined with bispecific antibody blinatumomab

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Abstract

Although cytokine Induced Killer (CIK) cells have shown anti-tumor activity *in vitro* and *in vivo*, there remains a need to increase their efficacy and specificity. This could be reached using either bispecific antibodies or chimeric antigen receptor (CAR) modifications. The aim of this study was to compare the *in vitro* function of CIK cells combined with soluble CD3xCD19 bispecific antibody blinatumomab (CIK+Blina) or modified with two different anti-CD19 CAR molecules (CARCIK-MNZ and CARCIK-BG2, carrying the same anti-CD19 moiety with different signaling modules, CD28-OX40-CD3ζ and 41BB-CD3ζ, respectively). The CARCIK-MNZ are being used by our group in clinical trials (FT01CARCIK and FT03CARCIK)[1] and the CARCIK-BG2 was designed on the structure of Tisagenlecleucel CAR (Novartis)[2], cloned however in a transposon vector. We have successfully generated CARCIK cells expressing the two CAR molecules and demonstrated that there were only minimal differences in phenotype compared to untransduced CIK cells. Either CARCIK-MNZ, CARCIK-BG2 or CIK+Blina demonstrated to be cytotoxic against $CD19^+$ target cells, proliferate after antigen binding, secrete cytokines and activate the NFAT and NF-kB signaling pathways, with some differences in efficiency in the different functions. The two CAR structures have not generally shown significant differences in *in vitro* functional activity. Instead, it is worth noting that, in many assays, CIK cells with blinatumomab have demonstrated to be more active than CARCIK-CD19 cells, especially for the cytotoxic activity and the proliferative ability. Future results in animal models will complete the work presented here and verify

whether specific *in vitro* and *in vivo* activities correlate, and if so in what manner. Overall, the functional comparison demonstrates that all the three strategies to improve CIK cells are effective in redirecting the anti-tumor activity of CIK cells against a $CD19^+$ target.

Introduction

Cytokine-induced killer cells (CIK) are activated T lymphocytes expanded *in vitro* for about 3 weeks with recombinant human interleukin 2 (rhIL-2), after stimulation with interferon-γ (IFN-γ) and anti-CD3 antibody. CIK cells are $CD3^+CD56^+$ double positive T cells that have acquired phenotypic markers of natural killer (NK) cells and show HLA non-restricted cytotoxicity against tumor cells *in vitro* and *in vivo*.[3–7] Allogeneic HLA-matched, haplo-identical or unmatched CIK cells do not induce significant graft-versus-host disease (GvHD).[8–11] Donor-derived, autologous or unmatched allogenic CIK cells have been used in several clinical trials to treat patients with cancer and have shown therapeutic activity with limited toxicity. The International Registry on CIK cells has reported a mean response rate of 39% and significantly increased OS after CIK cells infusion, accompanied by an improved quality of life and with minor side effects. Improvement of clinical parameters have been reported not only in hematological malignancies, but also in hepatocellular carcinoma, gastric cancer, renal cell carcinoma and non-small cell lung cancer.[12] Since CIK cells alone have shown activity mostly in a low-tumor burden context, they still need an improvement in terms of activity and anti-tumor specificity.

Previously, our as well as other groups have shown that CIK cells activity can be redirected towards tumor targets by the combination with bispecific antibodies (BsAb) simultaneously targeting a CIK surface antigen (e.g. CD5 or CD3) and a tumor antigen.[13–16] Two different anti-CD5 BsAbs have been demonstrated to redirect CIK cells activity against their target tumor cells. The CD20xCD5 BL-01 BsAb has shown to efficiently redirect $CD5^+$ CIK cells against $CD20^+$ tumor cells *in vitro* and *in vivo* in a diffuse large B cell lymphoma xenograft.[17] The HD37xT5.16 BsAb (CD19xCD5), when combined with CIK cells, has shown high cytolytic activity *in vitro* against $CD19^+$ B lymphoma cell lines.[18] Moreover, Golay et al. have demonstrated the *in vitro* and *in vivo* activity of CIK cells combined with the BsAb blinatumomab for the control of B cell Acute Lymphoblastic Leukemia (B-ALL) growth.[19] Blinatumomab is a non-IgG like Bispecific T cell Engager antibody (BiTE) composed of two single chain fragment variables (scFvs) against CD19 and CD3 (CD19xCD3). This antibody acts as a bridge between $CD3^+$ CIK cells and the $CD19^+$ tumor target, activating CIK cells to proliferate and kill the latter. These data demonstrate that the combination of CIK cells with BsAbs is broadly applicable against different targets and changing the antigen specificity may allow to redirect CIK cells against different tumors.

Another approach to redirect T cells is to modify them genetically with chimeric antigen receptors (CARs) directed against a tumor antigen. The first CAR-T clinical product that has been approved by FDA/EMA was based on a second generation anti-CD19 CAR, introduced into autologous T cells using viral transfection. First results

have reported significant remission rates in both adult and pediatric B-ALL patients.[20–22] Indeed, CARs with different signaling modules have already been introduced in the clinic.[23,24] However, manipulation of viral vectors requires high containment levels and extensive quality controls for safety. Another problem is that patient-derived T cells can fail to expand *in vitro* when derived from heavily pre-treated patients. To overcome these hurdles, another approach has been proposed by our collaborator in Monza, Magnani et al. The main innovation of this study is the use of allogenic CIK cells as effectors and of the Sleeping Beauty (SB) transposon system for stable CAR insertion into these cells. The Monza group thus validated the use of allogeneic CIK cells engineered with non-viral vectors that express a third generation anti-CD19 CAR. The phase I/IIa clinical trial has demonstrated that donor-derived SB-engineered CARCIK cells expand and persist *in vivo* in pediatric and adult B-ALL patients and achieve anti-leukemic activity without severe toxicities.[1,25,26]

Given these different possible approaches to enhance CIK cells activity, we have set out to compare the *in vitro* activities of CIK cells either combined with soluble CD3xCD19 bispecific antibody binatumomab (CIK+Blina) or modified with two different anti-CD19 CAR molecules carrying different signaling modules but the same anti-CD19 moiety. CAR-MNZ is the same anti-CD19 CAR linked to CD28-OX40-CD3ζ signaling used by our groups in clinical trials (FT01CARCIK and FT03CARCIK; Eudract n. 2017-000900-38 and 2020-005025-85)[1], whereas CAR-BG2 recapitulates the Tisagenlecleucel product (Novartis)[2] carrying the 4-1BB-CD3ζ signaling modules, cloned however in a transposon vector. These

three strategies have been compared to unmodified CIK cells in terms of proliferation, cytotoxicity, cytokine release and NFAT and NF-kB signaling *in vitro*.

Materials and Methods

Cell lines and primary cells

The B-ALL cell line REH, Burkitt lymphoma Daudi, acute T cell leukemia Jurkat and cutaneous T lymphocyte HuT 78 were maintained in culture in RPMI 1640 medium (Euroclone, Wetherby, West Yorkshire, UK) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Euroclone), 2mM L-Glutamine (Euroclone) and 100 μM gentamycin (PHT Pharma, Milano, Italy).
Peripheral Blood Mononuclear Cells (PBMCs) from normal donors' buffy coats were obtained by Ficoll Hypaque (Lympholyte-H; Cedarlane, Burlington, Canada) gradient centrifugation after informed consent.

Transposon plasmids

The anti-CD19 CAR-MNZ Sleeping Beauty (SB) transposon plasmid expresses the human third generation anti-CD19-CD28-OX40-CD3ζ CAR under the pTMNDU3 promoter. The CAR coding sequence is flanked by the recognition sites for the sleeping beauty transposase SB11.[1] The anti-CD19 CAR-MNZ plasmid together with the plasmid encoding the SB11 transposase were obtained from the Tettamanti Research Center (Monza, Italy).

The anti-CD19 CAR-BG2 Sleeping Beauty (SB) transposon plasmid expresses the human second generation anti-CD19-4-1BB-CD3ζ CAR, based on the published sequence[2] under the EF1α promoter. This sequence was synthetized by GeneArt (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and subcloned into the pT4 vector, in which the CAR coding sequence is flanked by the recognition sites for the sleeping beauty transposase SB100X. The plasmid encoding the more efficient SB100X transposase pCMV(CAT)T7-SB100 was a gift from Zsuzsanna Izsvak (Addgene plasmid #34879; http://n2t.net/addgene:34879; RRID:Addgene_34879).[27]
Plasmids were verified by sequencing and purified using the Maxiprep plasmid isolation kit (Invitrogen, Thermo Fisher Scientific) following the manufacturer's instructions.

Generation of unmodified CIK cells and anti-CD19 CARCIK cells

CIK cells were generated starting from PBMCs, cultured in RPMI advanced medium (Gibco, Thermo Fisher Scientific) with 10% FBS (Euroclone), 2mM L-Glutamine (Euroclone) and 100 μM gentamycin (PHT Pharma). Cells were stimulated on day 0 with 1000 U/ml IFN-γ (Clinigen Healthcare Ltd, Burton upon Trent, UK) and on day 1 with 50 ng/ml anti-CD3 (OKT-3, TakaraBio, Kyoto, Japan) and 300 U/ml rhIL-2 (Proleukin, Clinigen Healthcare Ltd), the latter being included in the medium from day 1 onwards. Expansion was performed for 21 days maintaining cells concentration at 0.75×10^6 cells/ml.
CARCIK-CD19 cells were generated as previously described.[1] Briefly, 10×10^6 PBMCs were transfected at day 0 with 10-15 μg CAR plasmid and 1 or 5 μg SB11 or SB100X transposase plasmid,

respectively, using the Human T cell nucleofector kit (Lonza, Basel, Switzerland) and the Amaxa IIb nucleofector device (Lonza). After transfection, cells were transferred to a 12-well plate containing 3 ml of pre-warmed RPMI medium with 20% FBS and 2mM L-Glutamine. Four hours after transfection 1 ml of medium was removed from each well and replaced with fresh complete medium (RPMI medium with 10% FBS, 2mM L-Glutamine and 100μM gentamycin) supplemented with 1000 U/mL IFN-γ. Twenty-four hours after IFN-γ addition, 300 U/ml rhIL-2 and 50 ng/ml OKT-3 were added to each well. Fresh medium and rhIL-2 were added twice a week and cell concentration was maintained at $0.75x10^6$ cells/ml. Ten days after transfection, anti-CD19 CAR^+ cells were labeled with the poly-histidine tagged recombinant human CD19 protein (Acro biosystems, Newark, DE), followed by the anti-histidine FITC antibody and immunoselected by passage through an anti-FITC magnetic beads separation column (Miltenyi Biotec, Bergisch, Gladbach, Germany). The positive fraction was collected and maintained in culture as above until day 21.

Flow cytometry

CIK and CARCIK-CD19 cells were characterized with the following monoclonal antibodies (mAbs): anti-CD3-PerCP-Cy5.5 (SK7 clone), anti-CD56-BV510 (NCAM16.2 clone), anti-CD4-PE-Cy7 (SK3 clone), anti-CD8-APC-H7 (SK1 clone), anti-CD45RA-FITC (L48 clone), anti-CD62L-APC (SK11 clone) (all from BD biosciences, San José, CA). CAR detection was achieved by incubating cells first with the poly-histidine tagged recombinant human CD19 protein (Acro biosystems), followed by FITC, APC or PE-conjugated anti–histidine

antibody (Miltenyi Biotec). A FACScanto II flow cytometer device (BD Biosciences) was used to analyze the samples with BD FACSDiva Software.

Cytotoxicity assay

Cell lysis was evaluated using the calcein release assay as previously described.[28] Briefly, target cell lines, REH or Daudi, were labeled for 30 minutes at 37°C with calcein–AM at 3.5µM final concentration (Fluka, Sigma-Aldrich, Milan, Italy). After washing, labeled target cells were distributed in 96-well plates at $5x10^3$ cells per well. CIK or CARCIK-CD19 cells were then added at different effector-to-target (E:T) ratios in the presence or absence of 10ng/ml blinatumomab (Amgen, Thousand Oaks, CA). After 4 hours, the cells were sedimented by centrifugation, 100 µl of supernatant were transferred to new plates and calcein released in the supernatant was determined using a fluorescence microplate reader (FLUOstar OPTIMA, BMG LABTECH, Ortenberg, Germany) with excitation at 485nm and emission at 535nm. The percentage of specific calcein release was calculated using the following formula: percent specific lysis = (test release minus spontaneous release) times 100 divided by (maximal release minus spontaneous release). Maximal lysis was achieved by adding 1% Triton X-100 (Sigma-Aldrich) in positive control wells.

Proliferation assay

The proliferation of CIK cells and CARCIK-CD19 cells following target recognition was evaluated using the green fluorescent dye 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) (Sigma-

Aldrich). Briefly, CIK and CARCIK-CD19 cells were incubated in 1 mL of PBS containing 1 μM CFSE. After 10 minutes at 37°C, cells were washed twice with complete medium and plated at $0.4x10^6$ cells/well in presence or absence of target cells at 10:1, 1:1 and 1:10 E:T ratios. In the case of CIK cells, 10 ng/ml blinatumomab was added. After 4 days cells were collected and stained with CD3-PE, CD4-PE-Cy7 and CD8-APC-H7 antibodies (BD Bioscience) and CFSE expression analyzed by flow cytometry, using the ModFit LT™ software to calculate the proliferation index.

Intracellular cytokine staining

The ability of CIK or CARCIK-CD19 cells to produce cytokines in response to target cells was evaluated by intracellular staining and flow cytometry. Effector and target cells were co-cultured for six hours at a 1:1 E:T ratio in presence of BD GolgiStop solution (BD Bioscience). Cells were then collected, fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Bioscience) following the manufacturer's instructions and stained with CD3-PerCP-Cy5.5, CD4-PE-Cy7, CD8-APC-H7 (BD Bioscience), IFN-γ-FITC and IL-2-PE antibodies (Miltenyi Biotec). Specimens were then analyzed by flow cytometry.

Analysis of NF-kB and NFAT signaling

In order to compare the signaling capacity of the different CARs and of CIK cells, $1x10^6$ Jurkat and HuT 78 cells were co-transfected with 5 μg of CAR-MNZ or CAR-BG2 plasmids and 1 μg of SB11 or SB100X transposase plasmids. After 7-10 days expansion, stably

transfected CAR^+ cells were purified by immunoselection, as described above for PBMCs. Jurkat and Hut 78 cell lines stably expressing the two different CARs on >90% of cells were therefore obtained. These cell lines were used to assess NF-kB and NFAT signaling by transient transfection with the appropriate reporter plasmids, followed by stimulation with target cells. Briefly, 2.5 μg NFAT or NF-kB inducible secreted luciferase reporter plasmids, respectively pNifty3-T-Lucia and pNifty3-N-Lucia (InvivoGen, San Diego, CA), were transfected into Jurkat or Hut 78 wild type or the same cell lines expressing CAR-MNZ or -BG2, using the nucleofector kit V and Amaxa IIb nucleofector device (Lonza). After transfection cells were resuspended in complete medium and left to recover at 37°C for 24 hours. Cells were then counted and co-cultured with the $CD19^+$ REH target cell line at 1:1 E:T ratio. 10 ng/ml blinatumomab was also added to the wild type cell lines. As positive controls, cells were stimulated with 500 ng/ml PHA and 1 μg/ml Ionomycin (Sigma-Aldrich). After further 24 hours, 10 μl of the supernatant was transferred to a new plate, 50 μl of coelenterazine substrate were added to the wells and the light signal was quantified as relative light units (RLU) using a luminometer (FLUOstar OPTIMA, BMG LABTECH).

Statistical analyses

Results were compared using the Student's *t*-test. A p value <0.05 (*) was considered significant.

Results

Generation of CIK and CARCIK-CD19 cells and their characterization

First, we obtained or generated 2 different anti-CD19 CARs carrying the same anti-CD19 scFv sequence but different spacer, transmembrane and signaling domains. The two structures are represented in Figure 1A and are compared to the physiological TCR complex and co-stimulatory molecules (Figure 1B). CAR-MNZ, used by our group in CARCIK-CD19 clinical trial,[1] is composed of CD19 scFv followed by the hinge, CH2 and CH3 domains of human IgG1, CD28 transmembrane and signaling domains, OX40 and CD3ζ signaling domains. CAR-BG2 is based on the Tisagenlecleucel structure[2] and composed of CD19 scFv followed by the CD8 hinge and transmembrane domain, 4-1BB and CD3ζ signaling domains (Figure 1A).

We then optimized the nucleofection protocol in presence of SB plasmids to generate CIK cells expressing the two distinct anti-CD19 CARs. Both CARCIK-MNZ and CARCIK-BG2 cells showed efficient expansion, reaching after 21 days a mean $1300x10^6$ and $891x10^6$ total nucleated cells (TNC), respectively (Figure 2A). The expression levels of CAR-MNZ and CAR-BG2 were stable and after 21 days reached mean values of 35.9% and 17.7%, respectively (Figure 2B), with a Mean Fluorescent Intensities (MFI) of 4209 and 2850, respectively (Figure 2C). Thus CAR-BG2 is significantly less expressed than CAR-MNZ, either as percentage or as MFI ($p<0.05$).

The transfection and expansion only minimally altered the phenotype of the CARCIK-CD19 cell products, compared to unmodified CIK cells. The percentage of $CD3^{+}CD56^{+}$ double positive CIK cells was comparable between transfected and unmodified CIK cultures. The $CD8^{+}$ subpopulation was over-represented in unmodified CIK cells (80.4% on total CD3+ cells) compared to CARCIK-MNZ and CARCIK-BG2 cultures (respectively 51.6% and 55.9%) (Figure 2D). The effector memory phenotype at day 21 of culture was comparable between CIK, CARCIK-MNZ and CARCIK-BG2 cells for naïve, effector memory and EMRA subpopulations. Instead, central memory cell population was significantly higher for both CARCIK-CD19 products, being a mean of 35.7% for CARCIK-MNZ and 26.1% for CARCIK-BG2, compared to 6,5% for unmodified CIK cells ($p<0.05$; Figure 2E).

Taken together, these data demonstrate that the method of generating CARCIK-CD19 cells by transfection is effective, although CAR-MNZ and CAR-BG2 are expressed at slightly different levels. In light of this observed variability, we routinely purified CAR^{+} cells during culture for later comparative functional analyses. The efficiencies of CARCIK-MNZ and CARCIK-BG2 purification were comparable and final products expressed >90% of CAR-CD19 in all cases (Figure 2F). Flow cytometry histograms show an example of CAR expression on non-purified and purified cells (Figure 2G-H).

CIK cytotoxicity is enhanced in presence of blinatumomab or anti-CD19 CAR

Purified CARCIK-CD19 cells were used for subsequent functional studies and compared to unmodified CIK cells in presence of blinatumomab.

These different cell populations were first evaluated for their ability to mount an effective anti-leukemia cytotoxic response *in vitro* using two $CD19^+$ cell lines as target, the B-ALL REH and the B lymphoma Daudi cell lines, at three different E:T ratios, ranging from 10:1 to 1:1. The cytotoxic activity of CIK+Blina as well as the two different CARCIK-CD19 was significantly higher than that of CIK cells alone against both the REH and Daudi cell lines, with higher efficacy observed at the 10:1 than 1:1 E:T ratio ($p<0.05$; Figure 3A and B). This demonstrates that both blinatumomab and the 2 different CARs are able to redirect CIK cells to a $CD19^+$ target. Furthermore, cytotoxicity induced by CIK+Blina *in vitro* tended to be slightly higher than that observed with the 2 CARCIK-CD19 populations, although this was not always statistically significant (Figure 3A and B).

CIK+Blina and CARCIK-CD19 cells proliferate in presence of $CD19^+$ target

The proliferative response of CIK+Blina or CARCIK-CD19 cells to target cells was then evaluated using the CFSE assay, after co-culture with $CD19^+$ target cell line REH. CIK+Blina and CARCIK-CD19 cells showed higher proliferation indexes compared to unmodified CIK cells (Figure 4A). Proliferation was higher at low E:T ratio (1:10)

than at high E:T ratio (10:1), meaning that CIK cells proliferated significantly more in presence of higher amount of target antigen (at 1:10 E:T ratio, $p<0.05$). There was no significant difference between CIK+Blina and CARCIK-CD19 proliferation in response to this cell line. On the contrary, when cells were co-cultured with the Daudi cell line, the proliferation was modest in all cases and not significantly different from that of CIK cells alone (Figure 4B).

In order to determine whether CD19 expression levels may explain the different proliferation rate induced by REH compared to Daudi cell line, the level of CD19 expression of these cells was analyzed. Both cell lines were highly positive for CD19 expression (99.6% for REH and 96% for Daudi) with similar MFI (respectively 3356 and 3301) (Figure 4C-D), suggesting that CD19 expression level is not the explanation of the different response.

CIK+Blina and CARCIK-CD19 secrete IFN-γ and IL-2 after stimulation with targets

We next investigated several cytokines released by CIK+Blina or CARCIK-CD19 cells after stimulation with the target REH and Daudi cell lines. The release of IFN-γ was evaluated on $CD8^+$ cells. In presence of either REH or Daudi cell lines as stimulators, CIK+Blina tended to produce more IFN-γ than CARCIK-CD19 ($P<0.05$, Figure 5A). Also, CARCIK-MNZ tended to be more effective than CARCIK-BG2, but this was not statistically significant.

The release of IL-2 was evaluated on $CD4^+$ cells. IL-2 were induced by both CARs or blinatumomab in presence of either the REH or Daudi cell line. CARCIK-MNZ showed overall more IL-2 secretion

than CIK+Blina or CARCIK-BG2, although this was statistically significant only with the Daudi cell line as stimulator ($P<0.05$, Figure 4B).

Overall therefore, we can conclude that the pattern of IFN-γ secretion by $CD8^+$ cells and IL-2 production by $CD4^+$ cells varied, with highest IFN-γ induced by CIK+Blina whereas the highest IL-2 was produced by CARCIK-MNZ.

NFAT and NF-kB are activated by CIK+Blina and CARCIK-CD19 cells upon antigen binding

To complete the comparison of the differences in the mechanism of action between blinatumomab and CAR constructs, we have studied the activation of the transcription factors NFAT and NF-kB. To do this, we used the $CD3^+$ T lymphocyte HuT 78 cell line, either wild type or stably transfected and purified to express CAR-MNZ and CAR-BG2 on >90% of cells. After 24 hours of co-culture of HuT 78 with the REH target cell line, we assessed the activation of NFAT and NF-kB by transient transfection with reporter plasmids. All conditions seemed to induce both NFAT and NF-kB, but not always at a significant levels. Indeed, HuT 78 cells in presence of blinatumomab activated the highest level of NFAT ($p=0.06$, Figure 6A). NF-kB was activated in all cases, but induction was significant only for HuT 78 wild type cells plus blinatumomab and HuT 78 expressing CAR-BG2 (Figure 6B). These experiments will be repeated to reach statistical significance.

Discussion

CIK cells are *ex vivo* expanded and activated T lymphocytes with anti-tumor cytotoxicity, tumor specific homing *in vivo* and little if any GvHD activity.[6,29] The possibility to "redirect" CIK cells using BsAbs, to directly retarget the CIK cells against the tumors and the possibility to transduce the cells with a CAR transgene are all modalities to enhance the natural cytotoxic antitumoral nature of these cells.[19,26] Here, we propose and compare three methods to improve CIK cells *in vitro* activity and anti-tumor specificity against CD19$^+$ targets, the addition of a BiTE to unmodified CIK cells (blinatumomab) or the genetic modification of CIK cells with 2 different anti-CD19 CARs carrying different spacer and signaling modules. We demonstrate the superior activity of all three approaches compared to unmodified CIK cells. Furthermore, we analyze the functional similarities and differences *in vitro* between these 3 effector cells, in terms of cytotoxicity, proliferation, cytokine release and signaling via NFAT and NF-kB, in response to target cells.

Firstly, we generated CIK cells stably expressing CAR-MNZ (carrying IgG1 CH2-CH3 spacer, CD28 transmembrane and signaling, OX40 and CD3ζ signaling modules)[1] and CAR-BG2 (carrying the CD8 spacer and transmembrane, 4-1BB and CD3ζ signaling modules)[2], via co-transfection with a plasmid encoding the SB transposase. The 21-days culture method allowed to expand a comparable number of total nucleated cells in both cases. The anti-CD19 CAR-MNZ had a slightly higher expression level in terms of percentage and fluorescent intensity compared to CAR-BG2. The subpopulations and the effector memory phenotype were similar

between unmodified CIK cells and the two CARCIK-CD19 cells, except for the larger proportion of $CD8^+$ cells in CIK populations compared to CARCIK-CD19 cells, as well as a more central memory rather than effector memory subset in the CARCIK-CD19 cells. This was true for both anti-CD19 CAR versions.

Since CAR expression was variable between experiments and different between the 2 CARs, we routinely purified CAR^+ cells halfway during expansion by immunoselection, in order to functionally analyze similar final populations, always containing ≥90% CARCIK-CD19 cells. These cells were compared to unmodified CIK redirected to target cells with blinatumomab.

Regarding cytotoxicity, both blinatumomab and CARs were very effective in increasing efficacy of CIK cells against 2 different $CD19^+$ tumor targets, the REH B-ALL cell line and Daudi B-lymphoma cell line, with a small advantage of CIK+Blina. As expected, in all cases more effector cells compared to targets (higher E:T ratio) induced more effective cytotoxicity. The cytotoxicity observed is consistent with previous data with blinatumomab or CARs from our and other groups.[19,25] The cytolytic activity of CIK+Blina was generally superior to that of CARCIK-CD19, particularly at low E:T ratios. This may be due to the observed higher percentage of $CD8^+$ cells in CIK compared to CARCIK-CD19 populations.

Also proliferation was efficiently induced in all cases by the REH target cell line. As expected, proliferation of CIK cells was more effective with a higher number of target cells (and therefore target antigen) compared to CIK (1:10 E:T ratio). Interestingly in no case did the Daudi cell line stimulate significant proliferation. This was not due

to any significant difference in CD19 levels of expression between REH and Daudi cells and suggests that some inhibitory surface or secreted molecules are produced by Daudi cells, which are able to block CIK mediated proliferation, but not cytotoxicity. Similar data have been published by other groups who showed that some target cell lines induce proliferation of CAR-T cells, whereas other do not. These authors have demonstrated that blocking the PD-1/PD-L1 pathway improves CAR-T cell proliferation and, in some cases, also cytokine release.[30,31] Our preliminary data show that Daudi express more CD80, CD86, GITR and TIM-3, checkpoint inhibitors than REH (Zaninelli S., Data not shown). Future work will investigate whether these checkpoint inhibitors or other factors explain the block in CIK proliferation mediated by the Daudi compared to REH cells lines.

We also measured cytokine release by CIK cells in presence of targets. The highest levels of IFN-γ were released by $CD8^+$ CIK cells stimulated with blinatumomab, followed by CARCIK-MNZ and lowest CARCIK-BG2. In contrast $CD4^+$ CARCIK-MNZ released higher levels of IL-2 compared to either CIK+Blina or CARCIK-BG2. Other groups have already reported that CAR-T cells bearing CD28 signaling module, like CAR-MNZ, induces higher levels of cytokines secretion, in particular of IL-2, compared to 4-1BB based constructs, like CAR-BG2.[32]

We finally analyzed the signaling via NFAT or NF-kB activation, using the T cell line HuT 78 stably transfected with the CAR-CD19. Both NFAT and NF-kB were induced with a possible advantage of blinatumomab over CAR-CD19 for NFAT activation. As expected, CAR-BG2 had the lowest induction of NFAT, since the 4-1BB

costimulatory domain does not induce NFAT activation[33], while NF-kB levels were more similar between blinatumomab and both CARs since they all activate NF-kB.

These functional differences observed between CIK+Blina and CARCIK-CD19 can be explained by the different signals activated through antigen binding. Whereas blinatumomab activates CIK cells through CD3ε, the activation through the CARs is via CD3ζ. In addition to the contribution of CD3 ITAM domains, there is the phosphorylation of CD28 and OX40 for CAR-MNZ and of 4-1BB for CAR-BG2 (schematic structures are reported in Figure 1A and B).[34] These different signaling modules activate overlapping but not identical intracellular pathways and different promoters, inducing the expression of specific genes.[35,36] Signaling domain comparisons performed by other groups have suggested that 4-1BB CARs compared to CD28 CARs are less active *in vitro* but more persistent *in vivo* in the long term.[37] Nevertheless, different studies have suggested that the advantage given by a specific co-stimulus is strictly dependent on the target antigen. One group has demonstrated that, when targeting CD30$^+$ lymphomas, the combination of CD28-OX40 was associated with better activity and longer persistence *in vivo*, while in the case of GD2$^+$ neuroblastoma targets, the combination CD28-4-1BB was accompanied with an improved anti-tumor acitivity.[38,39]

It is important to take into account that the CAR activity could also be influenced by the different spacer and trans-membrane domains for the 2 CARs. Finally, the anti-CD19 scFv of blinatumomab sequence is different from that used for both CARs, which both derive from the FMC63 clone.[40]

Clearly, to have a full view of the systems used, a comparison of the anti-leukemic activity of these different therapeutic strategies in *in vivo* mouse models is necessary and these experiments are in progress. These results will allow to verify whether specific *in vitro* and *in vivo* activities correlate, and if so in what manner.

Overall, the functional comparison presented here demonstrates that all three strategies studied to improve CIK cells efficacy are functional and they all can successfully redirect the anti-tumor activity of CIK cells against a $CD19^+$ target. The two CAR structures have not generally shown significant differences in *in vitro* functional activity. Instead, it is worth noting that, in many assays, CIK cells with blinatumomab have demonstrated to be the most active, especially with regard to the cytotoxic activity and the proliferative ability. These differences however may be due to the higher percentage of $CD8^+$ cells in CIK, rather than CARCIK-CD19 populations. The CIK+Blina strategy has the advantage over CARCIK cells of not requiring genetic modification of the effector cells. Blinatumomab has a half-life of few hours and has been administered by continuous infusion.[41] This can be an advantage since it allows to rapidly stop the treatment at the onset of life-threatening side effects. Moreover, this platform is amenable to be used with other T cells redirecting BsAbs, against either leukemic targets or solid tumors. Of note is that 5 out of 6 approved BsAbs against cancer are directed against CD3 and a tumor antigen (CD19, EGFR, BCMA, etc).[42] These could all potentially be combined with unmodified CIK cells.

Nevertheless, CAR-T cells are well established immunotherapeutic drugs with 6 products already approved by FDA/EMA.[43] These cells

can overcome tolerance, synergize with the endogenous immune response, and persist long-term *in vivo*.[44,45] On the other hand, CAR-T cells still need improvement to reduce associated toxicities, be successful against solid tumors and to lower costs and time of production.[46,47] CAR-modified CIK cells, rather than standard T cells, constitute a new platform that may overcome some of the complications, in particular GvHD or insufficient tumor infiltration, posed by CAR-T cells, although formal proof of these advantages in humans are still lacking. The ability to use an allogenic source for CIK or CARCIK production is an important point of strength, since CAR-T cells from heavily pre-treated patients may fail to expand or be exhausted.[48] Allogenic CARCIK-CD19 cells have demonstrated to be safe in the phase I/IIa clinical trial, in which no GvHD have been reported and only mild toxicities have been described, confirming the CIK cells safety profile.[26] The non-viral Sleeping Beauty transposon platform simplifies the cells' production under GMP conditions. The transposon system has demonstrated to be effective in inducing CAR expression and integration sites are randomly distributed inside the genome, without preferential insertion regions.[26,49,50] These findings altogether suggest that CARCIK-CD19 cells are a functional and safe product for the management of B-ALL. Future results in animal models and in clinical trials will add important information to the work presented here.

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Tables and figures

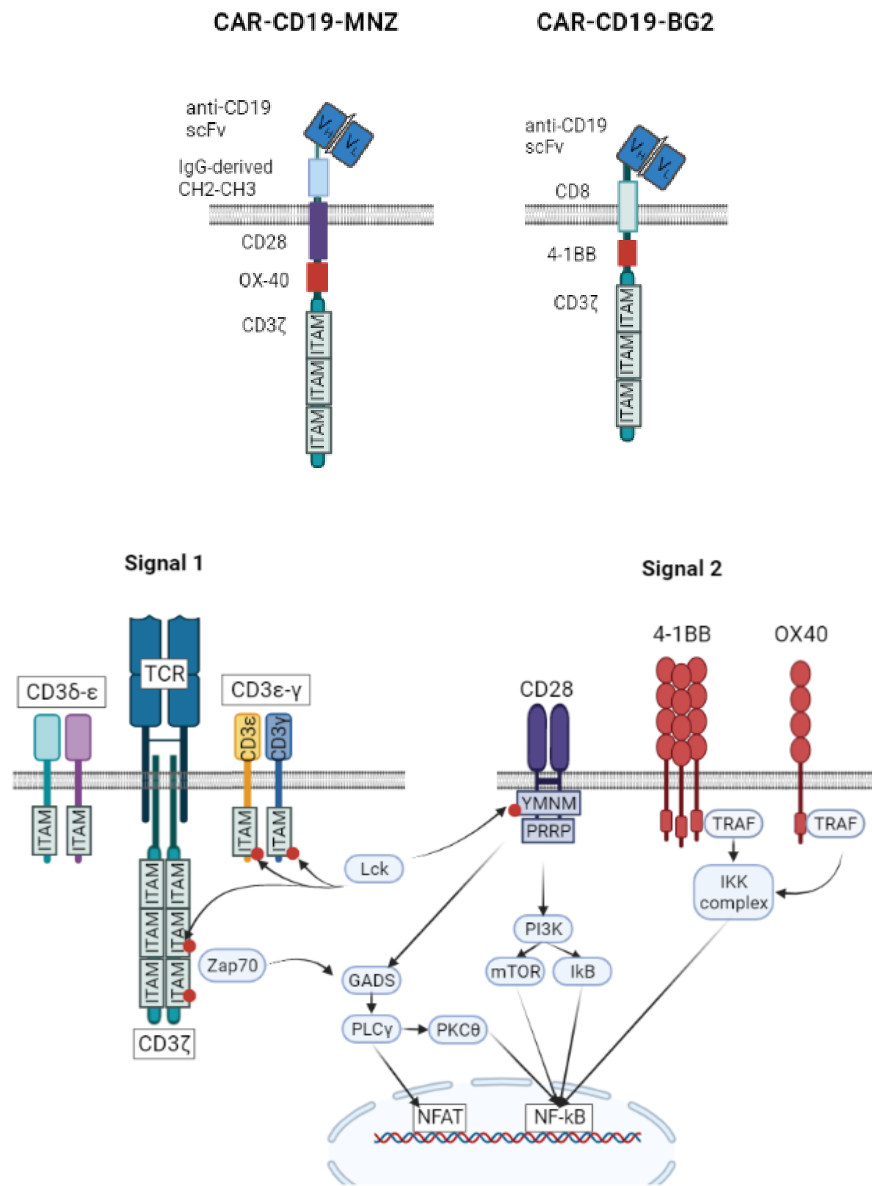

Figure 1 – CAR structures and TCR complex.
(A) Schematic representation of the two anti-CD19 CAR structures used. (B) Schematic representation of the TCR signaling, on the left, and of co-stimulatory receptors on the right. Images created with Biorender.com.

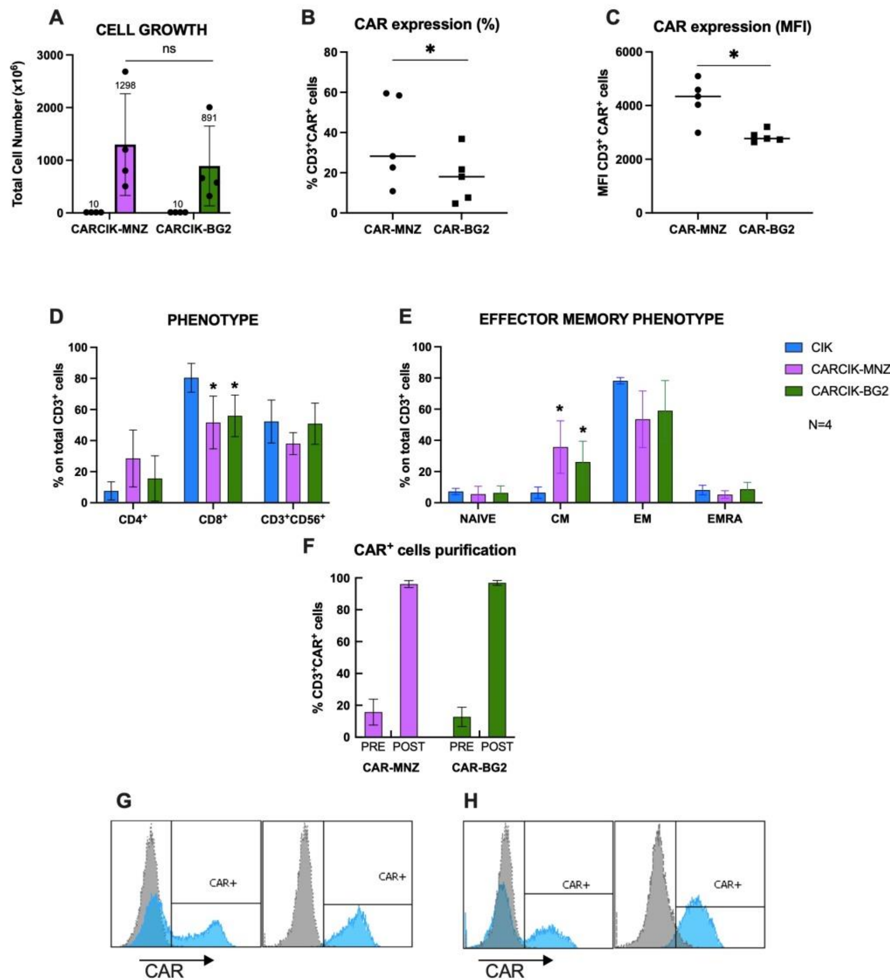

Figure 2 – Generation of CARCIK-CD19 cells and their purification and characterization.

(A) PBMCs were transduced with the CAR-MNZ and CAR-BG2 plasmids and expanded to CIK for 21 days. (C-F) CAR expression and immunophenotype, including effector-memory, were analyzed at the end of the cultures by flow cytometry on non-purified cells. (F) CAR^+ cells were purified at day 10-14 by immunoselection. (G-H) Phenotypes of non-purified and purified cells were analyzed using flow cytometry. The results are the means and standard deviations of 4 to 5 experiments using different donors as starting material. The statistics refer to the comparison between CARCIK-MNZ and CARCIK-BG2 with unmodified CIK (*$p<0.05$).

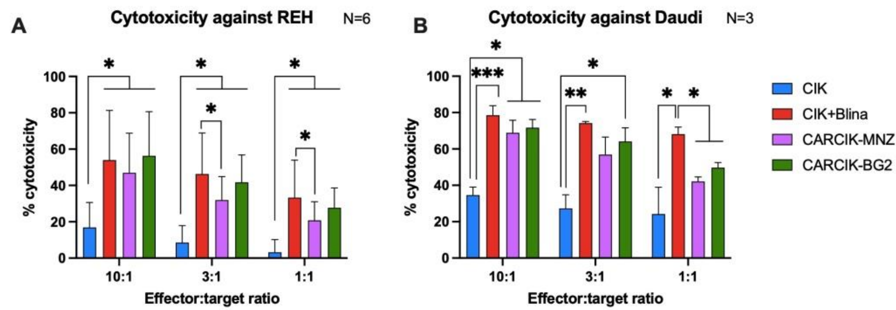

Figure 3 – CIK cytotoxicity in presence of blinatumomab or anti-CD19 CAR.

Comparison of the killing activity *in vitro* using Calcein release assay. CIK (blue bars), CIK cells in presence of 10ng/ml blinatumomab (red bars), CARCIK-MNZ (pink bars) and CARCIK-BG2 (green bars) at the end of culture were used as cytotoxic effector cells against the REH and DAUDI leukemia cell lines at a 10:1, 3:1 and 1:1 E:T ratio. (A) Cytotoxic activity against REH cell line. (B) Cytotoxic activity against DAUDI cell line. Percentage target lysis is shown as mean and standard deviation of six or three experiments, respectively (*p<0.05, **p<0.01, ***p<0.001).

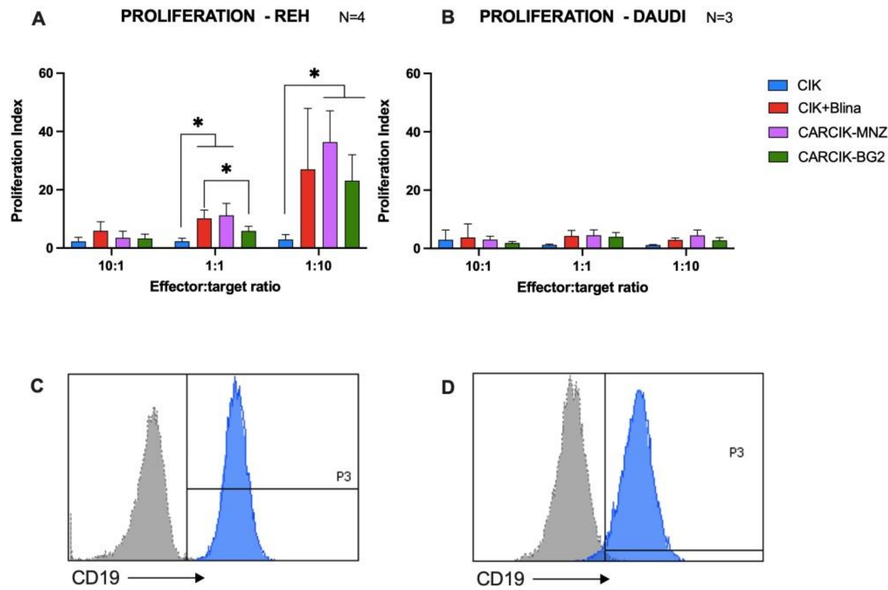

Figure 4 – Proliferation of CIK cells in presence of CD19^{+} target. Comparison of the proliferation ability *in vitro* (proliferation index) measured by flow cytometry on CFSE stained CIK cells. CIK (blue bars), CIK cells in presence of 10ng/ml blinatumomab (red bars), CARCIK-MNZ (pink bars) and CARCIK-BG2 (green bars) were co-cultured with target cells, REH and DAUDI cell lines, at a 10:1, 1:1 and 1:10 E:T ratio, for 4 days. (A) Proliferation in co-culture with REH cell line. (B) Proliferation in co-culture with DAUDI cell line. (C-D) Flow cytometry histograms of CD19 expression on REH (99,6% CD19^{+}, MFI 3356) and Daudi (96% CD19^{+}, MFI 3301) cell lines in blue and the isotype control in grey. Proliferation indexes are shown as mean and standard deviation of four or three experiments, respectively (*p<0.05).

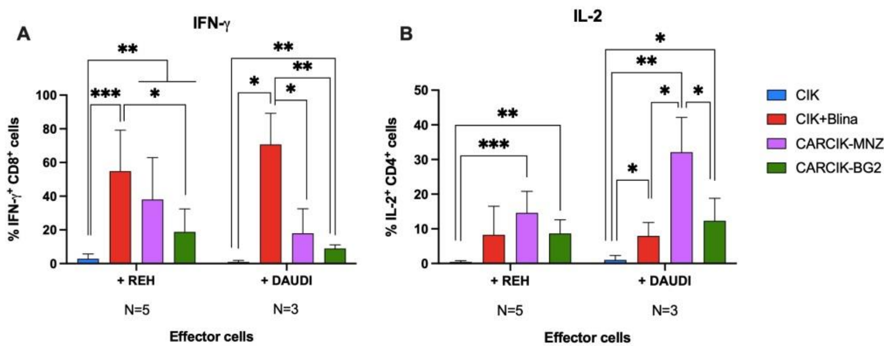

Figure 5 – IFN-γ and IL-2 release in presence of target cell lines. IFN-γ (A) and IL-2 (B) production was determined by intracytoplasmic flow cytometry after 6 hours co-culture at 1:1 E:T ratio of CIK (blue bars), CIK cells in presence of 10ng/ml blinatumomab (red bars), CARCIK-MNZ (pink bars) and CARCIK-BG2 (green bars) with REH and DAUDI cell lines. Percentages of positive cells are shown as mean and standard deviation of five experiments against REH cell line and three experiments against DAUDI cell line (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

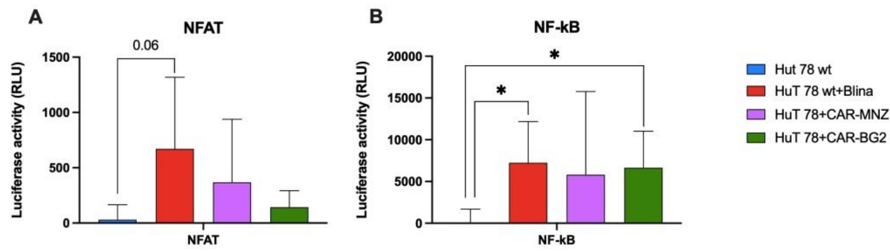

Figure 6 –NFAT and NF-kB intracellular signaling upon target binding. The NFAT (A) and NF-kB (B) signaling upon stimulation was measured by co-transfection of Lucia luciferase reporter plasmids into HuT 78 cell line wild type alone (as CIK, blue bars), or stimulated with 10ng/ml blinatumomab (red bars), or stably transfected with CAR-MNZ (pink bars) or with CAR-BG2 (green bars) and co-cultured with REH cell line for 24 hours. Relative luminescence units (RLU) are shown as mean and standard deviation of five experiments (*p<0.05).

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CHAPTER 3

Chimeric Antigen Receptor modified Cytokine Induced Killer cells against a Tumor Microenvironment Antigen for the treatment of solid tumors and lymphomas

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Abstract

Although chimeric antigen receptor T cells (CAR-T) technology has revolutionized cancer immunotherapy, CAR-T cells for solid tumors are still lacking. We set out to transfer CARCIK-CD19 technology, successfully used to treat B cell acute lymphoblastic leukemia (B-ALL) patients, to new CAR molecules against an extracellular matrix (ECM) molecule overexpressed in different tumor types. This ECM target molecule has been called TMA (Tumor Microenvironment Antigen) since its real identity cannot be revealed at present for patenting reasons. Firstly, we have studied TMA expression on putative targets, primary cells and cell lines. Two solid tumor cell lines positive for TMA expression were identified, HT-29 (colon adenocarcinoma cell line) and MDA-MB-231 (breast cancer cell line). In parallel, we have generated two cell lines expressing the TMA domain recognized by our CARs fused to a trans-membrane domain, called here TMA-TM. Stably transfected TMA-TM^{+} and naturally expressing TMA cell lines were then used to test CARCIK-TMA cells *in vitro*. Finally, 2 different anti-TMA CAR constructs were generated. These CARs shared the same anti-TMA scFv domain, based on a known anti-TMA monoclonal antibody (MAb), but differed in their backbone, the first recapitulating the signaling domains present in the anti-CD19 CAR Tisagenlecleucel (Novartis) and the second the CAR structure of CARCIK-CD19 cells developed by our collaborators at the Fondazione Matilde Tettamanti Menotti De Marchi Onlus. Both CARs were introduced into cytokine induced killer cells (CIK) by transfection together with the Sleeping Beauty transposase for insertion into genomic DNA. *In vitro* functional assays

demonstrated that anti-TMA CARCIK cells are cytotoxic against TMA$^+$ targets, proliferate in response to antigen binding and secrete the IFN-γ and IL-2 cytokines. *In vivo* animal models have been set up with the two solid tumor cell lines already tested *in vitro* as well as the B lymphoma cell line BJAB. In these models the tumor growth curves and TMA expression *in vivo* were analyzed. In addition, in the HT-29 *in vivo* model, a first pilot experiment infusing a single dose of CARCIK-TMA cells has been performed indicating lack of toxicity and transient anti-tumor activity. Additional *in vivo* experiments are in progress to verify and compare the efficacy of the two anti-TMA CARs.

Introduction

The tumor microenvironment (TME) is a highly heterogeneous environment that includes cellular components like fibroblasts, endothelial cells, adipocytes, immune and inflammatory cells, and a non-cellular component termed the extracellular matrix (ECM).[1,2] The ECM is a scaffold in which cells are embedded and together create a tissue. The ECM consists of four major components: type IV collagen, laminins, nidogen and the heparan sulphate proteoglycan perlecan.[3,4] The ECM has a great impact on the immune response, particularly in the immune cells migration into inflamed tissues and immune cells differentiation. In solid tumors the barrier function of the ECM can physically block the immune response.[5,6] Tumor markers overexpressed in the ECM are attractive molecules for the targeted delivery of therapeutics. ECM components are good candidate targets because they are easily accessible, have a low shedding profile, are

abundant and stable and in some cases overexpressed in tumors. Indeed several monoclonal antibodies (MAbs) have been developed against ECM components and some have reached the clinic.[7–13]

The Chimeric Antigen Receptor T cell (CAR-T) technology has revolutionized cancer immunotherapy. The most successful studies have led to the first approval of anti-CD19 CAR-T cells in patients with B cell malignancies already in 2017 and till now other 5 CAR-T products have been approved, all directed against hematopoietic tumors (CD19 and BCMA).[14,15] Whereas the adoptive transfer of CAR-T cells has provided a valid treatment for hematologic tumors, there are still many hurdles to applying this therapy for solid tumors. One limitation in developing CAR-T therapy for non-hematologic malignancies is the selection of target molecules specific for or overexpressed in solid tumors, as well as the need to favor the infiltration of the CAR-T cells inside solid tumors which are often poorly permissive to immune cells.[16,17]

Recently, cytokine induced killer (CIK) cells have been modified with an anti-CD19 CAR molecule to treat, in phase I/II trials, relapsed and refractory B cell Acute Lymphoblastic Leukemia (B-ALL) patients with allogenic anti-CD19 CARCIK cells (FT01CARCIK and FT03CARCIK; Eudract n. 2017-000900-38 and 2020-005025-85).[18–20] One innovation of this study was the fact that CIK were transduced with anti-CD19 CAR using electroporation of a Sleeping Beauty transposon system, thus avoiding viral transduction.

CIK cells are *in vitro* expanded $CD3^{+}CD56^{+}$ T cells characterized by some phenotypic markers of natural killer (NK) cells and by major histocompatibility complex (MHC)-independent cytotoxic activity

against tumor cells.[21,22] Moreover, CIK cells have been shown to infiltrate both hematopoietic and solid tumors, making these cells attractive as anti-cancer agents.[23,24] Finally, CIK cells also induce little if any graft versus host disease (GvHD) in allogenic context, making these cells potentially effective and safe effector for cancer treatment.[25,26] Modification of these cells with CARs enhance their anti-tumor potential.

Given the positive results obtained with CARCIK-CD19 in clinical trials of B-ALL patients[18], we set out to extend this technology to new CAR molecules against an ECM marker overexpressed in cancer and capable of targeting different tumor types. We will call our target protein TMA (Tumor Microenvironment Antigen) and we will not reveal its identity at this stage for patenting reasons. Starting from an existing MAb, previously already successfully tested *in vivo* in mice, we developed two novel anti-TMA CAR constructs. We then generated and tested CARCIK-TMA cells *in vitro* and produced first results of the effect of these cells in *in vivo* tumor models in mice.

Materials and Methods

Cell lines and primary cells

The human colon adenocarcinoma HT-29 and T lymphoblastic CEM cell lines were cultured in RPMI 1640 medium (Euroclone, Wetherby, West Yorkshire, UK) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Euroclone), 2mM L-Glutamine (Euroclone) and 100 μM gentamycin (PHT Pharma, Milano, Italy). The human breast cancer MDA-MB-231 and the embryonic kidney HEK-293 cell lines

were maintained in α-MEM medium (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% heat-inactivated FBS (Euroclone) and 100 μM gentamycin (PHT Pharma).

Peripheral Blood Mononuclear Cells (PBMCs) were purified by Ficoll Hypaque (Lympholyte-H; Cedarlane, Burlington, Canada) gradient centrifugation of normal donors' buffy coats, obtained after informed consent.

Monocytes were isolated from PBMCs, obtained after informed consent from healthy donors as previously described.[27] Briefly, $CD14^+$ monocytes were purified by immunoselection using anti-CD14 magnetic beads (Miltenyi Biotec, Bergisch, Gladbach, Germany). Purified cells were cultured in RPMI medium (Euroclone) supplemented with 20% FBS (Euroclone) for six to seven days in presence of different stimuli to drive M1 or M2 polarization. For M1 polarization, monocytes were cultured in 100ng/ml granulocyte-macrophages colony-stimulating factor (GM-CSF, Mielogen, Shering-Plough, Milan, Italy) and 1ng/ml lipopolysaccharide (LPS from *E. Coli*, Sigma Aldrich, Merck KGaA, Darmstadt, Germany) plus 100 U/ml interferon-γ (IFN-γ, Clinigen Healthcare Ltd, Burton upon Trent, UK) were added during the last 24 hours of culture. For M2 polarization, monocytes were cultured in presence of 20ng/ml of macrophage colony-stimulating factor (M-CSF, R&D systems, Minneapolis, MN, USA).

Mesenchymal stromal cells (MSC) were obtained from either healthy donors bone marrow (BM) or umbilical cord (UC), after informed consent, as previously described.[28–30] Briefly, for BM-MSC, cells

were isolated from washouts of discarded bags and filters, leftover after the filtration of whole BM explants, which is performed routinely before infusion to patients. Cells were plated in MSC expansion medium consisting of α-MEM medium (Gibco) enriched with 5% human platelet lysate (Biolife solutions, Bothell, Washington, USA), 50 μg/ml gentamicin (PHT Pharma) and 2 UI/ml Heparin (Pharmatex, Milan, Italy). For UC-MSC, fresh human UCs were collected from the Operating Room of the Obstetrics and Gynecology Unit after cesarean sections. The UCs were minced in very small fragments and seeded in the above MSC complete medium. After approximately 14 days, adherent MSCs were detached using TrypLE (Gibco) and replated in multilayered flasks for a maximum of two consecutive expansion steps.

Detection of TMA protein

To detect extracellular or intracellular TMA by flow cytometry, either live or fixed and permeabilized cells (Cytofix/Cytoperm kit, BD Bioscience, San José, CA), respectively, were stained with the murine anti-human TMA MAb followed by the FITC labeled goat anti-mouse IgG.

For in situ immunofluorescent staining, cells were seeded onto sterile cover slips in 6-well plates in complete medium for one to four days. After incubation, cells were fixed with 4% paraformaldehyde (Thermo Fisher) for 15 minutes at room temperature, washed three times with PBS and then blocked with PBS+5% FBS for 30 minutes. Cells were incubated for 2 hours with the mouse anti-human TMA MAb, followed by the Cy3-labeled donkey anti-mouse secondary antibody

(Jackson ImmunoResearch, Ely, Cambridgeshire, UK) for one hour and finally with 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI, Merck KGaA) for 10 minutes. The cells were washed three times in PBS after each step. At the end, the coverslips were mounted on slides and observed on an inverted fluorescence microscopy (Axio Vert A1, Zeiss, Oberkochen, Germany) and a confocal microscopy (SP8 lightning, Leica, Wetzlar, Germany).

Detection of TMA RNA

To quantify TMA expression, Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) was performed on the RNA extracted from different cell lines with the RNeasy mini kit (QIAGEN, Hilden, Germany). RNA was retrotranscribed using the Superscript IV VILO master mix (Thermo Fisher), following the manufacturer's instructions. Amplifications were carried out in Power SYBR green PCR Master Mix (Applied Biosystems, Thermo Fisher) and cycles included an initial 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. To verify the absence of nonspecific amplification, a melting curve was generated at the end of the amplification cycles. Primers were designed to detect TMA and two internal reference genes, the β-glucuronidase gene (GUS) and the human glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH). The relative expression levels of TMA among different cell lines were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Generation of cell lines stably expressing the TMA antigen on their surface

In order to generate target cells stably expressing the TMA antigen for functional analyses, we planned to stably transfect the CEM and the HEK-293 cell lines with the domain of the TMA protein recognized by the anti-TMA original MAb and CAR. A plasmid expressing the TMA domain fused to the trans-membrane and intracellular region of the Heparin Binding Epidermal Growth Factor (HB-EGF-TM) was synthesized by GeneArt (Thermo Fisher). The HB-EGF intracellular region was mutated to avoid the nuclear signaling, as already reported in literature.[31] The fused TMA domain-HB-EGF-TM cDNA, called TMA-TM, was cloned into the pT4 vector carrying the SB transposase recognition sequences (LIR-RIR) to give the pT4-TMA-TM plasmid.
To generate cell lines stably transfected with TMA-TM, CEM and HEK-293 cells were transfected with pT4-TMA-TM and SB100X plasmids, using the nucleofector kit V and Amaxa IIb nucleofector device (Lonza, Basel, Switzerland). After 7-10 days expansion, stably transfected TMA-TM$^+$ cells were purified by staining with a mouse anti-human TMA antibody and FITC-labeled goat anti-mouse IgG antibody, followed by immunoselection with anti-FITC magnetic beads (Miltenyi Biotec). After two rounds of immunoselection, CEM and HEK-293 cell lines stably expressing the TMA-TM fusion protein on >90% of cells were obtained. These cell lines were used as targets to assess CARs functional activity.

Anti-TMA CAR transposon plasmids

We generated two plasmids encoding two different anti-TMA CAR constructs. The two plasmids that express the same anti-TMA single chain fragment variable (scFv) fused to different trans-membrane and intracellular signaling domains were synthesized by GeneArt (Thermo Fisher). They were then subcloned into the pT4 vector, that carries the SB transposase recognition sequences (LIR-RIR) upstream and downstream from the CARs. Cloning was carried out using the NEBuilder HiFi DNA assembly cloning kit (NEB, Ipswich, UK), PCR primers and a high-fidelity polymerase to produce and assemble the two fragments (the CAR cDNA and the pT4 vector).

The final plasmids were called CAR-TMA-BG4 and CAR-TMA-BG5. The CAR-TMA-BG4 construct expresses the anti-TMA scFv cloned upstream of CD8 spacer and transmembrane sequence, followed by 4-1BB-CD3ζ signaling domains, under the EF1α promoter. The CAR-TMA-BG5 construct expresses the same anti-TMA scFv cloned upstream of the human IgG1 hinge and CH2-CH3 domain as spacer element, followed by CD28 transmembrane and CD28-OX40-CD3ζ signaling modules, under the pTMNDU3 promoter. The SB100X transposase plasmid pCMV(CAT)T7-SB100 was a gift from Zsuzsanna Izsvak (Addgene plasmid #34879; http://n2t.net/addgene:34879; RRID:Addgene_34879).[32]

Plasmids were verified by sequencing and purified with the Maxiprep plasmid isolation kit (Invitrogen, Thermo Fisher Scientific) following the manufacturer's instructions.

Generation of anti-TMA CARCIK cells

CARCIK-TMA cells were generated as previously described.[20] Briefly, $10x10^6$ PBMCs were co-transfected at day 0 with pT4-CAR-TMA-BG4 or pT4-CAR-TMA-BG5 and SB100X plasmids, using the Human T cell nucleofector kit (Lonza) and the Amaxa IIb nucleofector device (Lonza). After transfection, cells were transferred to a 12-well plate containing 3 ml of pre-warmed RPMI 1640 advanced medium (Gibco, Thermo Fisher Scientific), 20% FBS (Euroclone) and 2mM L-Glutamine (Euroclone). Four hours after transfection 1 ml of medium was removed from each well and replaced with fresh complete medium (RPMI advanced medium with 10% FBS, 2mM L-Glutamine and 100µM gentamycin) supplemented with 1000 U/mL IFN-γ (Clinigen Healthcare Ltd). Twenty-four hours after IFN-γ addition, 300 U/ml recombinant human interleukin 2 (rhIL-2, Proleukin, Clinigen Healthcare Ltd) and 50 ng/ml anti-CD3 (OKT-3, TakaraBio, Kyoto, Japan) were added to each well. Fresh medium and rhIL-2 were added twice a week and cell concentration was maintained at $0.75x10^6$ cells/ml. Ten days after transfection, anti-TMA CAR^+ cells were immunoselected by staining CAR^+ cells with the poly-histidine tagged recombinant human TMA protein (R&D systems), followed by the anti-histidine FITC antibody and anti-FITC magnetic beads (Miltenyi Biotec) and passage through a separation column. The positive fraction was collected and maintained in culture as above until day 21. The cell products were called CARCIK-TMA4 (in case of transfection with pT4-CAR-TMA-BG4 plasmid) or CARCIK-TMA5 (in case of transfection with pT4-CAR-TMA-BG5

plasmid). As control, unmodified CIK cells were expanded in parallel from the same donors without transfection step.

CARCIK-TMA immunophenotyping

CARCIK-TMA cells were characterized with the following MAbs: anti-CD3-PerCP-Cy5.5 (SK7 clone), anti-CD56-BV510 (NCAM16.2 clone), anti-CD4-PE-Cy7 (SK3 clone), anti-CD8-APC-H7 (SK1 clone), anti-CD45RA-FITC (L48 clone), anti-CD62L-APC (SK11 clone) (all from BD biosciences). CAR detection was achieved by incubating cells first with the poly-histidine tagged recombinant human TMA protein (R&D systems), followed by FITC, APC or PE-conjugated anti–histidine antibody (Miltenyi Biotec). A FACScanto II flow cytometer device (BD Biosciences) was used to analyze the samples with BD FACSDiva Software.

Cytotoxicity assay

Cell lysis was evaluated using the GFP-certified™ Apoptosis/Necrosis detection kit (Enzo Life Science, Farmingdale, NY, USA). TMA^{+} target cell lines were first labeled with the green fluorescent dye 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE; Sigma-Aldrich, Merck KGaA). The CEM and HEK-293 cell lines stably transfected with TMA-TM were stained with 0.5 μM CFSE immediately prior to testing, while the HT-29 and MDA-MB-231 cell lines, naturally expressing TMA, were stained with 1 μM CFSE, plated and allowed to reach confluency for four days in culture. CARCIK-TMA and unmodified CIK cells were then co-cultured with CFSE-labeled targets at either a 5:1 effector:target (E:T) ratio for 4

hours (for CEM/HEK-293-TMA-TM^+ targets) or a 1:1 E:T ratio for 24 hours (for TMA^+ HT-29 or MDA-MB-231 cell lines). At the end of the assay, cells were collected, washed and stained with the Apoptosis Detection Reagent (Annexin V-Enzo Gold) and the Necrosis Detection Reagent for 10 minutes. Finally, cells were analyzed by flow cytometry. The percentage of dead cells was determined by calculating the overall percentage of Annexin V^+ and Necrosis Detection $Reagent^+$ in $CFSE^+$ target cells co-cultured with the effectors minus the spontaneous lysis of target cells alone. Maximal lysis was achieved by adding Apoptosis Inducer (staurosporine) in positive control wells.

Proliferation assays

The proliferation of CARCIK-TMA cells following target recognition was evaluated using CFSE. Briefly, CIK and CARCIK-TMA cells were stained with 1 μM CFSE for 10 minutes at 37°C. After washing with complete medium they were plated at 0.4×10^6 cells/well in presence or absence of target cells at 10:1, 1:1 and 1:10 E:T ratios. To test proliferation in co-culture with HT-29 and MDA-MB-231 cell lines, target cell lines were plated overnight prior adding CARCIK-TMA to let them adhere to plastic and express TMA extracellularly. After 4 days co-culture, cells were collected and stained with CD3-PE, CD4-PE-Cy7 and CD8-APC-H7 antibodies (BD Bioscience) and CFSE expression was analyzed on different populations by flow cytometry, using the ModFit LT™ software to calculate the proliferation index.

Intracellular cytokine staining

The ability of CARCIK-TMA cells to produce cytokines in response to target cells was evaluated by intracellular staining and flow cytometry. HT-29 and MDA-MB-231 cells were plated for four days prior to testing to reach confluency and express TMA extracellularly. Effector and target cells were co-cultured for six hours at a 1:1 E:T ratio in presence of BD GolgiStop solution (BD Bioscience). Cells were then collected, fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Bioscience) following the manufacturer's instructions and stained with CD3-PerCP-Cy5.5, CD4-PE-Cy7, CD8-APC-H7 (BD Bioscience), IFN-γ-FITC and IL-2-PE antibodies (Miltenyi Biotec). The samples were then analyzed by flow cytometry.

TMA^{+} tumor models *in vivo*

Groups of 8 athymic nude mice (7 weeks-old females, Hsd:Athymic Nude-*Foxn1*^{nu}, Envigo, Indianapolis, Indiana, USA) were inoculated subcutaneously with 10x10^{6} HT-29 cells. When tumors reached about 200 mg, mice were randomized in four groups, one control group received PBS only, one 15x10^{6} unmodified CIK cells, the last two groups received CARCIK-TMA5 cells at 5 or 15x10^{6} cells/mouse. Mice were monitored for tumor growth and were humanely killed when tumors reached 2g of weight and autopsied.

Groups of 8 NOD-SCID mice (6 weeks-old females, NOD.CB17-*Prkdc*^{scid}/NCrHsd, Envigo) were also inoculated subcutaneously with 5x10^{6} MDA-MB-231 cells or BJAB cells. Formalin fixed paraffin embedded (FFPE) tumor specimens were analyzed by

immunohistochemistry to assess TMA expression. Experiments *in vivo* were authorized by the local ethical committee on animal experimentation and the Italian Ministry of Health. Animals were handled in accordance with European laws on animal experimentation.

Statistical analyses

Results were compared using the Student's *t*-test. A *p* value <0.05 (*) was considered significant.

Results

TMA expression in cell lines and primary cells

TMA is an extracellular matrix protein with a specific expression in adult tissues, related to inflammation, repair and tumors. In order to characterize TMA expression and identify primary cells and cell lines that could be used as targets for anti-TMA therapies, we have screened for TMA expression 20 cell lines derived from hematological tumors, either of B- and T-cell origin, and 6 cell lines derived from solid tumors. Moreover, we have tested 4 types of primary cells, specifically BM and UC derived MSCs and monocytes-derived macrophages, polarized with cytokines towards either the M1 or M2 phenotype. TMA expression was measured at the protein level by surface and intracellular flow cytometry and immunofluorescence and at the RNA level by RT-qPCR. The results of TMA expression analyses of cell lines and primary cells using these different methods are summarized in Table 1, 2 and 3, respectively.

Among hematological cell lines tested, all T lymphoma and B-ALL cell lines tested negative by surface flow cytometry (Table 1). In contrast, more mature B cell lines derived from chronic lymphocytic leukemia (CLL), B cell non-Hodgkin lymphoma (B-NHL) or multiple myeloma (MM) showed a variable pattern of surface TMA expression, with several cell lines showing weak to medium levels of expression. The highest TMA positivity was observed on the mantle cell lymphoma cell line (MCL) Jeko1 and Burkitt's lymphoma cell line BJAB. TMA surface expression was confirmed with two different anti-TMA MAbs targeting different epitopes of the protein. In contrast RT-qPCR tested negative for nearly all hematopoietic cell lines, except weak expression in the CLL and MCL cell lines (Table 1).

With regard to solid tumors, among the 5 cell lines tested, TMA positivity was observed by intracellular flow cytometry and in situ immunofluorescence in 2 cell lines: the colon carcinoma cell line HT-29 showed relatively weak expression, whereas the breast cancer cell line MDA-MB-231 showed high expression (Table 2). The confocal microscopy images for these two cell lines are shown in Figure 1. Finally, TMA was also clearly expressed at the RNA level by qPCR.

The primary cells investigated were all positive for TMA expression (Table 3), detected by flow cytometry and in situ fluorescent microscopy. The highest staining was detected on the BM-derived MSCs and M2 macrophages. RT-qPCR confirmed the high TMA RNA expression in BM-MSCs (Table 3).

Generation of stably transfected cell lines expressing TMA-TM fusion protein

In order to generate target cells stably expressing the TMA antigen on their cell surface, to be used in functional assays with CARCIK-TMA, the T lymphoma cell line CEM and the embryonic kidney HEK-293 cell lines were transfected with a plasmid carrying the cDNA for the TMA domain recognized by the anti-TMA MAb, fused to the HB-EGF transmembrane domain, called TMA-TM, and the SB transposase plasmid. After transfection and immunoselection of the positive cells, two cell lines were obtained stably expressing on more than 90% of the cells the TMA-TM fusion protein on their surface (Figure 2A-B). The TMA-TM expression was confirmed by surface flow cytometry and, for HEK-293, by immunofluorescent staining, following the same protocols and anti-TMA antibodies used for the detection of the native protein (Figure 2C).

Cloning of CAR-TMA carrying different signaling modules

In order to generate an anti-TMA CAR, we have cloned the V_H and V_L sequences from the sequence of anti-TMA MAb, fused together with a linker. This anti-TMA scFv was then inserted upstream of two different CAR backbones, already used in our laboratory, and carrying 2 different spacer, trans-membrane and intracellular signaling modules. The first construct, CAR-TMA-BG4, contains the CD8 hinge and trans-membrane domains and the 4-1BB and CD3ζ signaling domains. The CAR-TMA-BG4 structure is transcribed from the EF1α promoter and resembles the clinically approved anti-CD19

CAR designed by Novartis (Tisagenlecleucel)[33]. The second construct, CAR-TMA-BG5, has the human IgG1 hinge and CH2-CH3 domain as spacer element, followed by the CD28 trans-membrane and signaling domains and finally the OX-40 and CD3ζ signaling domains. The CAR-TMA-BG5 structure is transcribed from the pTMNDU3 promoter and recapitulates the anti-CD19 CAR used in the CARCIK-CD19 clinical trial[20] (Figure 3A). The cDNA coding for each CAR structure was successfully cloned into a pT4 vector in between the SB100X transposase recognition sites. The overall structure of the 2 anti-TMA CARs are shown schematically in Figure 3A.

Generation of CARCIK-TMA cells and their characterization

We next generated CIK cells genetically modified with the 2 different anti-TMA CARs, using the transfection protocol previously optimized for CARCIK-CD19 cells, already used in clinic. $10x10^6$ PBMCs were transfected by electroporation with the vectors carrying the 2 different CAR-TMA (CAR-TMA-BG4 and CAR-TMA-BG5) together with the SB100X transposase plasmid. Cells were then cultured in CIK conditions for about 21 days.

Both CARCIK-TMA4 and CARCIK-TMA5 cells showed efficient expansion, reaching after 21 days a mean $221x10^6$ and $233x10^6$ total nucleated cells, respectively (Figure 3B). The expression levels of CAR-TMA-BG4 and CAR-TMA-BG5 were stable and after 21 days reached values of 34.5% and 43.8%, respectively (Figure 3C), with a Mean Fluorescent Intensities (MFI) of 24655 and 11657, respectively (Figure 3D). CAR-BG4 is expressed at a slightly lower percentage,

but with a significantly higher fluorescent intensity than CAR-BG5 ($p<0.05$).

Considering the variability observed in CAR expression between experiments and between the two different CARs, we decided to routinely purify CAR$^+$ cells half-way during culture, in order to perform functional assays on CARCIK cells expressing comparable amounts of CARs. The efficiencies of CARCIK-TMA4 and CARCIK-TMA5 purifications were comparable, and products expressed >90% of CAR-TMA in all cases (data not shown). Figure 3E shows an example of CAR expression histograms by flow cytometry.

We next characterized the CARCIK cell products in terms of immunophenotype, in particular CD4, CD8, CIK and effector-memory phenotype. The CD4$^+$, CD8$^+$ and CD3$^+$CD56$^+$ subpopulations were comparable between both CARCIK-TMA and unmodified CIK cells (Figure 3F). Regarding effector-memory phenotype, CIK, CARCIK-TMA4 and CARCIK-TMA5 cells were similar for naïve and EMRA subpopulations. In contrast, the central memory (CM) population was significantly higher for both CARCIK-TMA, being a mean of 63.5% for CARCIK-TMA4 and 61.2% for CARCIK-TMA5, compared to unmodified CIK cells (30.3%) ($p<0.05$). The percentage of effector memory cells (EM) was lower represented in both CARCIK-TMA compared to unmodified CIK cells (respectively 28.4%, 35.2% compared to 67.6%, $p<0.05$, Figure 3G).

Taken together, these data demonstrate that the CARCIK-TMA cells culture method is effective, and that their phenotypes do not differ significantly from unmodified CIK cells, except for few parameters. In particular, the transfection of CARCIK-TMA, in either form,

induced a more central memory rather than effector memory phenotype, compared to untransduced CIK.

Purified CARCIK-TMA cells were used for subsequent functional studies and using unmodified CIK cells from the same donors as negative control.

Cytotoxic activity of CARCIK-TMA cells against TMA-TM$^+$ stably transfected cell lines

Firstly, CARCIK-TMA4 and CARCIK-TMA5 cells were evaluated for their ability to mount effective cytotoxic responses *in vitro* using the two TMA-TM$^+$ stably transfected cell lines as targets. Both CARCIK-TMA cells were cytotoxic to a similar extent against the T lymphoma cell line CEM TMA-TM$^+$ and the activity was in both cases significantly higher compared to unmodified CIK cells ($p<0.01$, Figure 4A). When tested against the embryonic kidney cell line HEK-293 TMA-TM$^+$, both CARCIK-TMA4 and CARCIK-TMA5 showed a lower cytotoxic activity compared to CEM TMA-TM$^+$ and only CARCIK-TMA5 cells were still significantly more cytotoxic than unmodified CIK cells ($p<0.05$, Figure 4A).

This demonstrate that CARCIK-TMA cells are able to bind and kill target cells expressing the recombinant antigen on the plasma membrane and that CARCIK-TMA5 cells are generally more cytotoxic than CARCIK-TMA4 cells.

Cytotoxic activity of CARCIK-TMA cells against cell lines naturally expressing TMA

We then tested the cytotoxic activity of CARCIK-TMA cells against the native form of TMA expressed by solid cancer cell lines. In order to do this, the colon adenocarcinoma HT-29 and the breast cancer MDA-MB-231 cell lines were first cultured for four days to allow TMA to be secreted in the cellular microenvironment and some ECM to be formed (see Figure 1). CARCIK-TMA cells were then added at a 1:1 E:T ratio and target cell killing evaluated after further 24 hours. Both CARCIK-TMA4 and CARCIK-TMA5 cells were significantly more cytotoxic than unmodified CIK cells against MDA-MB-231 cell lines ($p<0.05$, Figure 4B). In contrast, only CARCIK-TMA5 cells were significantly cytotoxic against the HT-29 cell lines, this activity being significantly higher compared either to unmodified CIK cells or CARCIK-TMA4 cells ($p<0.05$, Figure 4B).
These data demonstrate that both CARCIK-TMA4 and -TMA5 can be cytotoxic against tumor cell lines naturally expressing TMA and confirm a higher cytotoxic activity of CARCIK-TMA5 compared to CARCIK-TMA4.

Proliferation of CARCIK-TMA cells in response to TMA$^+$ target

The proliferative response of CARCIK-TMA cells to the TMA antigen was evaluated using the CFSE assay. CARCIK-TMA4 and CARCIK-TMA5 were labelled with CFSE and co-cultured with either TMA-TM$^+$ stably transfected CEM or native TMA$^+$ MDA-MB-231. In both cases CARCIK-TMA5 showed a higher proliferation index compared to unmodified CIK cells or CARCIK-TMA4 cells, although

the difference was not always significant (Figure 5A and B). Proliferation was in all cases higher at low E:T ratio (1:10) than at the higher E:T ratio (10:1), indicating that CARCIK-TMA5 cells proliferated significantly more in presence of higher amount of target cells and therefore of antigen. CARCIK-TMA4 showed only modest proliferative activity in all cases (Figure 5A and B).

IFN-γ and IL-2 release by CARCIK-TMA in response to TMA$^+$ target cells

The cytokines released by CARCIK-TMA4 and -TMA5 cells in presence of target antigen was evaluated after 6 hours co-culture with TMA-TM$^+$ stably transfected CEM or native TMA$^+$ MDA-MB-231 target cell lines. The release of IFN-γ was evaluated on CD8$^+$ cells. In presence of both CEM TMA-TM$^+$ and MDA-MB-231, CARCIK-TMA5 produced more IFN-γ than unmodified CIK cells ($p<0.05$, Figure 6A) and more than CARCIK-TMA4, although the latter was not significant in all conditions.

The release of IL-2 was evaluated on CD4$^+$ cells. There was a tendency for CARCIK-TMA5 to induce IL-2 more than CIK or CARCIK-TMA4 cells, but statistical significance was not reached (Figure 6B).

To summarize *in vitro* results, the data shown suggest that CARCIK-TMA5 cells are overall more effective *in vitro* than CARCIK-TMA4 cells in terms of cytotoxicity, proliferation, and cytokine release. Nonetheless, CARCIK-TMA4 cells are more effective than untransduced CIK cells in most of these functions.

In vivo TMA$^+$ models

In order to determine whether CARCIK-TMA cells are effective *in vivo* without inducing significant toxicity, we set up several human tumor models in immunodeficient mice. The original anti-TMA Mab cross-reacts with murine TMA (data not shown), so that anti-TMA CARs should recognize both murine TMA in the tumor microenvironment as well as human TMA secreted by human tumors *in vivo*.

Athymic nude mice were inoculated subcutaneously with HT-29 cell line, whereas NOD-SCID mice were used as recipient for MDA-MB-231 and BJAB cell lines. Tumor growth was monitored during time to assess the optimal time point for mice randomization and CARCIK-TMA cells infusion. The growth curves of the 3 models are shown in Figure 7A, demonstrating steady increase of all tumors during 25-30 days period following inoculation.

In a preliminary efficacy/toxicity pilot experiment, groups of mice inoculated with HT-29 were also treated with 2 different doses of CARCIK-TMA5 cells ($5x10^6$ and $15x10^6$), given when tumors had reached 200mg (day +7). Control mice were inoculated intravenously with $15x10^6$ non-transduced CIK cells. The first important observation is that no toxicity was observed in CARCIK-TMA5 treated mice compared to CIK or untreated controls, animals did not experience body-weight reduction and did not show any other sign of sufferance (data not shown). Moreover, mice which received CARCIK-TMA5 cells reported a significant delay in tumor growth at 3 days after cell infusion, compared to controls (Figure 7B). This effect was temporary and rapid tumor growth was observed in all animals at later time

points. This result is however a preliminary indicator that CARCIK-TMA cell may have anti-tumor activity. Based on these data, in the next experiments 2 or more doses of CARCIK-TMA will be administrated at 1 week interval and treatment will start as soon as tumors will become palpable.

Discussion

CAR-T cells have achieved impressive results in the treatment of B-ALL patients, however limited results have been obtained against solid tumors. Major limitations in developing CAR-T therapy for non-hematologic malignancies are the target molecule selection and the need for CAR-T cells to infiltrate efficiently the tumor, which is often poorly permissive to immune cells.[17] The TME is an immunosuppressive microenvironment in which cells and the ECM collaborate to avoid immune cells response against the tumor, either by inhibiting immune cells activation and by physically blocking their access to the tumor.[34]

We have selected as a novel target a molecule over-expressed at embryonic level and in adults in inflammatory context and for which antibodies tested *in vivo* were already available.[35–37] We have called this selected ECM molecule tumor microenvironment antigen (TMA), since its identity cannot be revealed at this stage for patenting reasons. Based on the available antibody sequence, we then developed two CAR constructs directed against TMA. We have chosen CIK as effector cells to be genetically modified with the novel CARs. Finally, we have produced and tested anti-TMA CARCIK cells *in vitro* and *in vivo*.

Firstly, we have screened different cell lines and primary cells to identify suitable targets for testing CARCIK-TMA activity. Most leukemia and lymphoma cell lines did not express TMA either on their surface or intracellularly or by PCR. Several mature B cell lines expressed surface TMA, but little TMA RNA was detected by PCR. Only 2 MCL cell lines also expressed TMA at both protein and RNA level. Among solid tumor cell lines tested, HT29 and MDA-MB-231 expressed TMA by both cytoplasmic staining and PCR, at low and high level, respectively. These 2 cell lines were therefore chosen as targets for CARCIK. Furthermore, to simplify the CAR testing in functional assays, we have also generated two cell lines, CEM and HEK-293, expressing on their surface a recombinant protein composed of the TMA domain, recognized by the CARs, fused to a trans-membrane domain. Finally, we have analyzed expression of TMA in primary cells likely to be present in the tumor microenvironment: MSCs and macrophages. We indeed confirm that these cells express TMA at quite high levels. Interestingly, M2 type macrophages show more intense staining than M1 cells. This can at least in part explain high expression of TMA in tumors since tumor infiltrating macrophages (TAM) are mostly M2.[38]

We have then constructed 2 different anti-TMA CARs, composed of the same anti-TMA scFv but different spacer, transmembrane and intracellular signaling modules. These two CARs were based on anti-CD19 CARs already used in the clinic, one based on the CARCIK-CD19 from the Monza group[20] (CAR-TMA5) and one on Tisagenlecleucel (Novartis)[33] (CAR-TMA4). These were stably introduced in CIK cells using transfection of transposon plasmids

expressing the CARs and SB100 transposase. Both CARs were expressed on transfected CIK cells, as shown by flow cytometry detection with a recombinant TMA fragment, although CAR-TMA4 was expressed at slightly lower levels. This result is similar to previous data generated with similar CARs, directed against CD19 (see chapter 2 of this thesis). In order to carry out functional assays, we routinely purified CARCIK-TMA cells during culture to work with effector cells >90% pure in all cases.

We then tested the *in vitro* activity of CARCIK-TMA cells in terms of cytotoxicity, proliferation in response to antigen binding and cytokines release in response to the fusion protein TMA-TM$^+$ and the native TMA$^+$ cell lines. Both CARCIK-TMA4 and 5 cells were highly cytotoxic against the stably transfected TMA-TM$^+$ cell lines, confirming the ability of the constructs to recognize TMA and mount an immune response. Both CARCIK-TMA cell products were also cytotoxic against the native TMA$^+$ cell lines, with higher efficacy of CARCIK-TMA5 over CARCIK-TMA4, particularly when TMA expression on the target cells was lower. Both CARCIK-TMA4 and 5 also proliferated in response to TMA$^+$ targets. Again, the response of CARCIK-TMA5 tended to be higher than that of CARCIK-TMA4, but this was not always statistically significant. Similar results were obtained for IFN-γ release by CD8$^+$ cells or IL-2 induction by CD4$^+$ cells. Also these results are in line with those obtained with the similar CAR constructs directed against CD19 (see chapter 2 of this thesis). This confirms that *in vitro* response of the construct based on the molecule developed in Monza is more efficient than that of the

Novartis-like molecule. Whether this higher activity *in vitro* will translate *in vivo* is unclear and remains to be determined.

The CAR-TMA-BG5 construct contains the intracellular signaling domains from CD28, OX40 and CD3ζ, while the CAR-TMA-BG4 contains the 4-1BB and CD3ζ signaling modules. Comparison of the activity of different anti-CD19 CAR-T cells signaling domains have been reported in the literature.[39,40] In agreement with these studies, CD28 induces a higher release of cytokines compared to 4-1BB *in vitro*, while 4-1BB based constructs have a lower activity *in vitro*, but is associated to a better persistence *in vivo* in patients.[41,42] *In vivo* experiments to test our two CARCIK-TMA products are in progress, to verify this hypothesis.

In a first phase we have set up several *in vivo* models using tumor cell lines, which tested positive for TMA expression *in vitro* and were known to be highly infiltrated with macrophages *in vivo*. The 3 models, using B-lymphoma line BJAB and TMA^+ solid cancer cell lines HT29 and MDA-MB-231 have been successfully set up.

In the case of HT-29 model in nude mice, the *in vivo* efficacy and toxicity of CARCIK-TMA5 cells was also tested in a preliminary experiment. A single infusion of $15x10^6$ CARCIK-TMA5 cells/mouse induced a specific but short-lived block in tumor growth for the first three days after treatment. Furthermore, $15x10^6$ CARCIK-TMA5 cells/mouse did not induce any observable toxicity in nude mice, even though the anti-TMA CARs cross-reacts with murine TMA. CARCIK-TMA activity *in vitro* and preliminary results *in vivo* suggest therefore that TMA is a good candidate target antigen for solid tumors. The efficacy of the two different anti-TMA CARs will

be evaluated *in vivo* against the three human tumor models that we have set up, planning at least two CARCIK-TMA cell infusions to control tumor growth. Also, specific analyses will be performed to verify infiltration of the tumor by CARCIK-TMA cells and confirm the lack of on-target off-tumor toxicities of the treatment in mice.

Other published studies are aimed at targeting the tumor microenvironment using CAR-T cells. For example, CAR-T cells have been developed against cancer associated fibroblasts (CAFs) targeting the fibroblast associated protein (FAP), a membrane-bound protease overexpressed by CAFs.[43] These studies demonstrate the importance and the feasibility of targeting the TME. Indeed, the advantage of targeting the TME is the possibility to find an antigen overexpressed on many solid tumors, which usually lack specific tumor antigens. Furthermore, targeting the TME may revert the solid tumor “cold” status, since CAR-T or CARCIK cells may release cytokines that can attract and activate other immune cells inducing inflammation. Finally, it is possible to develop in the future a multiple targeting strategy based on anti-TMA CAR that could associate TME antigen with a specific tumor antigen, in order to reach the tumor and overcome the physical barrier thanks to the TME antigen and hit at the same time specifically tumor cells.

Acknowledgments

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Tables and Figures

Table 1 – TMA expression on hematopoietic cell lines in suspension.

Disease	**Cell line**	**Flow Cyt.**	**PCR**
		Non-perm	**RNA**
Acute leukemia	REH *(B-ALL)*	-	-
	TOM-1 *(B-ALL)*	-	-
	697 *(B-ALL)*	-	-
Chronic leukemia	MEC1 *(CLL)*	+	+/-
B-NHL	GRANTA 519 *(MCL)*	+	+/-
	JEKO1 *(MCL)*	++	+
	DOHH2 *(DLBCL)*	++	-
	DHL4 *(DLBCL)*	-	-
	Albanes *(Burkitt)*	-	-
	NAMALWA *(Burkitt)*	+	-
	BJAB *(Burkitt)*	++	-
	AS283A *(Burkitt)*	+	-
	IM9 *(EBV-LCL)*	-	-
Plasma cells neoplasia	KMS 12 *(MM)*	-	-
	JJN3 *(MM)*	+	-
	RPMI 8226 *(MM)*	-	-
	OPM2 *(MM)*	-	-
T cell lymphoma	HH *(CTCL)*	-	nd
	Karpas 299 *(ALCL)*	-	-

	HuT 78 *(TCL)*	-	nd

Legend: nd: not done; Flow Cyt: flow cytometry; B-ALL: B cell acute lymphoblastic leukemia; CLL: chronic lymphocytic leukemia; B-NHL: B cell non-Hodgkin lymphoma; MCL: mantle cell lymphoma; DLBCL: diffuse large B cell lymphoma; EBV-LCL: Epstein-Barr virus derived lymphoblastoid cell line; MM: multiple myeloma; CTCL: cutaneous T cell lymphoma; ALCL: anaplastic large cell lymphoma; TCL: T cell lymphoma.

Table 2 – TMA expression on solid tumor cell lines in adhesion.

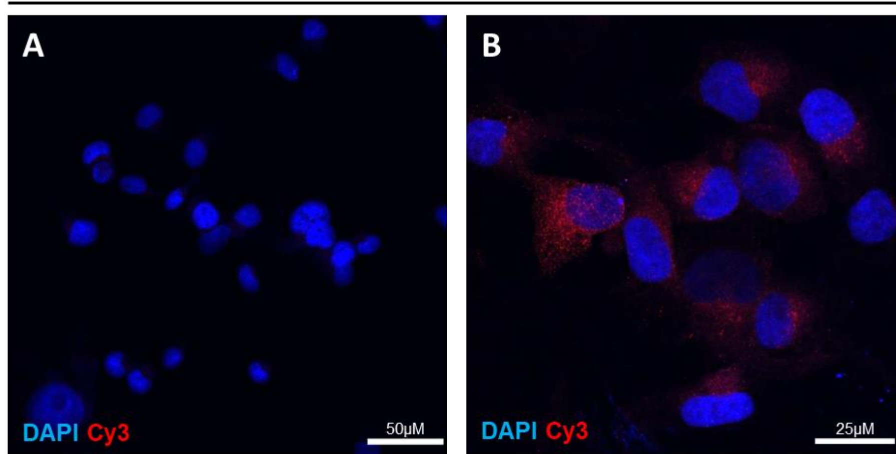
Disease	Cell line	Flow Cyt.	IF	PCR
		Perm	Non-Perm	RNA
Solid tumors	HT-29 *(colon adeno-carcinoma)*	+	+	+
	MDA-MB-231 *(breast cancer)*	++	++	++
	HepG2 *(hepatic carcinoma)*	-	-	nd
	HT-1080 *(fibrosarcoma)*	-	nd	nd
	MCF7 *(breast cancer)*	-	nd	nd
	3T3 *(mouse fibroblast)*	-	nd	nd

Legend: Flow Cyt: flow cytometry; IF: immunofluorescence on fixed cells; nd: not done.

Table 3 – TMA expression on primary cells.

Primary cells	Flow Cyt.	IF	PCR
	Perm	**Non-Perm**	**RNA**
BM-MSC	++	++	++
UC-MSC	+	+	nd
Macrophages M1	+	+	nd
Macrophages M2	++	++	nd

Legend: Flow Cyt: flow cytometry; IF: immunofluorescence on fixed cells; nd: not done; BM-MSC: bone marrow derived mesenchymal stromal cells; UC-MSC: umbilical cord derived mesenchymal stromal cells.



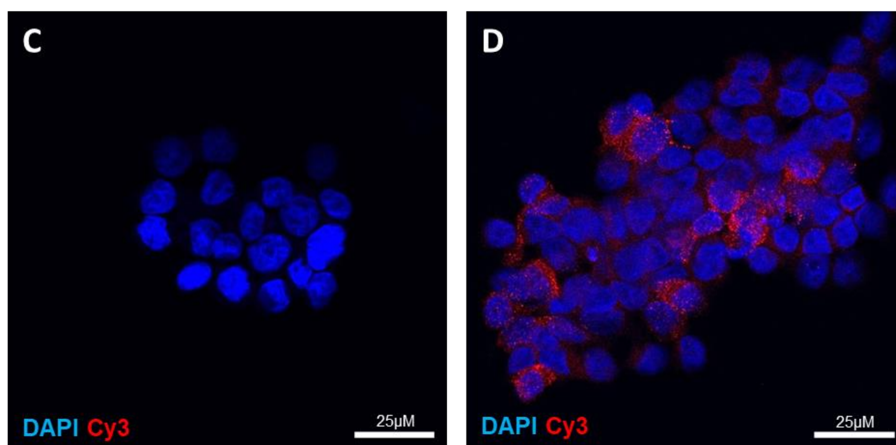


Figure 1 – TMA protein visualization.

In situ immunofluorescent staining of extracellular TMA expression on MDA-MB-231 (A-B) and HT-29 (C-D) cell lines. In red is Cy3 fluorochrome for TMA staining and in blue is DAPI for nuclei visualization. Cells have been cultured four days prior staining to obtain protein secretion and extracellular matrix deposition. Figure A and C are negative controls.

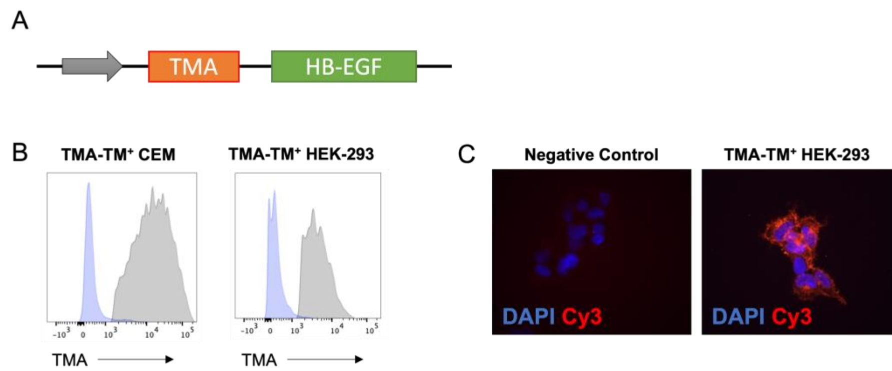


Figure 2 – Generation of two TMA-TM$^+$ cell lines. (A) TMA-TM plasmid construct schematic representation. (B) Flow cytometry histograms of TMA-TM expression on stably transfected cell lines in grey and the isotype control in blue. (C) Immunofluorescent staining of TMA-TM$^+$ HEK-293 cells, Cy3 staining (in red) is for TMA and DAPI (in blue) is for nuclei visualization. Images have been acquired with the inverted fluorescent microscope (63X).

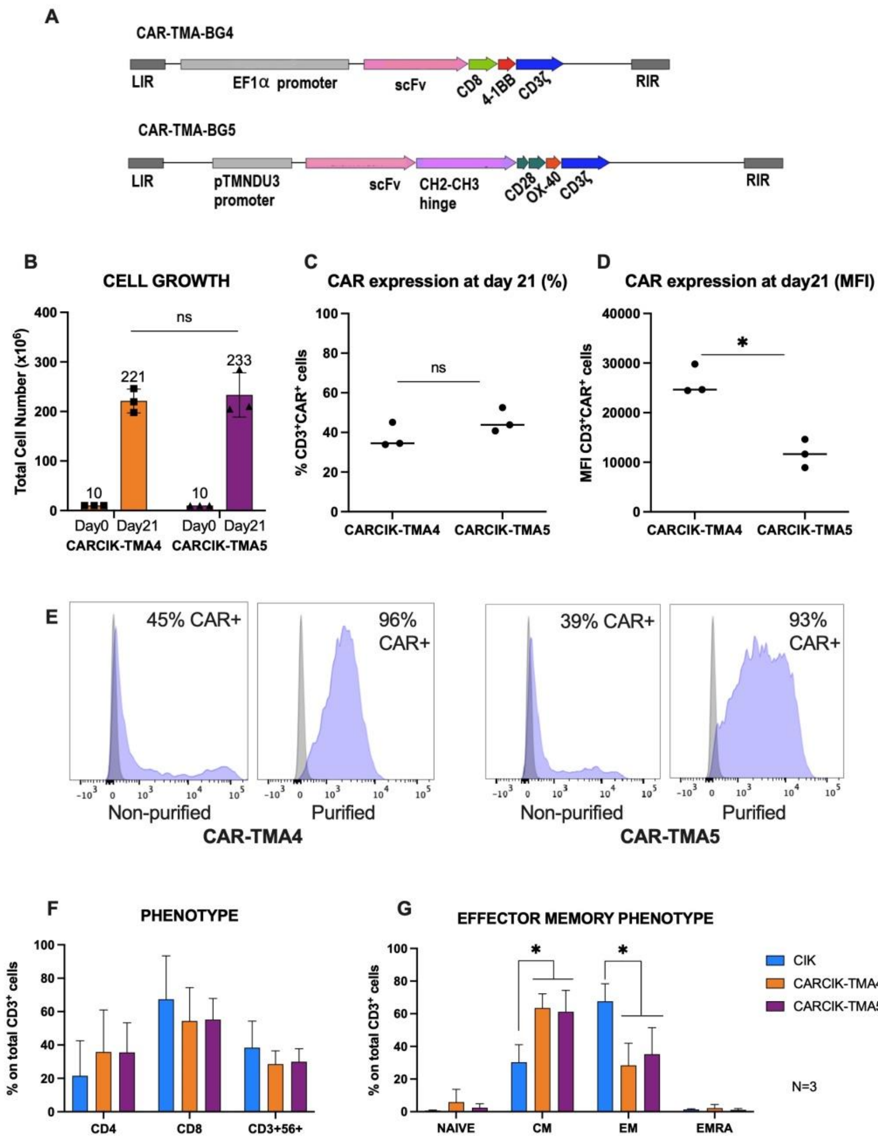

A
CAR-TMA-BG4
LIR
EF1α promoter
scFv
CD8
4-1BB
CD3ζ
RIR
CAR-TMA-BG5
LIR
pTMNDU3 promoter
scFv
CH2-CH3 hinge
CD28
OX-40
CD3ζ
RIR
B
CELL GROWTH
Total Cell Number (x10^6)
ns
10
221
10
233
Day0
Day21
Day0
Day21
CARCIK-TMA4
CARCIK-TMA5
C
CAR expression at day 21 (%)
% CD3+CAR+ cells
ns
CARCIK-TMA4
CARCIK-TMA5
D
CAR expression at day21 (MFI)
MFI CD3+CAR+ cells
*
CARCIK-TMA4
CARCIK-TMA5
E
45% CAR+
96% CAR+
39% CAR+
93% CAR+
Non-purified
Purified
CAR-TMA4
Non-purified
Purified
CAR-TMA5
F
PHENOTYPE
% on total CD3+ cells
CD4
CD8
CD3+56+
G
EFFECTOR MEMORY PHENOTYPE
% on total CD3+ cells
NAIVE
CM
EM
EMRA
CIK
CARCIK-TMA4
CARCIK-TMA5
N=3

Figure 3 – Generation of CARCIK-TMA cells and their characterization.

(A) CAR-TMA schematic structure. (B) PBMCs were transduced with the CAR-TMA-BG4 and CAR-TMA-BG5 plasmids and expanded to CIK for 21 days. (C-D) CAR expression was analyzed at the end of culture by flow cytometry. (E) Examples of histograms of purified or non-purified CARCIK-TMA4 or -5 are shown. (F-G) Immunophenotype of the purified cellular products at the end of the culture, including effector-memory, was analyzed by flow cytometry. The results are the means and standard deviations of 3 experiments using different donors as starting material. The statistics refer to the comparison between CARCIK-TMA4 and CARCIK-TMA5 with unmodified CIK (*$p<0.05$).

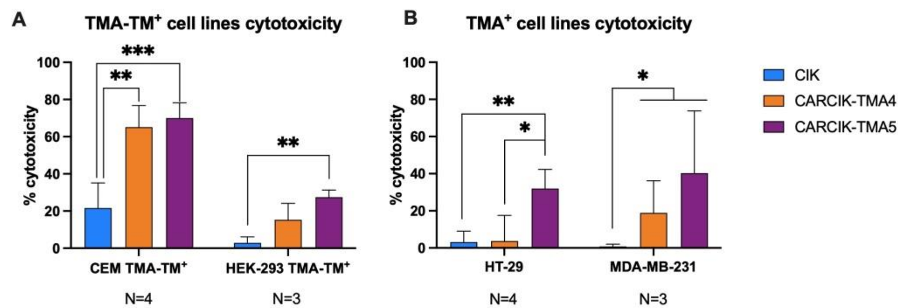


Figure 4 – Cytotoxic activity of CARCIK-TMA cells against the TMA$^+$ cell lines.

(A-B) Comparison of the killing activity *in vitro* using the apoptosis/necrosis detection assay. Unmodified CIK (blue bars), or purified CARCIK-TMA4 (orange bars) or CARCIK-TMA5 (purple bars) at the end of culture were used as cytotoxic effector cells against CEM TMA-TM$^+$ and HEK-293 TMA-TM$^+$ at 5:1 E:T ratio for 4 hours and against HT-29 and MDA-MB-231 at 1:1 E:T ratio for 24 hours. Percentages of cytotoxic activity are shown as mean and standard deviation of 3 to 4 experiments (*P<0.05, **P<0.01, ***P<0.001).

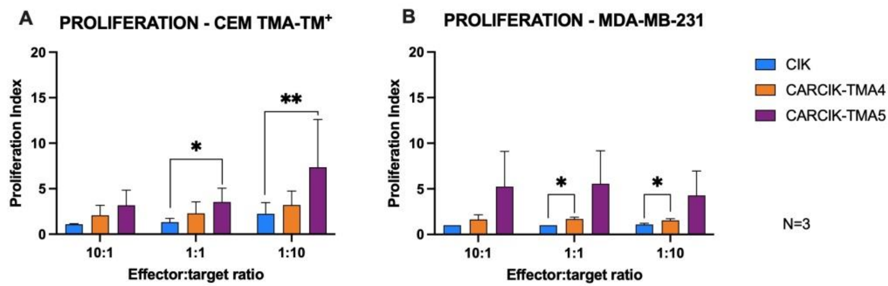


Figure 5 – Proliferation of CARCIK-TMA cells in response to TMA^{+} targets.

Comparison of the proliferation ability *in vitro* measured with CFSE staining by flow cytometry. Unmodified CIK (blue bars), or purified CARCIK-TMA4 (orange bars) or CARCIK-TMA5 (purple bars) products were co-culture with target cells for four days at 10:1, 1:1 and 1:10 E:T ratios. (A) Proliferation induced by CEM TMA-TM^{+}. (B) Proliferation induced by MDA-MB-231. Proliferation indexes are shown as mean and standard deviation of three experiments (*P<0.05, **P<0.01).

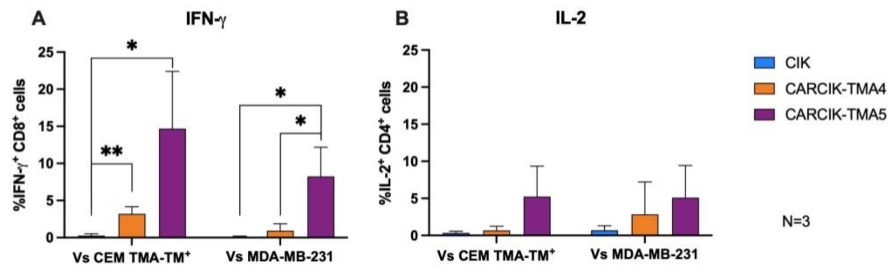


Figure 6 – IFN-γ and IL-2 release by CARCIK-TMA in response to TMA$^+$ target cells

IFN-γ (A) and IL-2 (B) production was determined by intracytoplasmic staining upon 6 hours co-culture at 1:1 E:T ratio of CIK (blue bars), purified CARCIK-TMA4 (orange bars) or CARCIK-TMA5 (purple bars) with CEM TMA-TM$^+$ and MDA-MB-231 cell lines. Percentages of positive cells are shown as mean and standard deviation of three experiments (*P<0.05, **P<0.01).

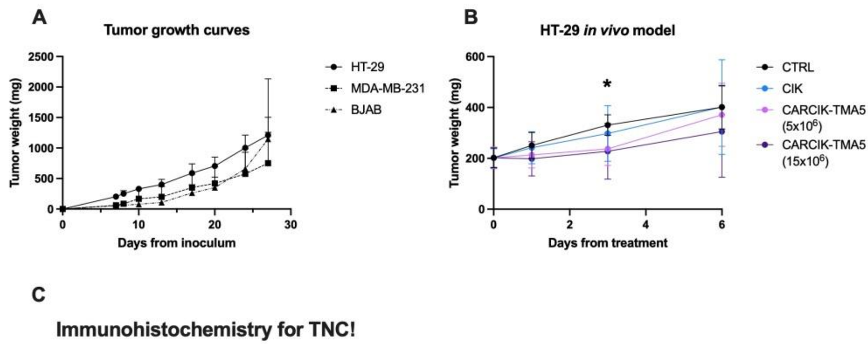


Figure 7 – *In vivo* TMA$^+$ models.

(A) Tumor growth curves of HT-29, MDA-MB-231 and BJAB cell lines subcutaneously inoculated in immunodeficient mice (athymic nude or NOD-SCID). (B) Mean tumor growth of HT-29 tumor model for the first six days after CIK or CARCIK-TMA5 cell infusion (n=8). P-values indicated refer to the differences between CARCIK-TMA5 treatments (5 and 15x10^6) compared to untreated control (*P<0.05).

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CHAPTER 4

Summary, conclusion and future perspectives

This PhD thesis project aimed to compare the functional activity of two strategies to improve cytokine induced killer (CIK) cells, bispecific antibodies (BsAbs) and Chimeric Antigen Receptor (CAR) genetic engineering. Firstly, we have compared these strategies against $CD19^+$ target cells, then a novel CAR has been developed to redirect CIK cells against solid as well as hematopoietic tumors, targeting an extracellular matrix (ECM) molecule.

Clinical trials have demonstrated that CIK cells infiltrate tumors *in vivo* and can lyse a broad array of tumor targets, inducing little if any graft versus host disease (GvHD).[1,2] Nonetheless, they have shown therapeutic activity only in low tumor burden context, so strategies to increase the therapeutic efficacy of CIK cells are required.[3,4]

In the first part of the work, we have compared the *in vitro* activity of CIK cells redirected against $CD19^+$ target cells through the BsAbs blinatumomab (CD3xCD19) or genetically engineered to express two different anti-CD19 CAR structures. The first CAR structure (CAR-CD19-MNZ) recapitulates the CAR expressed by CARCIK-CD19 cells developed by our collaborators at the Fondazione Matilde Tettamanti Menotti De Marchi Onlus[5,6], while the second (CAR-CD19-BG2) recapitulates the structure of the anti-CD19 CAR Tisagenlecleucel (Novartis)[7,8]. Both CARs were inserted in CIK cells using the Sleeping Beauty transposon system. CARCIK-MNZ, CARCIK-BG2 or unmodified CIK cells in presence of blinatumomab demonstrated to be more active *in vitro* compared to unmodified CIK cells alone. Functional activity was tested in terms of cytotoxicity against $CD19^+$ target cells, proliferation after antigen binding, cytokines secretion and activation of the NFAT and NF-kB signaling

pathways. The two CAR structures were generally similar in terms of activity, instead, CIK cells with blinatumomab seemed to be more active *in vitro* than CARCIK-CD19 cells, especially for the cytotoxic and proliferative activity. Experiments in animal models to compare these strategies *in vivo* are ongoing. Future results will complete this work and verify whether specific *in vitro* and *in vivo* activities correlate, and if so in what manner.

Similar strategies combining in vitro expanded T cells with BiTEs or other bsAbs have been proposed by others, either co-administered or stably transfected into T cells.[9–12]

Based on the findings obtained with CARs directed against CD19, already used in the clinic, we have extended the approach to apply such "optimized" CIK cells to target solid tumors. In the second part of the thesis, we therefore focused on an ECM protein, overexpressed in different tumor types, that we called TMA (tumor microenvironment antigen), whose identity cannot be revealed at present for patenting reasons. We developed two novel anti-TMA CAR constructs based on the two anti-CD19 CARs initially developed and studied also in our laboratory, described above. The CARs shared the same anti-TMA single chain fragment variable (scFv), derived from an existing monoclonal antibody (MAb), and differed in their backbone, the first recapitulating the structure and signaling domains of CAR-CD19-BG2, called CAR-TMA-BG4 (based on the Tisagenlecleucel sequence), and the second carrying the same structure and signaling domains of the locally developed CAR-CD19-MNZ, called in this case CAR-TMA-BG5. This was done with the idea that *in vitro* and *in vivo* results with anti-CD19 CARs should be

then verified on a different CAR against a tumor antigen present in the ECM. Both structures were again used with the transposon platform Sleeping Beauty and were expressed on the surface of transfected CIK cells. *In vitro* functional assays demonstrated that both anti-TMA CARCIK cells (BG4 and BG5) are cytotoxic against TMA^{+} targets, proliferate in response to antigen binding and secrete the IFN-γ and IL-2 inflammatory cytokines. *In vitro*, CARCIK-TMA5 seemed to be more active than CARCIK-TMA4, nevertheless *in vivo* comparisons will be performed to verify and compare their efficacy. We presented preliminary set up experiments of three tumor models to test the CARCIK-TMA cells *in vivo*. The experimental results presented using anti-TMA CARs, demonstrate that targeting the ECM is possible, that such CARCIK-TMA cells can reach and kill tumor cells, at least *in vitro*, even if the antigen is not necessarily attached to their plasma membrane but expressed in the ECM, that accumulates after several days of culture of adherent cells. The confirmation of efficacy *in vivo* needs to await the results of planned experiments with the models already set up. However, the major obstacle in developing immunotherapies against solid tumors is to avoid on-target off-tumor toxicity.[13–16] In our case *in vivo* experiments will also help in determining the possible off-tumor activity of the strategy, since our CARs recognize also murine TMA. Preliminary data suggest lack of toxicity with a single dose of CARCIK-TMA5. Nonetheless these data need to be confirmed in more experiments.

If we confirm the anti-TMA activity of CARCIK-TMA cells and the absence of severe toxicity, we will then also try and improve the anti-tumor specificity and efficacy of our cells. This is likely to be

necessary, since the target antigen is not fully tumor specific and is not necessarily bound to the tumor cell surface. In particular, based on the promising results of CIK cells plus blinatumomab *in vitro* and *in vivo*[10] as also described in this thesis, we are planning to combine CARCIK-TMA cells with a T cell activating BsAb that will target a tumor antigen, such as the EGFRxCD3 BiTE for solid tumors. Preliminary data *in vitro* suggest that this approach is feasible and that adding a BiTE can improve the cytotoxic activity of CARCIK-TMA cells (Zaninelli S., data not shown). Using this combination, we can take advantage of TMA expression on the tumor stroma to reach the tumor site and activate CARCIK cells, while the BsAb will specifically bind tumor cells and increase both specificity and efficacy. Similar approaches have been proposed by other groups using CAR-T cells.[17–20]

Another aspect that will need to be investigated more accurately, before further clinical development, is the specificity of CAR-TMA using human tumor and normal tissues. This could be used in a first approach by staining different murine and human tissues with the recombinant extracellular portion of CAR-TMA.

The data presented overall shown how development of different strategies in one tumor background ($CD19^+$ neoplasms) can be used to develop similar strategies against solid tumors, for which effective immunotherapies are still lacking, due to lack of specificity of tumor target antigens and often poor infiltration of effector T cells into solid tumor tissues.[21,22] It is hoped that the use of novel targets and of CIK cells which may infiltrate tumor tissues more effectively that standard

T cells,[3] may overcome these hurdles. Hopefully, the new CAR-TMA will be able to reach clinical development in the future.

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