UNIVERSITA' VITA-SALUTE SAN RAFFAELE

CORSO DI DOTTORATO DI RICERCA INTERNAZIONALE IN MEDICINA MOLECOLARE

Curriculum in Basic and Applied Immunology and Oncology

DEVELOPMENT AND VALIDATION OF TCR-BASED IMMUNOTHERAPIES FOR GASTROINTESTINAL TUMORS

DoS: Prof. Chiara Bonini

Second Supervisor: Prof. Hana Algül

Tesi di DOTTORATO di RICERCA di Martina Spiga

Matr. 017137
Ciclo di Dottorato XXXVI
SSD MED/15

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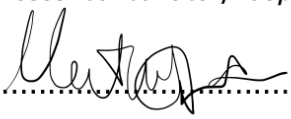
Data /Date …30/09/23…… Firma /Signature …………

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All the results presented here were obtained by myself, except for:

1) The computational analyses of NGS (Results, Chapter 1, Figure 7-8) and of ultra-deep whole exome sequencing (Results, Chapter 2, Figure 9-11) were performed in collaboration with Dr. Stefano Beretta, SR-TIGET, Gene Transfer technologies and new gene therapy strategies unit, San Raffaele Scientific Institute, Milan, Italy.

2) The computational analyses of RNAseq data collected from CRC and PDAC patients enrolled at Ospedale San Raffaele (Results, Chapter 2, Figure 17) were performed in collaboration with Dr. Chiara Balestrieri, Immunology Division, Experimental Hematology unit, San Raffaele Scientific Institute, Milan, Italy.

All sources of information are acknowledged by means of reference.

A mio padre, mia madre e mia sorella

ABSTRACT

Colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC) are major unmet medical needs, with a high mortality rate mainly related to the onset of metastases, hindering the therapeutic efficacy of conventional treatments. Adoptive Cell Therapy (ACT) with TCR-edited T cells is a promising therapeutic strategy for patients with late-stage tumors, but its applicability is limited by the paucity of TCRs targeting relevant tumor antigens and the presence of an immunosuppressive tumor microenvironment. Moreover, the safety of the therapeutic cellular products, generated by genome editing tools, is fundamental for an effective and rapid clinical translation. With the aim of improving the safety profile of T-cell based therapeutic products manufactured by genome editing, we evaluated the efficacy and safety of cytosine base editor (CBE). We disrupted the endogenous TCR to redirect T cells specificity toward tumor cells, and TIGIT, a major inhibitory molecule in gastrointestinal tumors to enhancing T cell ability to counteract immunosuppression. Beyond the high efficiency of CBE in simultaneously disrupting the target genes, we observed only marginal sgRNA-dependent and -independent off-target events as measured by analyzing the top predicted off-target loci and by ultra-deep whole exome sequencing, respectively. In addition, unlike CRISPR/Cas9-treated cells, we could not detect translocations in CBE edited T cells. To select the antigens to target by engineered T cells, we investigated published datasets and identified 19 relevant tumor-associated antigens. By clonal tracking of the TCR repertoire of T cells stimulated with autologous APCs loaded with the selected antigens, we isolated 5 different TCRs specific for AGR2, DKK1, MET, MSLN and MUC5AC. Importantly, these antigens are expressed in our cohort of primary and metastatic PDAC and CRC patients. We generated TCR-edited T cells displaying anti-tumor activity against PDAC cell lines, CRC and PDAC patient-derived organoids and sparing HLA-unrelated and antigen-negative controls. Although all TCRs showed a comparable tumor killing ability, AGR2-, DKK1- and MSLN-T cells were prioritized for their ability to produce cytokines upon antigen recognition. To boost the efficacy of anti-tumor T cells, we coupled TIGIT disruption to TCR editing. By challenging TCR-edited $TIGIT_{KO}$ T cells *in vitro* and in an orthotopic model of CRC liver metastases, we observed that TIGIT disruption can ameliorate the ability of tumor-specific T cells to control tumor growth, based on the anti-tumor TCR expressed by T cells. Our findings suggest that CBE can be exploited to efficiently generate genetically engineered T cell products with an optimal safety profile. Moreover, we found that TIGIT knockout has the potential to improve the anti-tumor efficacy of TCR-edited T cells for gastrointestinal tumors.

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ACRONYMS AND ABBREVIATIONS

ABE	adenine base editor
ACT	adoptive T cell therapy
APC	antigen presenting cell
CAF	cancer associated fibroblast
CAR	chimeric antigen receptor
CBE	cytosine base editor
CDR1	complementarity determing region 1
CDR2	complementarity determing region 2
CDR3	complementarity determing region 3
CMV	citomegalovirus
CRC	colorectal cancer
CRISPR	clustered regularly interspaced short palindromic repeats
CRS	cytokine release syndrome
DSB	DNA double strand breaks
ECM	extracellular matrix
FBS	fetal bovine serum
HD	healhty donor
hSPCs	hematopoietic pluripotent stem cells
ICANS	immune effector cell-associated neurotoxicity syndrome
ICB	immune checkpoint blockade
IFNg	interferon g
IL-15	interleukin 15
IL-2	interleukin 2
IL-21	interleuikin 21
IL-7	interleukin 7
IR	inhibitory receptor
LOH	loss of heterozigosity
MDSC	myeloid derived suppressor cells
MHC	major histo compatibility complex
MLR	mixed lymphocyte reaction
NGS	next generation sequencing
NHEJ	non homologous end joining
NSCLC	non small cell lung cancer
OT	off target
PBMCs	peripherla blood mononuclear cells
PBS	phosphate buffered saline
PDAC	pancreatic ductal adenocarcinoma
sgRNA	single guide RNA
SNV	single nucleotides variant
SP	subpools
TAA	tumor associated antigens
TALEN	transcription activator-like effector nucleases
TAM	tumor associated macrophages
TCR	T cell receptor
TIL	tumor infiltrating lymphocyte
TME	tumor microenvironment
TNFa	tumor necrosis factor alpha
unr	unrelated
us	unstimulated

WES	whole exome sequencing
ZFN	zinc finger nucleases

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INTRODUCTION

1. COLORECTAL CANCER AND PANCREATIC DUCTAL ADENOCARCINOMA

1.1 Colorectal cancer and pancreatic ductal adenocarcinoma are metastatic diseases with limited therapeutic options

Colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC) are among the deadliest malignant diseases, representing 5.8% and 4.7% of cancer-related deaths worldwide in 2020, respectively (GLOBOCAN 2021, Sung et al. 2021).

Colorectal cancer

Colorectal cancer accounted for 1.88 million new cancer diagnoses in 2020, becoming the third most common tumor diagnosis after breast and lung cancer (Sung et al. 2021). Thanks to the improvements of screening methods and the development of novel therapeutic strategies, the 5-year survival rate now reaches the 65% (US). Surgical resection still represents the election therapy; indeed, when the tumor mass is resectable the overall survival increases to 91% (Siegel et al. 2022). However, CRC is a metastatic disease: in addition to the 20% of patients that already display distant metastases at the first diagnosis, an additional 25% of patients with localized tumors will eventually metastasize within 5 years (SEER). In this case, the survival rate lowers to 12%, making metastatic CRC an unmet clinical need (Biller e Schrag 2021).

Surgical resection combined with systemic chemotherapy is still the best therapeutic option for metastatic patients to reduce or eliminate the lesions and clear the organ from micro-disseminations (Dekker et al. 2019; Xie, Chen, e Fang 2020). However, the introduction of tumors' molecular characterization can further ameliorate patients' prognosis, allowing for the selection of a proper targeted therapy. Anti-EGFR antibodies (cetuximab and panitumumab) coupled with a standard chemotherapy regimen have been exploited for the treatment of patients with wild-type KRAS signaling pathway and proved able to extend patients' survival. The combination of anti-VEGF antibodies (bevacizumab, aflibercept, and ramucirumab) with chemotherapeutics improved patients' progression-free survival independently from RAS status and origin of the lesion (right- or left-side colon). Bevacizumab is also an attractive option as second-line, as well as maintenance, treatment. Patients with mutant BRAF (5-10%) might experience clinical benefits

from combinatorial approaches that include BRAF inhibitors, anti-EGFR, and chemotherapy. Different clinical trials are now investigating the efficacy of ERBB2 and HGF/MET inhibitors (Dekker et al. 2019; Xie, Chen, e Fang 2020; Biller e Schrag 2021), which are upregulated in most patients. In addition to targeted therapy, immunotherapy with anti-CTLA4 and anti-PD1 antibodies have been tested for CRC. Unfortunately, immune checkpoint blockers proved effective only for those patients characterized by microsatellite instability. Indeed, microsatellite unstable tumors are highly immunogenic and infiltrated by an endogenous repertoire of tumor-specific T cells, that potently re-activate upon immune checkpoint blockade administration (Overman et al. 2017; 2018).

Pancreatic ductal adenocarcinoma

In 2020, 495'773 new cases of pancreatic cancer (90% defined as PDAC) were detected and 466'003 deaths were reported, meaning that more than 90% of patients diagnosed with pancreatic cancer ultimately die from the disease. Indeed, the mean 5-year survival rate is 11%, reaching only 42% when the tumor is still localized (Sung et al. 2021).

The only treatment with curative potential is represented by surgical resection followed by adjuvant chemotherapy, but this strategy can be applied only to patients bearing a localized lesion (15-20%). Unfortunately, most patients with a resectable lesion will relapse developing metastases (Kleeff et al. 2016). In the metastatic setting, systemic chemotherapy regimens consist of FOLFIRINOX (5-FU, irinotecan, and oxaliplatin) or gemcitabine/nab-paclitaxel, which are interchangeable first- and second-line treatments. Given their efficacy, the administration of these chemotherapeutic agents is being tested as a neo-adjuvant strategy (Sohal et al. 2017; Milella et al. 2022). Beyond chemotherapy, genetic mutations druggable by targeted therapies are rare in PDAC. Only a small fraction of patients is characterized by BRCA1/2 or PALB2 mutations, and therefore can benefit from the employment of PARP inhibitors. Alone or in combination with platinum-based therapies, PARP inhibitors displayed the potential to improve the outcome of those patients (Ruess et al. 2017). Different and novel biological agents, e.g targeting mutant p53, CDKN2A, CDK4/6, ERK, TGFβ, JAK1 and epigenetic alterations, are now under investigation for their anti-tumor activity (Ruess et al. 2017). Standard immunotherapies, represented by immune checkpoint inhibitors, failed in treating PDAC (Royal et al. 2010; Brahmer et al. 2012). Indeed, PDAC is a "cold" tumor, in which the low mutational burden,

the fibrotic stroma and the immunosuppressive microenvironment contribute to T cell exclusion (Karamitopoulou 2019).

1.2 The immunosuppressive TME and liver resident cells collaborate in dampening anti-tumor immunity

A common feature between CRC and PDAC is the dissemination process, that frequently is directed to the liver. 50% and 90% of CRC and PDAC advanced patients, respectively, present hepatic lesions (Park, Chawla, e O'Reilly 2021; H. Zhou et al. 2022), which are associated with a poor prognosis in both gastrointestinal tumors (Engstrand et al. 2018; Sahin et al. 2018). Liver colonization by CRC and PDAC cells is facilitated by anatomical proximity to the primary site, and liver sinusoids' fenestrations, and is controlled by a precise remodeling of the liver microenvironment (Obenauf e Massagué 2015). First, cancer and stromal cells secrete exosomes and soluble factors that participate in the generation of a pro-metastatic niche (Houg e Bijlsma 2018; H. Zhou et al. 2022). This pro-metastatic niche consists of activated hepatic stellate cells, which create the fibrotic environment by secreting aberrant amounts of fibrillar proteins, and immune cells recruited by either hepatocytes or tumor cells. The coordinated action of different cell populations generates metabolic and hypoxic conditions that promote tumor cell proliferation and inhibits the host anti-tumor immune responses, allowing tumor outgrowth (Obenauf e Massagué 2015; Houg e Bijlsma 2018; H. Zhou et al. 2022).

Primary and distant lesions share common features of the tumor microenvironment (TME) that can limit the activity of both endogenous and adoptively transferred T cells.

Primary as well as metastatic tumors are characterized by the deposition and remodeling of a stiff ECM, that represents a physical barrier to T cell entry into the tumor bed (Lanitis et al. 2017). This barrier is generated by ECM-producing cells, namely cancer-associated fibroblasts (CAFs) (Sahai et al. 2020) and hepatic stellate cells which acquire a myofibroblast-like phenotype (Costa-Silva et al. 2015). In addition to ECM production, CAFs support tumor progression by secreting growth factors and cytokines, that sustain cancer cell proliferation, recruit immunosuppressive cellular populations and directly inhibit T cell activation (Sahai et al. 2020).

Besides physical barriers, Tumor-associated macrophages (TAMs), regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSCs) populate the TME

generating an immunosuppressive environment. These cells, which are recruited by cancer cells and CAFs or polarized from pre-existing immune cells, cooperate to drive tumor immune evasion (Binnewies et al. 2018; Labani-Motlagh, Ashja-Mahdavi, e Loskog 2020).

TAMs are the most represented and better characterized component of the immune infiltrate in solid tumors. Different reports correlate their abundance with a reduced survival rate in many tumor types, highlighting their crucial role in cancer progression (Qiong-wen Zhang et al. 2012; Jung et al. 2015). By secreting growth factors and remodeling the ECM, TAMs promote cancer cell proliferation and dissemination. Moreover, TAMs dampen the effector functions of intra-tumoral cytotoxic lymphocytes, as well as adoptively transferred T cells, by producing immunosuppressive molecules (e.g IL-10, TGFβ, IL-1β, PGE2), activating inhibitory cascades through the expression of immune checkpoint ligands and sustaining other immunosuppressive cell populations (Ostuni et al. 2015; Mantovani et al. 2017). Moreover, by generating a syngeneic mouse model bearing CRC with synchronous liver metastases it was observed that liver-resident TAMs can also directly interact with tumor-specific T cells, induce T cell apoptosis via FasL-Fas axis, depleting them from the circulation (J. Yu et al. 2021).

In addition to TAMs, MDSCs represent a heterogeneous cellular population that includes progenitor cells, mononuclear MDSCs (M-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs) and that frequently infiltrate solid tumors. The two MDSC differentiation states share the immunosuppressive mechanisms exerted by TAMs and tumor-associated neutrophils (TANs), respectively. MDSCs originate from the bone marrow and are recruited by cancer cells to the tumor site, where they sustain cancer progression by inhibiting cytotoxic T lymphocytes and stimulating Treg differentiation and expansion (Gabrilovich, Ostrand-Rosenberg, e Bronte 2012).

Treg accumulation is also a common feature of several solid tumors (Alvisi et al. 2022), where they exert their suppressive functions and actively participate in the creation of an immunosuppressive TME. High levels of interleukin-2 high-affinity receptor alpha chain (IL-2Rα) expressed on Treg surface (Sakaguchi et al. 1995) allows Tregs to efficiently consume IL-2 and subtract this cytokine from effector T cells, indirectly affecting their proliferation. In addition, by consuming ATP through the CD39-related pathway, Tregs produce adenosine, a potent inhibitor of T cell effector functions (Deaglio et al. 2007). Nevertheless, to fully exert their suppressive function, Tregs need specific antigen recognition and TCR signaling (Levine et al. 2014). Tumor-infiltrating Tregs TCR repertoire only marginally overlaps with

circulating Treg or conventional T cell repertoire and displays specific reactivity against tumors and tumor-associated antigens (TAAs) (Ahmadzadeh et al. 2019). Following the recognition of tumor antigens exposed by antigen presenting cells (APCs), T regs suppress APCs by CTLA-4-mediated engagement and secrete immunosuppressive cytokines as well as lytic granules (Togashi, Shitara, e Nishikawa 2019). An additional mechanism of T regs immunosuppression was described in a mouse model of liver metastases. Specifically, upon recognition of the target antigen, T regs populating liver metastases affected anti-tumor T cells localized within the subcutaneous tumor, stimulating the recruitment of MDSCs at the primary site and thus creating a systemic immunosuppressive state (J. C. Lee et al. 2020).

Overall, adoptive T cell therapy (ACT) represents a promising opportunity for the treatment of CRC and PDAC advanced patients. However, the tumor microenvironment characterizing the primary and the hepatic lesions places several challenges that need to be overcome to achieve clinical benefits.

2. ADOPTIVE T CELL THERAPY

2.1 From tumor infiltrating lymphocytes to TCR-edited T cells

The idea of employing adoptively transferred T cells to treat cancer arose in mid 1900s, when T cells were firstly demonstrated able to mediate tumor rejection in preclinical settings (Delorme 1964). The difficulties in finding appropriate culture conditions to maintain T cells *ex vivo* initially held back their application as therapeutic products. Only in 1976, with the identification of a conditioned medium containing a "T cell growth factor", later defined as interleukin-2 (IL-2), T cells were successfully expanded (D. A. Morgan, Ruscetti, e Gallo 1976; Gillis e Smith 1977). Accordingly, the addition of IL-2 to fragments derived from malignant lesions retrieved from mouse models (S. A. Rosenberg, Spiess, e Lafreniere 1986) or cancer patients (Muul et al. 1987), mediated the proliferation of specific T cell subsets infiltrating the tumor mass and displaying tumor-killing abilities. In metastatic patients, the administration of autologous T lymphocytes, together with IL-2, resulted in cancer regression (Steven A. Rosenberg et al. 1985). These studies identified T cells as key players in anti-tumor responses and paved the way for investigating which T cell populations and molecular mechanisms were involved.

The first ACT strategy employing tumor-infiltrating lymphocytes (TILs) was designed for the treatment of melanoma patients. After being isolated from the

resected lesions, TILs were expanded *ex vivo* with IL-2 and tested for their anti-tumor activity before being infused back into the patient (S. A. Rosenberg et al. 1988). Results across different clinical trials showed a 41% overall response rate, and 56 complete responders out of a total of 410 treated patients (Dafni et al. 2019). Given the promising results obtained in the context of melanoma, TILs have been isolated from other solid tumors, i.e sarcoma, NSCLC, cervical carcinoma, renal and colorectal cancer. This widening revealed some limitations in the TILs approach: (i) only 10% of TILs is tumor-reactive, making their specific expansion technically difficult (Simoni et al. 2018; Scheper et al. 2019); (ii) T cells enriched at the tumor site are generally hyporesponsive, with impaired effector functions and reduced cytokine production (Zhen Zhang et al. 2020); (iii) tumors characterized by a low mutational burden, and therefore a reduced immunogenicity, are T cell excluded (Camus et al. 2009).

To overcome these issues, novel strategies have been developed employing autologous peripheral blood-derived T cells, modified to target tumor antigens in the absence of a pre-existing tumor-specific T cell population. Inducing the expression of a chimeric antigen receptor (CAR) or a tumor-specific T cell receptor (TCR), T cells can recognize both neoantigens and tumor-associated antigens (TAAs). CARs are synthetic molecules built in a modular fashion, composed by an antigen binding domain, a transmembrane domain and up to 3 different intracellular signaling domains. The antigen binding portion derives from the variable chain of monoclonal antibodies, thus allowing the CAR to recognize surface antigens independently from the major histocompatibility complex (MHC) presentation (Sterner e Sterner 2021a). On the other hand, TCRs are physiological heterodimers formed by an α and β chain, which are responsible for the antigen recognition, associated with the CD3 subunits (ϵ/γ/δ/ζ) that convey the intracellular signal. Each individual has a unique TCR repertoire, generated by a multitude of genetic rearrangements. The TCR α and β genes are divided into a number of variable, constant and junction (the TCR β locus contains also the diversity segment) regions that are recombined during T cell maturation. This process, called V(D)J recombination, is an error-prone mechanism that generates the CDR3 region, the key element of TCR variability and antigen recognition. In this way, TCRs can recognize up to 10^{11} different epitopes, derived either from surface or intracellular proteins, in the form of peptides exposed on MHC complexes (Mahe, Pugh, e Kamel-Reid 2018).

Tumor-specific TCRs were first retrieved from TILs isolated from 3 melanoma patients (Kawakami, Eliyahu, Delgado, Robbins, Sakaguchi, et al. 1994; Kawakami, Eliyahu, Delgado, Robbins, Rivoltini, et al. 1994): TILs were challenged against HLA matched cell lines expressing a cDNA library derived from autologous primary melanoma cells. Upon stimulation, by measuring IFNγ production, T cell reactivity was observed toward MART-1 and gp100 (Kawakami, Eliyahu, Delgado, Robbins, Sakaguchi, et al. 1994; Kawakami, Eliyahu, Delgado, Robbins, Rivoltini, et al. 1994). Another approach for TCR identification relied on the serial stimulation of patients' peripheral T cells with autologous melanoma cells (Brichard et al. 1993) and led to the identification of tyrosinase-specific T cells. By exploiting similar strategies, several anti-tumor TCRs were isolated, and thanks to the optimization of TCR gene transfer technology (DembiĆ et al. 1986), cloned and transduced into host lymphocytes (i.e gp100- , NY-ESO-1- and MART-1-specific TCRs) (R. A. Morgan et al. 2003; Hughes et al. 2005; Y. Zhao et al. 2005). The encouraging results on TCR-T cells efficacy and safety in preclinical models led to the first trial employing TCR-transgenic T cells specific for MART-1 for the treatment of metastatic melanoma patients (R. A. Morgan et al. 2006).

Since then, other approaches have been designed to increase the number of tumor-specific TCRs, thus broadening their availability for the treatment of different malignancies. Human antigens, presented as proteins (Cohen et al. 2005) or viral vectors (Scheikl-Gatard et al. 2017), have been employed to immunize HLA-A2 transgenic mice with the aim of originating human-reactive murine TCRs. Moreover, tumor-specific T cells can be generated *in vitro* by a mixed lymphocyte reaction (MLR). Accordingly, healthy donor's peripheral blood mononuclear cells (PBMCs), as source of T lymphocytes, were stimulated with dendritic cells either pulsed with antigenic peptides (Ho et al. 2006; H. J. Lee et al. 2012; Stromnes et al. 2015; Tsukagoshi et al. 2018) or transduced with RNAs encoding a TAA to select and expand T cell specificities (Wilde et al. 2009). In a similar fashion, tumor-specific TCRs that target acute myeloid leukemia (Ruggiero et al. 2022) and colorectal cancer (CRC) (Potenza et al. 2023) have been recently isolated. Briefly, PBMCs from healthy donors were sequentially stimulated with autologous antigen presenting cells (APCs) pulsed with 9-mers peptides, that can span the entire length of one specific antigen, i.e WT-1, (Ruggiero et al. 2022) or derive from different proteins (Potenza et al. 2023). Notably, tumor-specific T cells progressively enrich overtime allowing the identification of the proper TCR α and β pairing by high throughput TCR sequencing (Ruggiero et al. 2015).

The progresses that have been made on anti-tumor TCRs identification, as well as CARs design, coupled with gene transfer and gene editing technologies led to the development of innovative therapeutic cellular products that widened the applicability of ACT beyond melanoma.

2.2 Upsides and pitfalls of ACT for hematological and solid tumors

The first clinical investigations of ACT with genetically engineered T cells was directed to metastatic melanoma (R. A. Morgan et al. 2006; 2013; Linette et al. 2013), synovial cell carcinoma (Robbins et al. 2011), colorectal cancer (Parkhurst et al. 2011), esophageal cancer (R. A. Morgan et al. 2013) and multiple myeloma (Linette et al. 2013). So far, 197 and 1258 clinical trials have employed TCR-T and CAR-T cells, respectively (clinicaltrial.gov). However, only six CAR-T cells products targeting CD19 or BCMA (Tecartus, Kymriah, Yescarta, Breyanzi, Abecma, Carvykti) obtained FDA approval and are currently administered to patients affected by B-cell malignancies (Sheridan 2017; Alnefaie et al. 2022).

Despite encouraging results on therapeutic efficacy, these clinical studies also highlighted several limitations of ACT strategies. First, adoptively transferred T cell activation can cause life-threatening toxic events, i.e cytokine release syndrome (CRS), and on-target off-tumor recognition, potentially impairing the integrity of healthy tissues. In addition, patients can experience tumor recurrence or resistance to the treatment following antigen loss. Finally, the tumor microenvironment (TME) in solid tumors hinders the therapeutic efficacy of ACT by restraining T cell infiltration, activation, and effector function.

<u>Detrimental toxicities mediated by T cell products</u>

One of the biggest limitations to ACT is the manifestation of toxic, sometimes life-threatening events. The most common ACT related toxicities, emerged in several CAR-T trials, are cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). In a phase 1 clinical trial employing anti-CD19 CAR-T cells (Maher et al. 2002), upon adoptive transfer, the potent tumor lysis not only reinforced CAR-T cell proliferation but activated bystander immune cells, i.e monocytes. Subsequently, the massive release of inflammatory cytokines, to which CRS owes its name, provokes a systemic inflammatory state associated with fever, hypoxia and organ dysfunction. ICANS can occur following CRS, when CAR-T cells cross the blood-brain barrier and accumulate in the central nervous system (Morris et al. 2022). Notably, 1/3 and 1/2 of (CD19) CAR-T treated patients showed signs of CRS and ICANS, respectively. The neurotoxic events, as well as heart and lung

failure, were irreversible and resulted fatal in some patients (Schuster et al. 2017; Donnadieu et al. 2022). The CAR construct (Ying et al. 2019; Bove et al. 2023), the cellular phenotype (Arcangeli et al. 2022) and the CD4/CD8 ratio in the therapeutic product (Bove et al. 2023) can modulate CRS incidence, event that is completely independent from the recognition of the target antigen in normal tissues (Morris et al. 2022).

While CRS has been observed exclusively upon CAR-T cell administration, off-target antigen recognition also occurred in the context of TCR-T cells and was first characterized in a study including 36 metastatic melanoma patients treated with MART1- specific and gp100-specific TCR T cells. Being MART-1 and gp100 tumor associated antigens, their expression can be detected at low levels also in healthy tissues, which can therefore be recognized by TCRs. For this adverse event, 90% of treated patients experienced toxicities localized to the skin, ears and eyes (L. A. Johnson et al. 2009). Analogous events were reported for CEA (Parkhurst et al. 2011) and MAGE-A3 (R. A. Morgan et al. 2013) specific TCRs. Upon injection of (CEA) TCR-T, 3 metastatic colorectal cancer patients experienced life-threatening colitis determined by CEA recognition on healthy colon cells (Parkhurst et al. 2011). Similarly, (MAGE-A3) TCR-T cells bound to epitopes of different proteins belonging to the same family as MAGE-A3, which are expressed by neurons. This recognition led to the death of 2 patients (R. A. Morgan et al. 2013). Moreover, TCR-T cells can potentially cross-react with peptides derived from unrelated antigens with a similar sequence. A phase 1 clinical trial employing T cells harboring an HLA-A*01 MAGE-A3 specific TCR was interrupted after the death of the first two treated patients occurred few days after T cell infusion. The toxicity was due to the recognition by (MAGE-A3) TCR-T cells of a distinct protein expressed by cardiac striated cells, causing heart failure (Linette et al. 2013).

A common feature between all TCRs that led to toxic reactions, is the presence of modifications to increase their avidity. MART-1- and gp100-specific TCRs harbored a murine constant region that improved CD3ζ recruitment and functional avidity (L. A. Johnson et al. 2009). Both the TCRs targeting MAGE-A3 (R. A. Morgan et al. 2013; Linette et al. 2013) and CEA (Parkhurst et al. 2011) were edited by insertional mutagenesis directed to the CDR3 region, unleashing the recognition of peptides with similar sequences, and enabling the attack of healthy tissues with low antigen densities. Hence, attempts were made to improve the functional avidity without impairing TCRs' safety profile. To this extent, Greenberg's group modified CDR1 and CDR2 TCR regions, involved in the stability of MHC binding, enhancing functional

avidity without safety concerns. Indeed, the TCR activation threshold can be reduced by ameliorating MHC molecules engagement (Schmitt et al. 2013). However, it was just recently observed that an intermediate functional avidity positively correlates with an optimal anti-tumor activity. In an elegant study employing a mouse model in which T cells can interact with target cells with different strengths, it was observed that high-affinity bindings resulted in T cell disfunction, while low-affinity ones led to functional quiescence, underlining the need of a fine-tuned TCR signal strength (Shakiba et al. 2021). In addition, low-affinity T cells are known to generate immunological memory, as showed in the context of chronic viral infections (Straub et al. 2023). Taken together, these data elucidate why physiological TCR constructs should be preferred to modified ones.

Of note, CAR-T cells can also mediate on-target off-tumor recognition. Liver toxicity was observed in renal cell carcinoma patients upon treatment with CAIX CAR-T due to the expression of the antigen in the bile duct (C. H. Lamers et al. 2013). By the same mechanism, ERBB2-specific (R. A. Morgan et al. 2010) and MSLN-specific CAR-T cells (Haas et al. 2023) caused the death of two patients due to acute pulmonary toxicity. CAR-T cells were found in the lungs, where low levels of antigen expression were detected.

Balancing the need of avoiding off-target events while targeting relevant oncogenic pathways is necessary to achieve therapeutic efficacy and prevent tumor recurrence by antigen loss. However, to date the preclinical models available to investigate toxicity do not fully recapitulate the human context and cannot predict all possible adverse events.

Antigen escape

A key mechanism of immune escape employed by cancer cells is the dysregulation of antigen presentation, which can completely nullify ACT efficacy. Tumor establishment is associated with antigen loss, downregulation of MHC molecules and intracellular components of the antigen processing pathway. These events eventually lead to antigen escape (Reeves e James 2017; Sterner e Sterner 2021b). Targeting native proteins independently from MHC presentation, CAR-T cells are resistant to some of these immune evasion mechanisms. However, native antigens' expression can also be altered during tumor progression by post-translational modifications, eluding CAR-T cells mediated recognition. For example, solid tumor cells are characterized by an aberrant expression of N-glycans, that

protects surface proteins from CAR-T targeting, masking the immunogenic epitopes and affecting the formation of a proper immunological synapse (Greco et al. 2022).

Besides tumor-intrinsic mechanisms, antigen escape can occur following the administration of T cell – based therapies due to the installed selective pressure. FDA-approved CAR-T cells products target lineage antigens, i.e CD19 and BCMA, which are upregulated in B-cell malignancies and expressed only on non-essential healthy tissues. However, despite a good safety profile, these antigens have a marginal role in tumor growth and progression and can be easily lost overtime, dampening CAR-T therapeutic efficacy. Indeed, follow-up data on (CD19) CAR-T or (BCMA) CAR-T treated patients evidence antigen loss as a common mechanism for tumor relapse (Majzner e Mackall 2018). The same event was observed in glioblastoma patients, in which antigen loss upon injection of EGFRvIII- (O'Rourke et al. 2017) or IL13Ra2-specific CAR-T (Brown et al. 2016) led to resistance.

A simple approach developed to overcome antigen escape is the simultaneous targeting of two different antigens. In clinical trials, the use of tandem CARs with dual specificity, i.e CD19/CD22 or CD19/BCMA, showed promising efficacy results and prevented antigen escape (Hossain et al. 2018).

Despite dual-targeting, the reduced capacity of CAR-T cells to recognize low antigen densities often contributes to tumor recurrence. In many patients treated with CAR-T cells, tumor relapse occurred for the outgrowth of antigen low variants rather than complete antigen loss (Fry et al. 2018). Several works demonstrated that CAR-T cells require thousands of molecules to trigger T cell activation; this feature is independent from the strength of antigen binding but associated with the correct recruitment of intracellular signaling proteins (K. Watanabe et al. 2015; Majzner et al. 2020; Salter et al. 2021). In an opposite fashion, TCRs can recognize even a single peptide:MHC complex (Sykulev et al. 1996). The higher sensitivity proper of native TCRs is linked to the assembly of the signaling machine, downstream to antigen recognition, and the correct establishment of a stable immunological synapse (Salter et al. 2021). To keep the MHC-independence advantage proper of CARs, while leveraging on the physiological ability of TCRs to properly activate the downstream signaling pathways, Sadelein's group generated the HIT receptors (Mansilla-Soto et al. 2022). HIT receptors are hybrids molecules formed by a CAR extracellular domain fused with the intracellular CD3 complex. This construct improved by 10-fold the sensitivity of the CAR-mediated antigen recognition. Further evaluations are needed to address how HIT receptors impact on T cell functionality and long-term persistence.

CAR targetable antigens include around 30% of the entire proteome, limiting the possibility to select molecules involved in key oncogenic mechanisms. Beyond their higher sensitivity, the repertoire of antigens that can be targeted by TCRs is wider since they can recognize peptides, presented by all HLA elements, either derived from intracellular proteins and surface proteins, or neoantigens, thus reducing the risk of antigen escape (Shafer, Kelly, e Hoyos 2022). In addition, it has been recently observed in ACT-treated melanoma patients that tumor-specific TCRs harbor the intrinsic potential to recognize multiple antigens. When tested *in vitro,* these TCRs recognized peptides derived from different TAAs restricted to the same HLA molecule. Importantly, by eliminating multiple target cells at the same time, these TCRs display an improved tumor killing ability compared to conventional ones (Dolton et al. 2023).

The main limitation to TCR-based therapies is represented by the requirement of HLA molecules to present the target epitope. Similarly to the mechanism of antigen escape, TCR-T-treated nonresponding or relapsing patients generally lose the expression of MHC class I molecules. The loss of HLA heterozygosity (LOH), determined by loss of chromosomes containing HLA loci or by epigenetic silencing, has been reported in clinical trials as a key event in tumor recurrence after TCR-T cell infusion (Tran et al. 2016; Paulson et al. 2018; Lo et al. 2019).

2.3 Generating tumor-specific T cells: genome editing technologies at the service of therapeutic cellular products

The efficacy of transferred tumor-specific TCRs can be influenced by the presence of the endogenous repertoire. First, the competition for surface expression and interaction with the CD3 complex between the endogenous and the engineered TCR hinders T cell anti-tumor function (Provasi et al. 2012; Manfredi et al. 2020). In addition, the endogenous TCR chains can mispair with the tumor-specific ones, leading to the generation of new, undesired specificities and, in turn, potential off-target events (Bendle et al. 2010; van Loenen et al. 2010). TCR mispairing was observed in the context of viral-specific TCRs (van Loenen et al. 2010) and confirmed in syngeneic mouse models, where severe and lethal autoimmunity reactions arouse upon adoptive transfer of TCR-transgenic T cells (Bendle et al. 2010).

Even though such events were not described in clinical trials with TCR-transgenic T cells, different methods have been elaborated to prevent the risk of TCR mispairing. These include engineering of TCRs with murine constant regions, modification of precise amino acids to improve proper pairing, or genetic disruption of the endogenous TCR (Shafer, Kelly, e Hoyos 2022).

Gene disruption is performed by using artificially modified nucleases, which upon recognition of a target DNA sequence mediate DNA double strand breaks (DSBs) that are often repaired by the activation of the NHEJ pathway. By introducing or removing random nucleotides, the NHEJ process can install premature stop codons or frameshift mutations, permanently suppressing gene expression (Doudna 2020). Zinc fingers nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are the first genome editing tools that have been developed, followed by CRISPR/Cas9. The disruption of the endogenous TCR genes to fully redirect T specificity has been successfully promoted by ZFNs (Provasi et al. 2012; Mastaglio et al. 2017) and TALENs (Berdien et al. 2014; Rasaiyaah et al. 2018).

Despite their high specificity, the complexity of protein design, as well as the difficult delivery into host cells, slowed down ZFNs and TALENs application for therapeutic cellular products.

Compared to ZFNs and TALENs, CRISPR/Cas9 (Jinek et al. 2012) represents a flexible and easy-to-use genome editing tool, in which the Cas9 nuclease activity is driven by a 20-nucleotide RNA fragment (single guide RNA) designed to be complementary to a target DNA sequence. Besides gene disruption, CRISPR/Cas9 is also employed for targeted integration, which occurs by favoring the high-fidelity homologous direct repair (HDR) pathway through the administration of a donor DNA template. The great advantage in employing CRISPR/Cas9 for ACT is the possibility to contemporarily edit multiple genes. Indeed, the multiplexing potential of CRISPR/Cas9 has been successfully exploited to simultaneously disrupt the endogenous TCR α and β chains and redirect T cell specificity toward tumor cells (Stadtmauer et al. 2020; Ruggiero et al. 2022; Potenza et al. 2023). By combining lentiviral vectors (Stadtmauer et al. 2020; Potenza et al. 2023) or a donor DNA template (Ruggiero et al. 2022) delivery of a tumor-specific TCR with the disruption of the endogenous TCR, TCR-T cell products with potent anti-tumor activity against solid tumors (Stadtmauer et al. 2020; Potenza et al. 2023) and liquid malignancies (Ruggiero et al. 2022; Lahman et al. 2022) were successfully generated. TCR-T genetically engineered by CRISPR/Cas9 are now under clinical investigation for the treatment of patients with refractory tumors (Stadtmauer et al. 2020), NCT05066165). Overtime, CRISPR/Cas9 – mediated gene disruption was applied to several different targets to potentiate the activity of cellular products, mainly targeting inhibitory receptors and downstream effector proteins with the aim of counteract the immunosuppressive TME (Hou, Chen, e Chen 2021; Potenza et al. 2023).

Despite successful, the described genome editing systems can present important toxicities risks related to genetic mutations produced by the double-strand break induction. Recently, T cell genetic manipulation has been implemented by using base editors and prime editors. Base editors are constituted by a modified form of the Cas9 protein, fused with a deaminase domain. The Cas9 domain, as for CRISPR/Cas9, directs the deaminase activity to a target DNA sequence recognizing the sgRNA. Importantly, base editors mutate the target sequence by converting a single nucleotide into a different one, without introducing DNA DSBs (Anzalone, Koblan, e Liu 2020). Base editors are divided in two categories: (i) cytosine base editors (CBE), first described in 2016, that mediate C to T substitutions by cytosine deamination (Komor et al. 2016); (ii) adenine base editors (ABE), described one year later, that mediate A to G substitutions by adenine deamination (Gaudelli et al. 2017). Initially designed to target pathogenic point mutations responsible for genetic disorders, the potential of base editors was immediately widened to gene disruption. To this aim, targeted nucleotide conversion can be achieved by producing premature stop codons (Kim et al. 2017), disrupting the splicing acceptor and donor sites (Gapinske et al. 2018; Webber et al. 2019a) or mutating the transcription start site (Wang et al. 2020).

CBEs have been efficiently employed to engineer T cells: the disruption of the endogenous TCR was coupled with the knockout of inhibitory receptors (Webber et al. 2019a) and other therapeutic relevant molecules (Webber et al. 2019a; Georgiadis et al. 2021). In May 2022, base edited allogenic CAR-T cells reached the clinical stage, and 3 patients with T-cell leukemia have been treated so far (Georgiadis et al. 2021; Chiesa et al. 2023). Specifically, CBE were used to disrupt TRBC, CD7 and CD52 genes in peripheral blood T cells derived from healthy donors, that were then transduced with a CD7-specific CAR. The triple disruption aimed at avoiding GvHD mediated by the expression of the endogenous TCR (TRBC), preventing fratricide mediated by the anti-CD7 CAR, and inhibiting the recognition of CAR-T by alemtuzumab, that is often used as lymphodepleting agent and acts by binding CD52.

Nowadays, the possibility to choose from a toolbox of different genome editing strategies widen the applicability of gene editing for ACT. Nevertheless, different issues need to be addressed, among all the off-target events mediated by genetic manipulation that ultimately affect the safety profile of therapeutic cellular products.

2.4 Arming T cells to counteract the immunosuppressive microenvironment

In hematological malignancies, the absence of physical barriers surrounding the tumor facilitates T cell encounter with cancer cells. This is not true for solid tumors, where the tumor microenvironment (TME) poses several obstacles to ACT, preventing T cell infiltration and dampening T cell anti-tumor responses. First, a structural barrier impedes the trafficking of adoptively transferred T cells to the tumor. This physical shield is actively shaped by cancer cells that, through the secretion of soluble factors and the recruitment of cancer-associated fibroblasts (CAFs) (Valkenburg, de Groot, e Pienta 2018), and endothelial cells (Nagl et al. 2020), mount a desmoplastic environment surrounded by an aberrant vasculature. Moreover, the TME is populated of different immune cells with immunosuppressive capabilities, i.e tumor-associated macrophages (TAMs), regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSCs) that negatively regulate ACT efficacy. The interplay between a dysregulated tissue architecture and a tolerogenic microenvironment inhibits T cell activation, expansion, and persistence, hindering their therapeutic potential (Labani-Motlagh, Ashja-Mahdavi, e Loskog 2020). These TME characteristics represent obstacles to the achievement of a satisfactory therapeutic effect of both TCR-T and CAR-T cells, yet TME intrinsic features can be exploited to boost the efficiency and persistence of these innovative therapeutic products. Besides overcoming physical barriers, strategies to target immunosuppressive cells in TME need to be developed to fully unleash the anti-tumor potential of ACT. To this aim, it is possible to selectively deplete immunosuppressive cellular subsets from the TME, or to engineer T cells to disrupt inhibitory molecules, to express chimeric receptors able to block suppressive signals or to convey these signals to promote activation pathways.

To boost the therapeutic potential of ACT, T cell delivery can be combined with approaches aimed at neutralizing immunosuppressive cell subsets. Many strategies have been developed to deplete TAMs, Tregs and MDSCs, by different means and their beneficial effects were observed in preclinical mouse models (Hou, Chen, e Chen 2021). However, despite the improvement in ACT efficacy, these approaches involve the systemic administration of soluble molecules that can have a broader and unwanted action on different cell populations. Nowadays, great efforts are focusing on the design of reprogramming strategies, to exploit the presence of these cell populations in the TME (Hou, Chen, e Chen 2021).

Beyond cell-specific targeting modalities, several approaches have been designed to prevent T cell dysfunction caused by the action of immunosuppressive molecules and upregulation of immune checkpoints. The cellular components of the TME collectively release an array of immunosuppressive cytokines and overexpress multiple immune checkpoint ligands, which binding to their cognate receptor on T cells surface activate an exhaustion cascade. T cell exhaustion is a dysfunctional state characterized by the progressive loss of cytotoxic abilities, reduction in expansion potential and, as a result, expression of inhibitory molecules (such as PD1, CTLA4, TIM3, LAG3, CD39 and TIGIT) (Chow et al. 2022).

The development of immune checkpoint blockade (ICB) strategies represented a breakthrough in cancer treatment (Couzin-Frankel 2013). To date, monoclonal antibodies targeting PD1/PD-L1 axis and CTLA4 are approved for clinical use and greatly improve the prognosis of patients with immunogenic cancers. Many other molecules blocking different inhibitory receptors (IRs), i.e TIM3, LAG3 and TIGIT, are currently under clinical investigation (Rohatgi e Kirkwood 2021). However, ICB administered as a monotherapy proved unsuccessful in treating most patients bearing solid tumors (Ribas e Wolchok 2018). Hence, several clinical trials are evaluating the combination of tumor-specific CAR-T cells with anti-PD1/PD-L1 to boost ACT and simultaneously stimulate pre-existing T cell responses. Despite encouraging results, this therapeutic strategy harbors some limitations. First, the co-administration of ICB requires the injection of multiple doses overtime. Second, the therapeutic efficacy of ICB is limited by the drug dilution due to systemic delivery, and by a reduced penetration into the tumor (Heczey et al. 2017a; Adusumilli et al. 2019; Durgin et al. 2021). Last, ICB systemic administration induces a plethora of immune-related side effects, also with fatal outcomes (D. B. Johnson et al. 2016). To overcome these issues, adoptively transferred T cells have been genetically modified to secrete therapeutic antibodies upon antigen recognition, allowing their targeted delivery (Suarez et al. 2016; Rafiq et al. 2018).

A possible approach to prevent T cell exhaustion is the knockout of proteins that are involved in T cell inhibition. Different genome editing tools, as TALEN or CRISPR/Cas9, and more recently base editors, has been exploited to disrupt inhibitory receptors and downstream signaling proteins. Several studies demonstrated that $PD1_{KO}$ T cells display superior abilities in eliminating PD-L1+ tumors in vivo (Ren et al. 2017; Webber et al. 2019). However, at the same time, $PD1_{KO}$ T cells displayed a reduced persistence, as observed in three treated patients. More in details, two patients with advanced myeloma and one with metastatic

sarcoma were infused with autologous $PD1_{KO}$ TCR-T cells. At four months after treatment, the proportion of PD1 disrupted T cells in patient peripheral blood was reduced from 25% to 5% (Stadtmauer et al. 2020). CD39 is another inhibitory receptor participating in the production of adenosine, a key T cell suppressive metabolite (Allard et al. 2017). It was recently observed that TCR-edited T cells disrupted in CD39 display an improved tumor killing ability in an orthotopic mouse model of CRC liver metastases (Potenza et al. 2023). Disruption of other ligands, such as TIM3 (Jafarzadeh et al. 2021) and LAG3, have been tested yet the latter did not show an improvement of CAR-T cell functionality (Y. Zhang et al. 2017).

Among inhibitory receptors, the T cell immunoreceptor with Ig and ITIM domains (TIGIT), is an important exhaustion marker upregulated on T lymphocytes during chronic infections and upon chronic antigen stimulation in multiple solid tumors (Johnston et al. 2014). Two T cell inhibitory mechanisms have been identified as determined by TIGIT engagement. First, interacting with antigen presenting cells, it can affect cytokine production and induce tolerogenic dendritic cells (X. Yu et al. 2009). Second, TIGIT prevents DNAM-1 dimerization, independently from the presence of APCs, dampening T cells cytotoxic abilities, adhesion properties and IFNγ production (Shibuya et al. 1996; Joller et al. 2011; Johnston et al. 2014). TIGIT upregulation was observed in tumor-infiltrating cytotoxic T cells, concomitantly to an exhaustion signature, in different solid tumors but particularly in PDAC (Steele et al. 2020; Cui Zhou et al. 2022). In addition, spatial analyses performed on 31 PDAC samples showed a specific overexpression of Nectins (TIGIT ligands), supporting the rationale for the development of therapeutic strategies aimed at inhibiting TIGIT signaling axis in T cells (Cui Zhou et al. 2022). Indeed, as for other inhibitory receptors, TIGIT blockade by monoclonal antibodies is under clinical investigation for NSCLC (Rohatgi e Kirkwood 2021). To date, the effect of TIGIT disruption in T cells has not been explored.

3. GENETIC MANIPULATIONS INFLUENCE THE SAFETY PROFILE OF THERAPEUTIC PRODUCTS

3.1 Limitations and safety concerns of CRISPR/Cas9

Genome editing tools are often exploited for the development of ACT strategies for cancer treatment, to fully redirect T cell specificity and to improve T cell persistence or ability to overcome the tumor microenvironment (Hou, Chen, e Chen 2021). Thanks to the high editing efficiency, TALENs (Qasim et al. 2017) and

CRISPR/Cas9 (Stadtmauer et al. 2020) were first used for the manufacturing of T cells for clinical applications. However, TALENs and CRISPR/Cas9 activity was soon associated with important off-target events related to the introduction of DNA double strand breaks and the activation of classical DNA repair mechanisms. Specifically, DNA double strand breaks can induce structural chromosomal rearrangements that include translocations (Webber et al. 2019; Stadtmauer et al. 2020; Turchiano et al. 2021), large deletions and chromosomes loss (Kosicki, Tomberg, e Bradley 2018; Turchiano et al. 2021; Tsuchida et al. 2023), and other complex outcomes (Leibowitz et al. 2021; Turchiano et al. 2021).

Translocations are the best characterized off-target event occurring upon endonuclease-mediated editing. Translocations, which consist in the recombination of two different genomic portions following DNA cleavage, can take place between two (or more) on-target sites or can involve distal off-target sequences. In anti-CD19 CAR-T cells, the simultaneous disruption of *TRAC* and *CD52* by TALENs produced all four possible translocations (translocation chromosomes 1p-14p, 1p-14q, 14p-1q, 14q-1q) with different frequencies (Poirot et al. 2015; Qasim et al. 2017). These translocations did not confer a proliferative advantage to CAR-T *in vitro* (Poirot et al. 2015) but were still detectable in treated patients many days after T cell infusion (Qasim et al. 2017). CRISPR/Cas9 activity can cause translocations as well. Hematopoietic pluripotent stem cells (hPSCs) engineered with a sgRNA targeting CCR5 produced translocations between the target gene and an off-target site with up to 0.5% frequency (Turchiano et al. 2021). In T cells, translocations were observed upon multiplex editing with around 2% frequency (Webber et al. 2019; Stadtmauer et al. 2020). Notably, in cancer patients treated with TCR-T cells, the translocation frequency showed a progressive reduction, suggesting that those off-target events affect T cell fitness rather than promote an oncogenic-like expansion (Stadtmauer et al. 2020).

In addition to the study of translocations, many efforts have been focused on detecting off-target editing at distal sites. To this aim, several approaches have been designed to identify off-target *loci*: (i) computational strategies can be employed to predict potential off-target regions based on sequences similarities to the sgRNAs (CasOT, Cas-OFFinder, CHOPCHOP, CRISPOR); (ii) cell-based methods can label off-target sites by using either marked oligos (GUIDE-seq) or antibodies (DISCOVER-seq) upon genetic manipulation;(iii) omics approaches, like whole exome and whole

genome sequencing, which can give a complete overview of edited off-target regions (Guo et al. 2023).

However, common undesired events locate far (more than 1kb distant) from cut sites and cannot be recognized by these tools, among these the generation of large deletions. A broad investigation of the effects of CRISPR/Cas9 activity outlined that, upon editing toward a specific target gene, 1/3 of all resulting alleles bear deletions ranging from 250bp to 6kb in length in different cell populations (Kosicki, Tomberg, e Bradley 2018). The disruption of a target gene can be achieved by directing the sgRNA against an intronic region, which causes damage to the nearest exon, supporting the frequency of the production of large deletions (Kosicki, Tomberg, e Bradley 2018). Large deletions at the *TRBC locus* occurred also during the manufacturing procedure of NY-ESO-1-specific T cells and were stably maintained by all treated patients even 170 days after T cell infusion (Stadtmauer et al. 2020). Similarly, large deletions of around 15kb were observed in $CD34^+$ cells targeted for *CCR5* disruption with 1.5% frequency and led to the loss of a proximal gene (Turchiano et al. 2021).

Large deletions can lead to pathogenic consequences, eventually determining the disruption of nearby genes (Turchiano et al. 2021) and partial or complete loss of the involved chromosome (Cullot et al. 2019; Leibowitz et al. 2021; Tsuchida et al. 2023). CRISPR-Cas9 mediated editing toward the *UROS* encoding gene produced large deletions with 10% frequency, and ultimately affected the entire chromosome's terminal region causing chromosome truncation (Cullot et al. 2019). Upon *TRAC* disruption, healthy donor derived T cells showed partial or complete chromosome loss at similar frequencies (Tsuchida et al. 2023). In addition, loss-of-heterozygosity (LOH) can result from CRISPR/Cas9 activity by the induction of chromothripsis, a genome rearranging event involved in cancer and genetic disorders (Leibowitz et al. 2021). Importantly, the frequency of these off-target events does not depend on the disruption efficacy or the targeted gene (Tsuchida et al. 2023).

Differently from deletions, additional chromosomal alterations can occur at the target site, i.e inversions, duplications, and insertions, even though with lower frequencies (Kosicki, Tomberg, e Bradley 2018; Turchiano et al. 2021).

Beyond physical alterations induced at genomic levels, CRISPR/Cas9 activity can also impair cell fitness by triggering the p53 pathway. Indeed, DNA DSBs resulting from CRISPR/Cas9 activity stimulate p53, causing either cellular death (Ihry

et al. 2018; Schiroli et al. 2019) or the selection of P53 defective clones with potential oncogenic properties (Haapaniemi et al. 2018; Enache et al. 2020).

Distal off-target editing and p53 activation can be modulated by employing high-fidelity CRISPR/Cas9 constructs (Anzalone, Koblan, e Liu 2020) and transient p53 inhibition (Haapaniemi et al. 2018; Schiroli et al. 2019), respectively. On the other hand, large deletions, chromosome damage, and other large rearrangements can be prevented only by avoiding DNA cleavage. By promoting gene disruption without the induction of DNA DSBs, base editors can come in help. Nevertheless, the activity of base editors has been also associated with diverse undesired events, which can impact the safety profile of cellular products.

3.2 Base editors mediate off-target events

Cytosine and adenine base editors (CBE and ABE, respectively) can correct pathogenic mutations and provoke gene disruption, by substituting a single nucleotide pair through a deaminase domain. In addition to the deaminase domain, base editors are constituted by Cas9 variants, whose endonuclease activity is completely removed (dead Cas9, dCas9) or modified to create single strand nicks (nickase Cas9, nCas9). The use of these Cas9 variants allows to direct base editors' activity while avoiding the generation of DNA double strand breaks and their associated off-target events including translocations, large deletions, and chromosome loss (Rees e Liu 2018; Webber et al. 2019a; Georgiadis et al. 2021).

However, the potential DNA and RNA off-target deamination represent a limit to their therapeutic application. Off-target DNA modification represents an important safety concern during the manufacturing of engineered cells. This event is mainly associated with the activity of cytosine base editors and rarely depends on the misrecognition of sgRNA similar sequences (Kim et al. 2017; Rees et al. 2017). Indeed, off-target sites predicted on the bases of sgRNA identity were edited with less than 1% and 2% frequency in T cells (Preece et al. 2020) and a human cell line (H. K. Lee et al. 2020). Similarly, base edited T cells bearing multiple gene disruption targeted to TRBC, CD7 and CD52, were screened for off-target editing at the top 20 predicted sites for TRBC and CD7 sgRNAs. Among the 40 checked sites, only two CD7 off-target regions displayed C-T substitutions with above 2% frequency, and were localized in intronic sequences (Georgiadis et al. 2021).

On the other hand, CBE activity is frequently related to spurious genome deamination. Compared to CRISPR/Cas9 or ABE, CBE-mediated editing increased

single nucleotide variations (SNVs) counts spanning the entire genome, both in mouse embryos (C. Zhou et al. 2019; H. K. Lee et al. 2020) and rice (Jin et al. 2019). In addition, specific enrichment of C-to-T substitutions was observed by whole genome sequencing in HEK293T upon cytosine base editing (Gaudelli et al. 2020). Such pronounced off-target activity relies on the rAPOBEC1, which is the cytidine deaminase domain, intrinsic ability to bind single strand nucleic acids. By recreating *in vitro* the transient exposure of DNA regions as single strands, an event that typically occurs during transcription or replication, it was observed the random deamination of 40% and 2.2% of tested *loci* by CBE compared to ABE (Y. Yu et al. 2020). By a similar technique, it was observed that CBE treatment only determines off-target DNA editing, at levels in the range between 0.4 and 25% (Doman et al. 2020).

Beyond DNA off-target editing, both base editor constructs interact and modify RNA molecules. Independently from the sgRNA sequence, ABE and CBE can produce thousands of RNA modifications (C. Zhou et al. 2019; Gaudelli et al. 2020) and affect their own transcript with up to 30.4% frequency (Grünewald, Zhou, Garcia, et al. 2019). Once again, CBE RNA off-target activity is more prominent compared to ABE one, due to the RNA deaminase function of rAPOBEC1. Indeed, upon CBE-mediated editing of two different human cell lines, half of the transcripts were modified with at least one C-U conversion. Importantly, those DNA positions were left untouched (Grünewald, Zhou, Garcia, et al. 2019).

While RNA off-target edits are temporary and progressively lost due to RNA degradation, DNA off-target editing can potentially occur at proto-oncogenes and tumor suppressor genes raising concerns about the safety profile of such technologies. Both off-target activities can be limited by optimizing the base editor constructs (Grünewald, Zhou, Iyer, et al. 2019; Grünewald, Zhou, Garcia, et al. 2019; Doman et al. 2020; Y. Yu et al. 2020) or the delivery method, reducing the cell exposure to its activity (mRNA vs plasmid) (Rees et al. 2017). Addressing eventual toxicities and possible safety issues at the pre-clinical stage is fundamental for driving cellular products to clinical translation.

AIM OF THE WORK

This project aims at developing effective TCR-edited T cells for the Adoptive Cell Therapy of PDAC and CRC, by endowing T cells to potently recognize tumor cells and counteract inhibitory signals. Thus, we aimed at:

(i) Optimizing the gene manipulation procedure to engineer T cells by employing a cytosine base editor, evaluating it in terms of efficacy and safety.

(ii) Developing a workflow for the identification of relevant tumor antigens to isolate novel anti-tumor TCR specificities.

(iii) Generating TCR-edited T cells disrupted in a key inhibitory molecule to boost their tumor killing activity.

RESULTS

1. INCREASING THE SAFETY PROFILE OF ENGINEERED T CELL PRODUCTS BY BASE EDITING

1.1 CBE is highly efficient in simultaneously disrupting TRAC, TRBC1, TRBC2, and TIGIT in T cells

With the aim of improving the safety profile of T-cell based therapeutic products manufactured by genome editing, we exploited base editors and compared them to CRISPR/Cas9 in their efficacy to generate engineered T cells. In fact, recent studies demonstrated that CRISPR/Cas9 activity can result in important off-target events, i.e translocations (Webber et al. 2019a; Stadtmauer et al. 2020), complex chromosomal rearrangements (Leibowitz et al. 2021; Turchiano et al. 2021), chromosome loss, and large deletions (Kosicki, Tomberg, e Bradley 2018; Tsuchida et al. 2023), ultimately impairing the safety profile of the manipulated cellular products.

To redirect T cell specificity and endow them with the ability to counteract the immunosuppressive TME, we simultaneously disrupted the genes encoding for the endogenous TCR and TIGIT. Indeed, TIGIT represents a key T cell inhibitory molecule engaged by PDAC and CRC tumor cells (Johnston et al. 2014; H. Zhou et al. 2022). We assessed the performance of both cytosine (BE4max) and adenine (ABE8.20m) base editors and tested multiple sgRNAs, designed to introduce premature stop codons or disrupt the splicing sites within *TRAC*, *TRBC1*, *TRBC2* and *TIGIT*.

To disrupt the endogenous TCR we selected: (i) the two most efficient single guide RNAs already published by Webber BR et al. (Webber et al. 2019a) targeting the TCR alpha chain (*TRAC* gene); (ii) 5 newly designed sgRNAs to knockout the TCR beta chain (*TRBC1* and *TRBC2* genes). Each sgRNA was co-administered with BE4max or ABE8.20m mRNA by nucleofection to activated healthy donor T cells. 12 days after the editing procedure, we measured TCR protein loss by flow cytometry and collected DNA samples to quantify the editing efficiency at the genomic level (**Figure 1**).

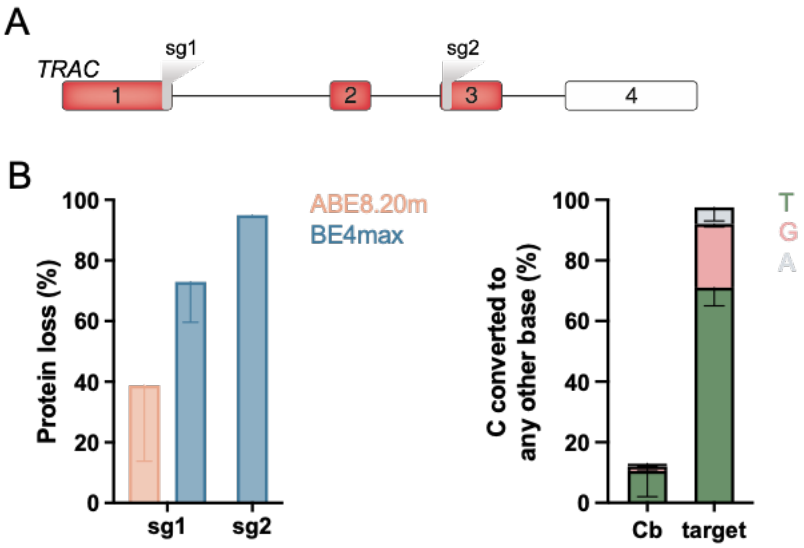

Figure 1. Evaluation of gene disruption efficiency of guide RNAs targeting the TRAC locus. (A) Schematic representation of the TRAC locus showing the region targeted by each tested sgRNA (Webber et al. 2019a). Colored boxes indicate protein coding regions. (B) The frequency of TRAC protein loss mediated by BE4max or ABE8.20m was measured through the assessment of surface CD3 expression by flow cytometry (left). Assessment of C to T/A/G conversion of target base and bystander cytosines for sg2 (right) were quantified by Sanger sequencing and EditR. Data are shown as mean ± s.e.m; n=2 independent donors. sg=single guide RNA, Cb=bystander cytosine, target=target cytosine.

Both sgRNA1 and sgRNA2 targeting *TRAC* are directed toward the splicing sites and disrupt a splicing donor site (GT:CA) and a splicing acceptor site (AG:TC), respectively (**Figure 1A**). sgRNA1 is compatible with CBE and ABE activity, while sgRNA2 is recognized only by CBE. We measured CD3 expression as a surrogate marker of the endogenous TCR by flow cytometry and observed that co-delivery of sgRNAs with BE4max determined higher TCR protein loss compared to ABE8.20m (**Figure 1B, left**). As already reported (Webber et al. 2019a), the most efficient sgRNA targeted the exon 3 splicing acceptor site (sgRNA2) (mean 94.95% TCR loss). The conversion of the target C to any other base mediated by sgRNA2 coupled with BE4max resulted in appr. 100% disruption efficiency. Indeed, when targeting splicing sites, the precise C to T conversion (mean 71%) and the non-target editing, namely C to G (mean 21%) and C to A (mean 5.5%) substitutions, contribute to the target site disruption (**Figure 1B, right**). We also evaluated editing rates for bystander cytosines present in the BE4max editing window (position 3-11 of sgRNA) of sgRNA2. We detected the effective modification of a bystander C located before the target base (**Figure 1B, right**), although it was always followed by the knockout-producing mutation.

We next targeted the TCR β chain, which constant portion is formed by TRBC1 or TRBC2 selected during TCR recombination (Tunnacliffe et al. 1985). Since TRBC1 and TRBC2 are mutually expressed in individual T cell clones, it is important to target both genes to disrupt all possible TCR β chains. We designed 5 sgRNAs, four of which were predicted to disrupt splicing sites (sgRNA1-4) and one to introduce a premature STOP codon (sgRNA5). Importantly, sgRNA1 and sgRNA5 map a homology region between *TRBC1* and *TRBC2* genes (**Figure 2A**). Once again BE4max outperformed ABE8.20m in inducing TCR protein loss. The combination of BE4max with sgRNA5, which generates a premature STOP codon in exon 1 of both *TRBC1* and *TRBC2*, resulted in the highest TCR disruption (**Figure 2B**). As for *TRAC*, we quantified the sgRNA5-mediated editing rate at the genomic level. Importantly, the 96.5% of TRBC1 and 99.2% of TRBC2 mutations detected at the target base were C to T conversions, which are required for the correct introduction of the STOP codon (**Figure 2C**). Despite we found many nontarget editing events, due to the presence of multiple bystander cytosines within the editing window, most of them installed silent mutations. The only exception is the modification of the second cytosine of *TRBC1*, which causes a missense mutation with unknown effects on function (**Figure 2C, right**).

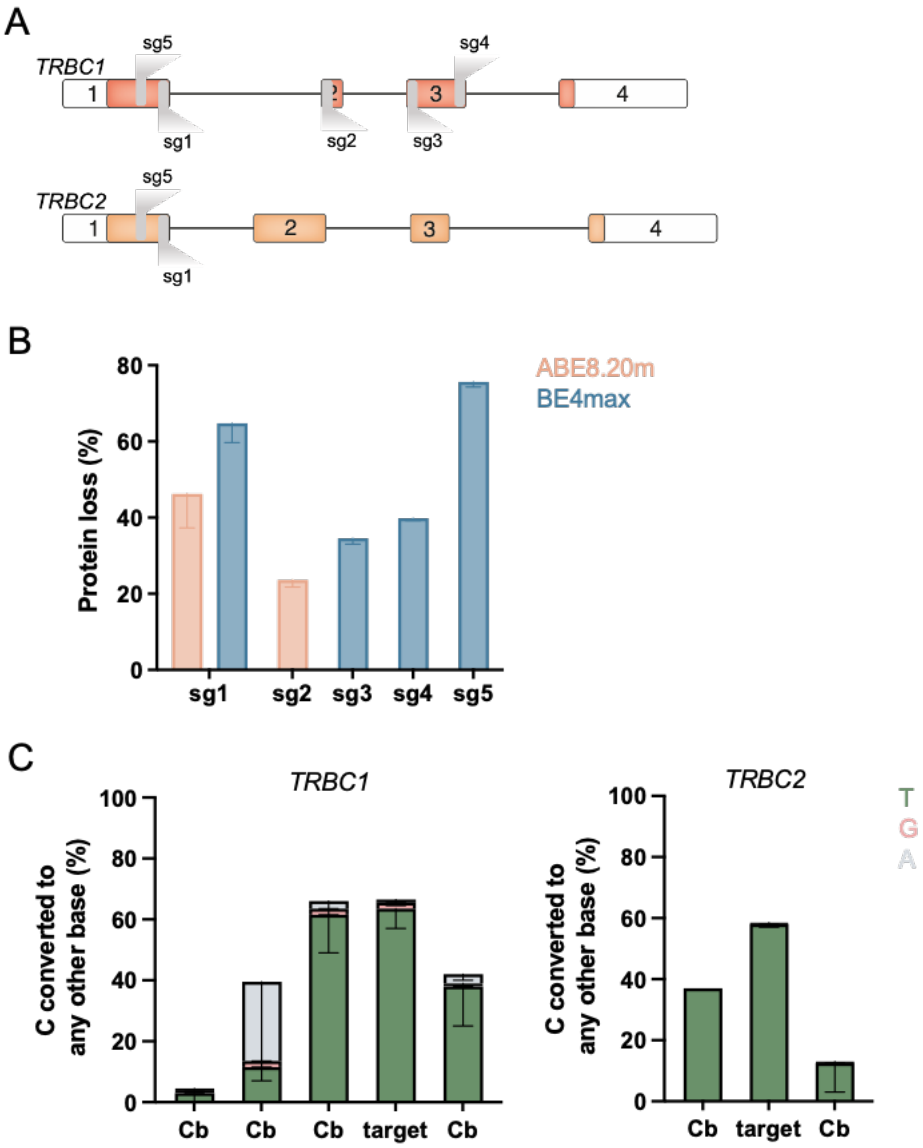

Figure 2. Evaluation of the efficiency of guide RNAs targeting TRBC1 and TRBC2. (A) Diagram of *TRBC1* and *TRBC2* loci showing the region targeted by each sgRNA designed. Colored boxes indicate protein coding regions. (B) The frequency of TRBC1/2 protein loss mediated by BE4max or ABE8.20m was measured through the assessment of CD3 expression by flow cytometry. (C) Assesment of C to T/A/G conversion of target base and bystander cytosines mediated by sg5 for *TRBC1* (right) and *TRBC2* (left). Target C to TA/G editing rates were assessed by Sanger sequencing and EditR. Data are shown as mean ± s.e.m, n=2 independent donors. sg=single guide RNA, Cb=bystander cytosine, target=target cytosine.

With a similar strategy, we designed 5 sgRNAs targeting TIGIT: one to disrupt a splicing site (sgRNA1), and four predicted to generate premature STOP codons (sgRNA2-5) (**Figure 3A**). We evaluated the editing efficiency at the genomic level and observed that sgRNA1 paired with ABE8.20m and sgRNA5 with BE4max exhibited comparable editing rates (**Figure 3B, left**). We further characterized the activity of sgRNA5, since sgRNAs selected to target *TRAC*, *TRBC1,* and *TRBC2* are all compatible

with BE4max. Precise C to T conversion was observed both on the target cytosine (mean 82%) and on a bystander one, which only resulted in a silent mutation (**Figure 3B, right**). To assess TIGIT protein loss, when T cell expansion reached the plateau, we stimulated them again with aCD3/CD28 magnetic beads. After 72 hours, we collected untreated and edited cells to check for TIGIT expression by flow cytometry. Consistently, sgRNA5 was highly efficient in causing TIGIT protein loss (mean 77.8% loss) (**Figure 3C, right**), being as efficient as a TIGIT-specific multi-guide approach based on CRISPR/Cas9 (**Figure 3C, left**).

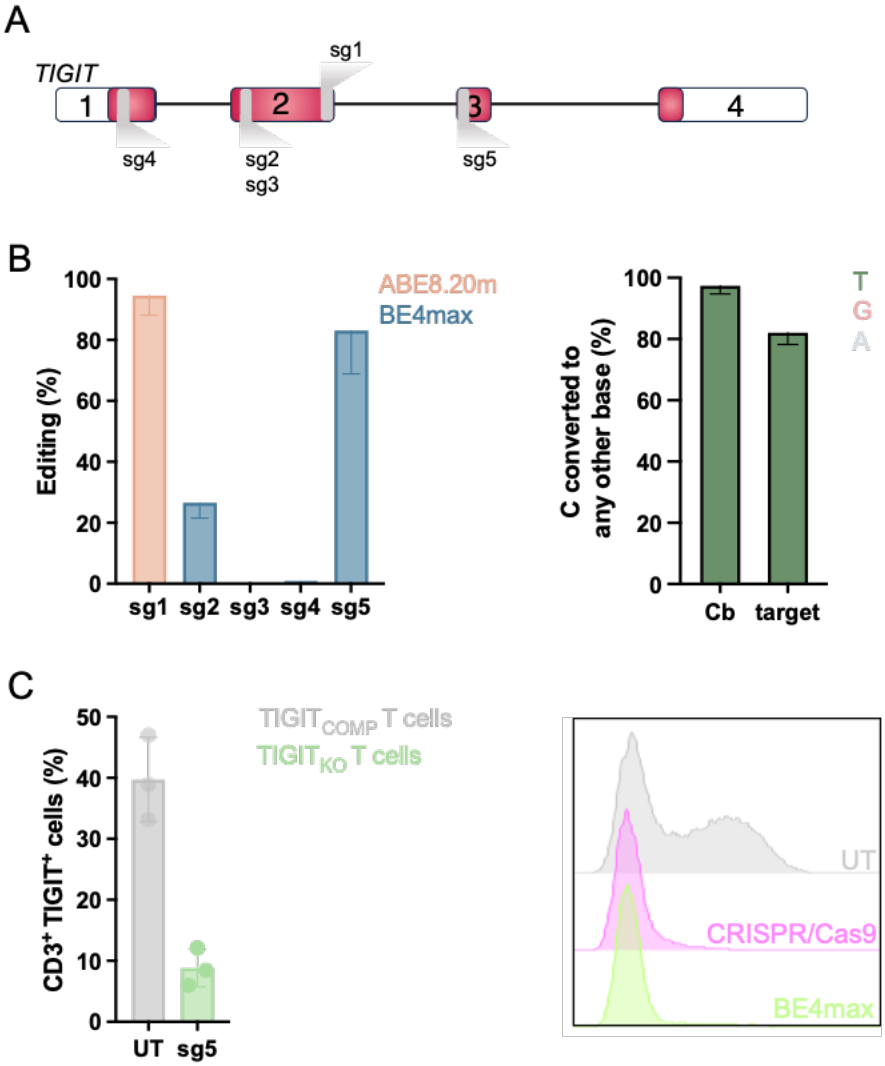

Figure 3. Evaluation of the efficiency of guide RNAs targeting *TIGIT*. (A) Diagram of *TIGIT* locus showing the region targeted by each sgRNA designed. Colored boxes indicate protein coding regions. (B) Quantification of C to T/A/G conversion and A to G/C/T of target base mediated by BE4max or ABE8.20m (left). Quantification of C to T/A/G conversion of target base and bystander cytosine mediated by sg5 (right). (C) Frequency of TIGIT protein loss mediated by sg5-BE4max measured by flow cytometry (left). Representative plot of untreated, TIGIT-targeting CRISPR/Cas9 or BE4max treated samples (right). Target C to T/A/G and A to G/C/T editing rates were assessed by Sanger sequencing and EditR. Data are shown as mean ± s.e.m, n=2 independent donors (B) and n=3 independent donors (C). sg=single guide RNA, Cb=bystander cytosine, target=target cytosine.

Based on these results, we selected the best-performing sgRNAs for each target gene: sgRNA2 targeting *TRAC*, sgRNA5 targeting both *TRBC1* and *TRBC2* and sgRNA5 targeting *TIGIT*.

Next, to generate our therapeutic cellular product we co-delivered our top sgRNAs to disrupt the TCR and TIGIT, both coupled with BE4max. CBE efficiently modified each target gene, and the multiplex setting did not affect its editing efficiency (mean 96.5% of TRAC disruption, 63.2% of TRBC1 disruption, 51.4% of TRBC2 disruption, and 82.4% of TIGIT disruption). Importantly, CBE activity did not introduce DNA double-strand breaks (**Figure 4A**). To redirect T cells specificity, base editing was successfully combined with LV transduction of a model tumor-specific TCR (Potenza et al. 2023), as outlined by the restoration of CD3 surface expression (**Figure 4B**) and the detection of the anti-tumor TCR specific Vβ (**Figure 4C**).

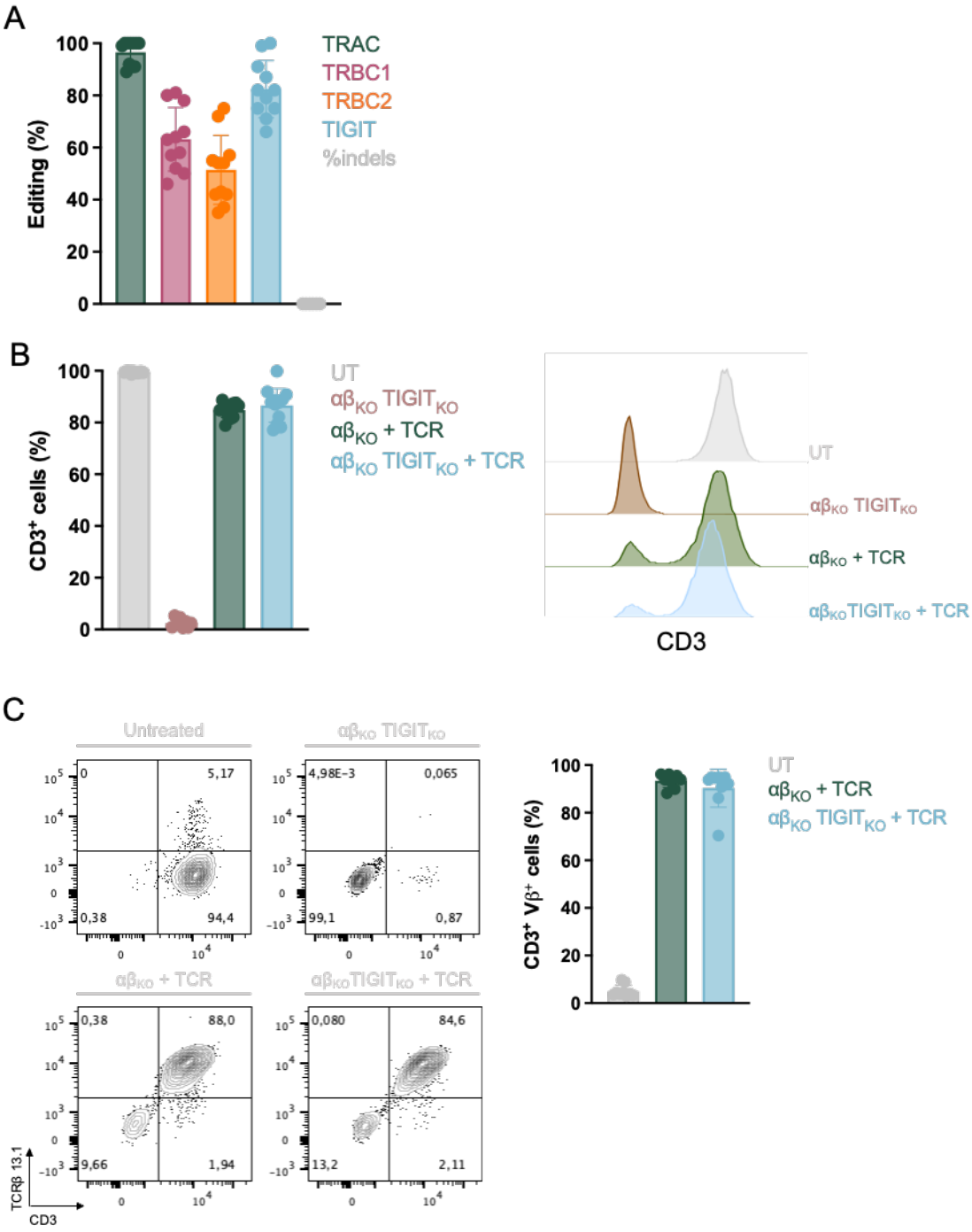

Figure 4. Generation of genetically engineered tumor-specific T cells by CBE. (A) Frequency of C•G-to-T•A conversion and INDEL introduction at on-target sites mediated by BE4max. (B) Frequency of CD3 expression in Untreated (UT), TCR knockout, and TIGIT disrupted T cells, as well as T cells with redirected specificity toward tumor cells (left). Representative plot of CD3 expression (right). CD3 was measured as a surrogate marker for endogenous TCR knockout. (C) Frequency of TCRβ 13.1 expression in Untreated (UT), base edited and redirected T cells (right); representative plots (left). Editing rates were assessed by Sanger sequencing and EditR. Indels were assessed by Sanger sequencing and ICE. Data are shown as mean ± s.e.m, n=11 independent donors. UT=untreated, KO=knockout.

Together, these data show that CBE can be exploited to efficiently engineer human T cells and can be coupled with lentiviral vector delivery of a tumor-specific TCR.

1.2 CBE mediates multiplex gene disruption introducing only minimal off-target events

To evaluate whether the use of a cytosine base editor can ameliorate the safety profile of our therapeutic products, we performed a side-by-side comparison of T cells engineered with CRISPR/Cas9 or BE4max. Specifically, we co-delivered our 3 sgRNAs (targeting *TRAC*, *TRBC1*, *TRBC2,* and *TIGIT*) together with BE4max mRNA or as ribonucleoprotein complexes with Cas9, thus comparing our optimized protocols for each genome editing tool. The two approaches were both highly efficient in simultaneously disrupting all target genes (mean 96.6% and 96.3% of TRAC disruption, 81% and 73.6% of TRBC1 disruption, 75% and 67.3% of TRBC2 disruption, and 89.3% and 90.7% of TIGIT disruption with CRISPR/Cas9 and BE4max, respectively) (**Figure 5A**), and did not compromise T cell expansion or memory phenotype differentiation (**Figure 5B**).

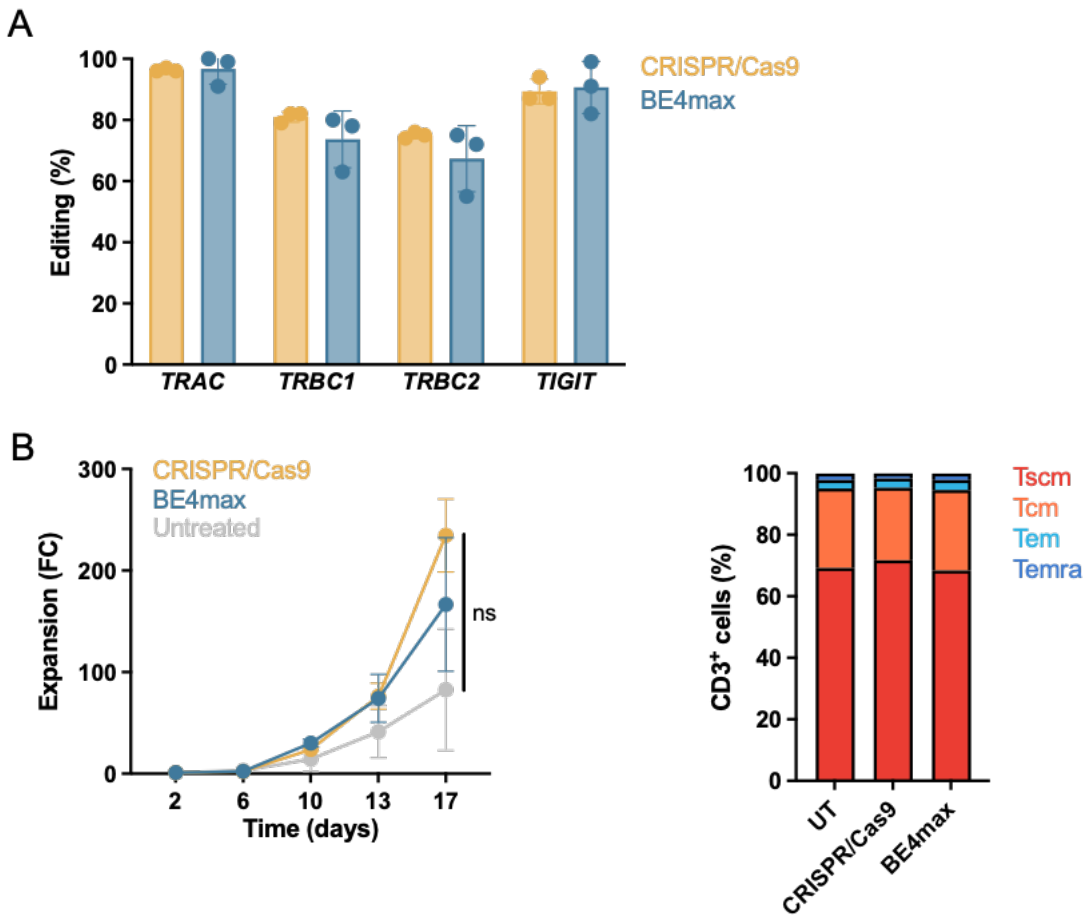

Figure 5. Comparative evaluation of multiplex editing on T cells by CRISPR/Cas9 or BE4max. (A) Quantification of indels (yellow) induced by CRISPR/Cas9 and C to T conversion (blue) mediated by BE4max represented as editing frequency at on-target loci evaluated 14 days from the editing procedure. (B) Expansion curve representing fold change of untreated, CRISPR/Cas9, and BE4max edited cells during in vitro maintenance. Editing was performed on day 2. (C) Distribution of CD3+ T-cell memory phenotypes described according to CD45RA and CD62L expression markers as T_{CM} (CD45RA-, CD62L+), T_{SCM} (CD45RA+CD62L+), T_{EMRA} (CD45RA+CD62L-) and T_{EM} (CD45RA-CD62L-) of untreated vs. genetically engineered T cells generated with CRISPR/Cas9 or BE4max. Editing rates were assessed by Sanger sequencing and EditR for BE4max. Editing rates were assessed by Sanger sequencing and ICE for CRISPR/Cas9. Data are shown as mean ± SD, n=3 independent donors. P-values are calculated by Two-way ANOVA. *P≤0.05, ** P≤0.00.1, *** P≤0.001, **** P≤0.0001. UT=untreated, FC=fold change.

Besides editing efficiency, we also analyzed the presence of undesired translocations between our 4 target *loci*. Indeed, CRISPR/Cas9 activity eventually led to translocations, that occur when a genomic region fuses to another chromosome following a DNA DSB (Webber et al. 2019a; Turchiano et al. 2021; Fiumara et al. 2023) (**Figure 6A**). On the other hand, the modified Cas9 which constitutes base editors only produces single strand nicks, reducing the amount of DNA DSBs and therefore decreasing the frequency of translocations (Webber et al. 2019a; Fiumara et al. 2023). To test this, we designed a droplet-digital PCR (ddPCR) assay to

determine the presence of translocations between the four target *loci* in BE4max and CRISPR/Cas9 treated cells. Upon CRISPR/Cas9 treatment, all tested translocations were detected at different frequencies, with TRAC-TRBC2 and TIGIT-TRBC2 being the highest ones (mean 0.31% and 0.35%, respectively). Conversely, only TIGIT-TRBC1 translocation, in addition to TRAC-TRBC1 in one single donor, was observed in BE4max edited T cells (**Figure 6B**).

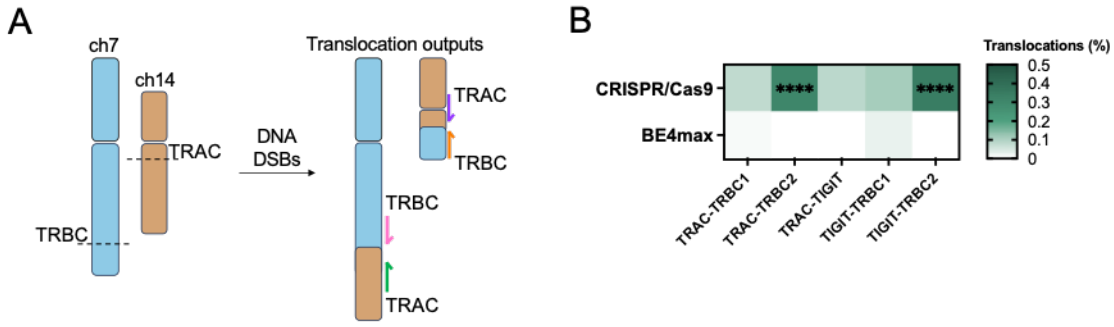

Figure 6. Translocation frequencies between TRAC, TRBC1, TRBC2, and TIGIT in T cells edited with CRISPR/Cas9 and BE4max. (A) Representative scheme of translocations resulting from genome editing approaches. (B) Heat map of translocation frequencies measured by ddPCR between our four target loci at 14 days from the editing procedure. ddPCR was performed on n=3 independent donors for each experimental group. Data are plotted as mean. P-values are calculated by Two-way ANOVA. *P≤0.05, ** P≤0.00.1, *** P≤0.001, **** P≤0.0001.

In conclusion, CBE, while maintaining the same editing efficiency of CRISPR/Cas9, induces fewer translocation events.

Like all genome editing tools, CBE can produce off-target events at the genomic level that go beyond translocations. For base editors, these off-target events are divided into sgRNA-dependent, which occur when the sgRNA recognizes DNA regions with a similar sequence, and sgRNA-independent, which occur when the base editor binds and modifies random regions temporarily exposed as a single strand.

To evaluate sgRNA-dependent off-target (OT) editing, by computational analyses, we first predicted the top off-target loci for each sgRNA that maps on coding regions and selected a total of 8 sequences to be evaluated for OT modifications (i.e OT1-3 for TRAC sgRNA, OT4 and 5 for TRBC sgRNA, OT6-8 for TIGIT sgRNA). Next, we electroporated T cells with the three sgRNA together with BE4max mRNA or with PBS and collected DNA samples 14 days after the manipulation procedure. To check for off-target editing, we performed targeted next-generation sequencing (NGS) of the selected off-target *loci* and the on-target ones as positive controls. We considered

only those modifications localized within the editing window (3-11 bases of the sgRNA). As reported in **Figure 7**, the mutation level varies between base edited and untreated samples at the target genes, while is conserved at off-target sequences. Collectively, this analysis showed the absence of alterations at off-target sites following the administration of sgRNAs and BE4max.

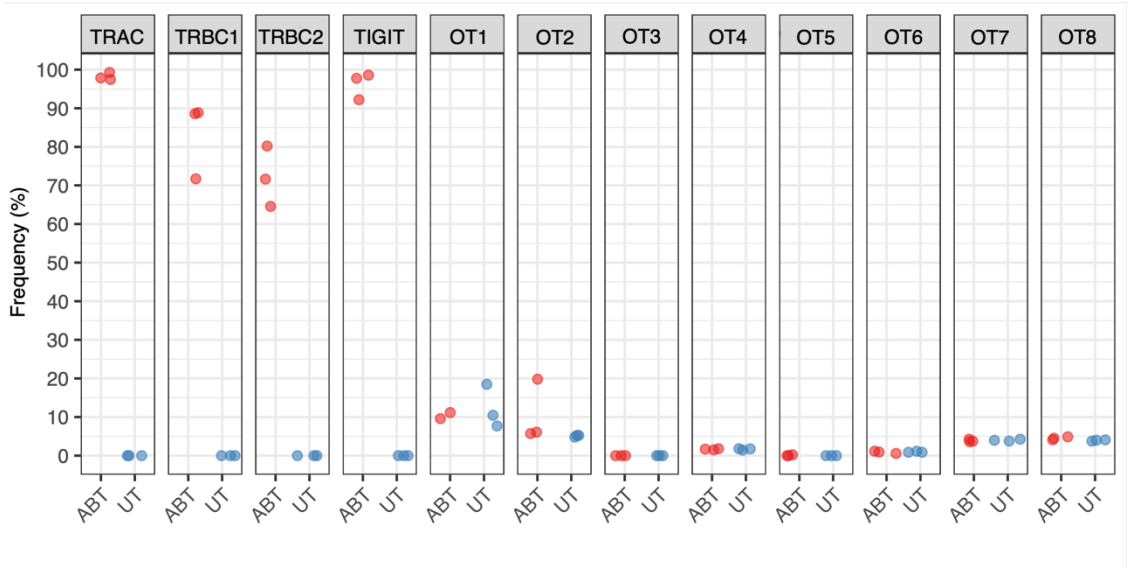

Figure 7. Mutation frequencies for target genes and off-target sites. Dot plot representing the sum of all mutations detected in base edited and matched untreated samples after 14 days from the editing procedure. 3 independent donors were treated with BE4max and the sgRNAs, or mock-electroporated. ABT= $\alpha\beta_{KO}$TIGIT$_{KO}$; UT=untreated

Moreover, we classified the modifications detected into three categories: (i) knockout events, which include those alleles containing the C to T substitution producing the knockout, (ii) deletions, among which are indels or large deletions, and (iii) other mutations, which are non-target nucleotide substitutions and small insertions. At on-target genes, deletions, which are generated by the induction of DBSs, were only detected at non-target bases with a mean frequency below 2%. Notably, knockout mutations represent the largest part of on-target modifications (mean 95.6% *TRAC* disruption, 78.1% *TRBC1* disruption, 68.04% *TRBC2* disruption, 93.5% TIGIT disruption) confirming the high editing efficiency achieved by CBE (**Figure 8**).



Figure 8. Evaluation of off-target base editing. Frequency of alleles falling into the four categories designed to classify mutations: knockout, deletions, other or wild type. Base edited and matched untreated samples were analyzed by NGS after 14 days from the editing procedure. 3 independent donors were treated with BE4max and the sgRNAs or just electroporated. ABT= $\alpha\beta_{KO}$TIGIT$_{KO}$; UT=untreated

We did not observe off-target editing except for one single donor at the *TRAC* OT2 site with a frequency of around 20%. These data show that CBE has a high on-target efficiency with minimal off-target activity.

To check for sgRNA-independent off-target editing, we performed ultra-deep whole exome sequencing (WES) of T cells treated with our pool of sgRNAs and BE4max or mock-electroporated as control. We collected DNA samples from bulk T cell populations, at the end of our manipulation procedure (typically between 16-18 days following the editing procedure), to have a snapshot of the safety profile of our therapeutic product as it would be in a translational setting. We performed WES at a depth of 500x on 4 independent base edited samples and their matched controls. Most alterations, detected from the reference genome, were germinal mutations since they were shared between the base edited and untreated samples. We filtered out germinal mutations and identified unique variants belonging to base edited or untreated samples. While the first ones are generated by the treatment, the latter are probably acquired during *in vitro* cell maintenance or caused by the electric pulse.

Notably, treatment-specific alterations were a restricted portion of all sequences (frequency up to 1.6%) (**Figure 9**).

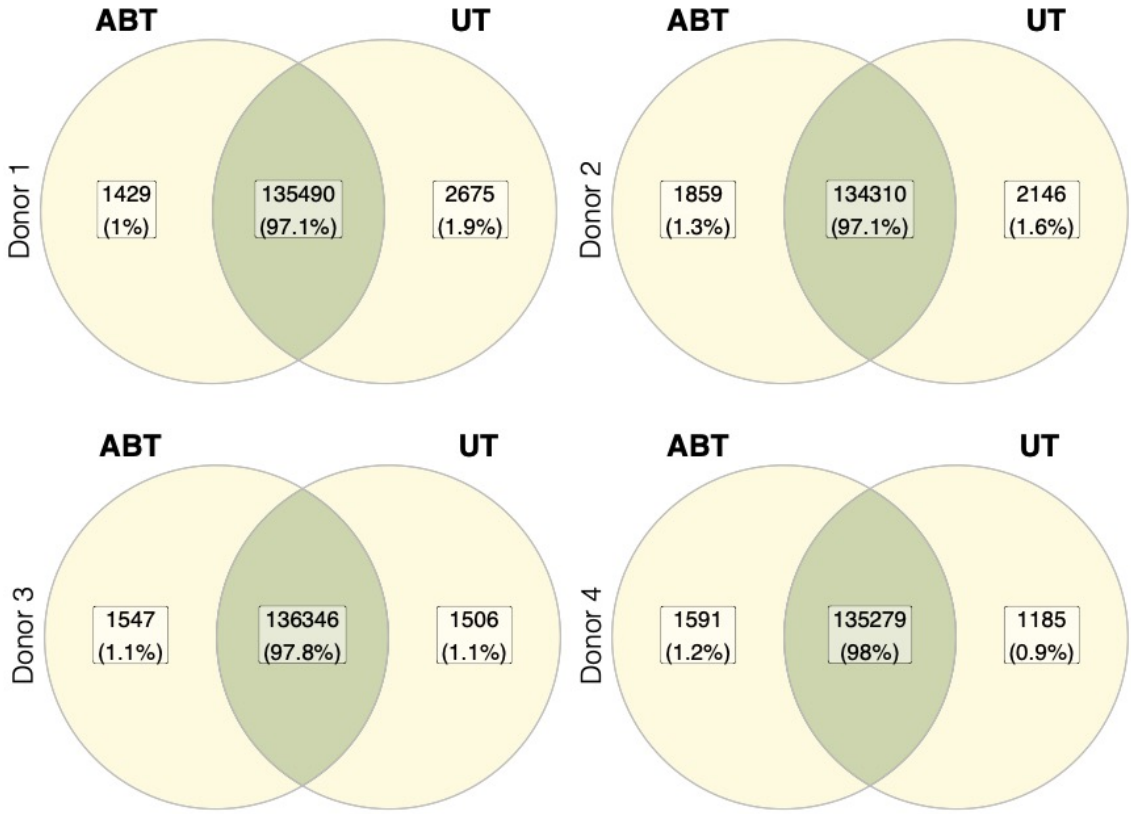

Figure 9. Venn diagrams of ultra-deep WES outputs in edited samples and matched untreated controls. WES sequencing results of 4 independent donors treated with the 3 sgRNAs targeting *TRAC*, *TRBC1*, *TRBC2* and *TIGIT*, combined with BE4max or mock-electroporated. Ultra-deep WES identified germinal variants, untreated- or base editor-specific mutations. ABT=$\alpha\beta_{KO}$TIGIT$_{KO}$; UT=untreated.

By cleaning samples for germinal mutations, no difference was observed in the proportions of variant types (**Figure 10A**) or specific nucleotide conversions (**Figure 10B**), upon treatment with the CBE.

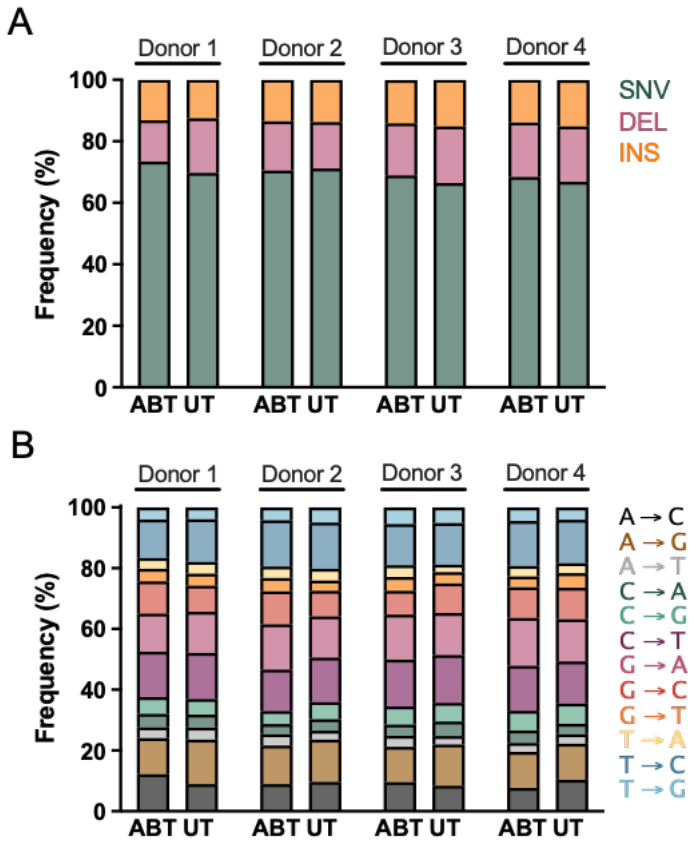

Figure 10. Characterization of CBE-induced or untreated-specific mutations. (A) CBE-induced or untreated-specific alleles were analyzed to classify variant types in single nucleotide variations (SNV), deletions (DEL) or insertions (INS) (B) CBE-induced or untreated-specific SNVs were quantified to determine the specific nucleotide conversion. ABT=$\alpha\beta_{KO}$TIGIT$_{KO}$; UT=untreated.

To further investigate the consequences of the CBE-based genome engineering approach, we deeply analyzed 7 different panels of genes associated with T cell functionality, senescence program activation, and malignant transformation. In general, the mutation profile was similar among the experimental groups (edited and untreated cells): we found substitutions, deletions, and insertions in all samples (**Figure 11**). Importantly, we detected 3 relevant hits, defined as missense mutations falling into coding regions, in 2 independent untreated samples. Only 1 base edited donor displayed 1 relevant mutation located to MUC17, which is included in the cellular senescence signature. However, we could not infer the biological effect resulting from this mutation, which is not annotated and did not cause fitness abnormalities during T cell *in vitro* maintenance (data not shown).

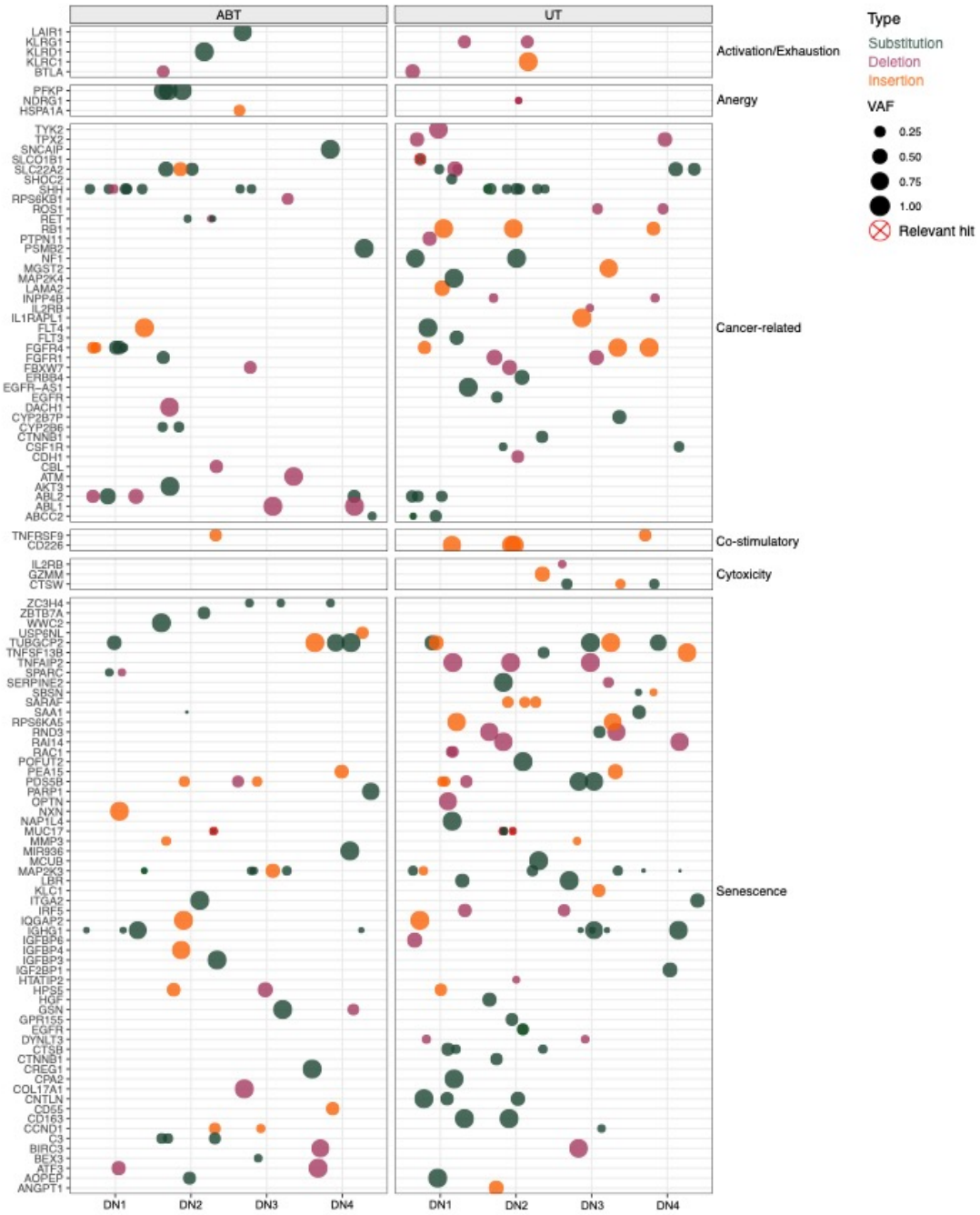

Figure 11. Analysis of signature genes related to T cell functionality, senescence, and malignant transformation. 7 different gene panels were investigated, including genes involved in T cell activation/exhaustion, T cell anergy, T cell cytotoxicity and effector functions, pro-inflammatory genes, costimulatory genes, genes participating in cellular senescence and associated with malignant transformation. Mutations from the reference genome were detected in 6/7 lists. ABT=$\alpha\beta_{KO}$TIGIT$_{KO}$; UT=untreated; VAF=variant allele frequency.

To summarize, by relying on CBE we optimized our multiplex genome editing approach achieving high on-target efficiency while reducing translocations and minimizing the introduction of off-target point mutations.

2. THE TCR HUNTING PIPELINE ALLOWS TO IDENTIFY NOVEL ANTI-TUMOR TCRS

2.1 A workflow for the identification of PDAC relevant antigens to be targeted by ACT

The first issue limiting ACT applicability in solid tumors is the paucity of TCRs targeting relevant tumor antigens. The high intra-tumoral and inter-patient variability represents a unique challenge in the selection of the optimal antigen to target. Antigen heterogeneity is even more challenging when both primary and metastatic lesions are present. Indeed, PDAC high mortality rate is related to the early development of metastases, that mainly occur in the liver (Kleeff et al. 2016). To overcome this limitation and identify relevant antigens to be targeted by ACT, we performed a deep investigation of datasets present in the literature to identify relevant PDAC TAAs (**Figure 12**). We focused on tumor-associated antigens for two reasons: (i) TAAs are often shared among patients and expressed by different tumor types, thus widening the applicability of an ACT strategy; (ii) PDAC has a low mutational burden (Halbrook et al. 2023), resulting in a reduced capacity to generate neoantigens. We analyzed 15 published gene expression profiles of PDAC primary samples (Buchholz et al. 2005; Lowe et al. 2007; Badea et al. 2008; Collisson et al. 2011; Goonesekere et al. 2014; Moffitt et al. 2015; Müller et al. 2015; Bailey et al. 2016; Mao et al. 2017; Klett et al. 2018; Tang et al. 2018; L. Zhao, Zhao, e Yan 2018; Maurer et al. 2019; Peng et al. 2019; Lin et al. 2020). From each dataset, we sorted upregulated genes using $\log_2$FC>1 as a threshold and eliminated those whose expression was associated also with stromal cell populations or healthy pancreatic cells. By merging all gene lists, we retrieved around 3000 unique genes upregulated by PDAC cells. We then categorized these genes based on the overexpression frequency, selecting as relevant only those genes present in at least 3 different datasets. We removed again genes expressed by stromal cells or constituting the extracellular matrix, resulting in 457 genes. Next, we filtered these genes based on different criteria: immunogenicity, oncogenicity, and annotated protein overexpression in PDAC. Protein immunogenicity is an essential feature to induce a T cell-mediated response and consequently isolate tumor-specific TCRs. To check this aspect, we examined the literature for the development of cancer vaccines exploiting these antigens or for the detection of antibody- or T cell-mediated reactivity toward them. We then selected genes whose overexpression in PDAC was confirmed at the

protein level and known to participate in key oncogenic mechanisms, to reduce the risk of antigen escape through antigen loss.

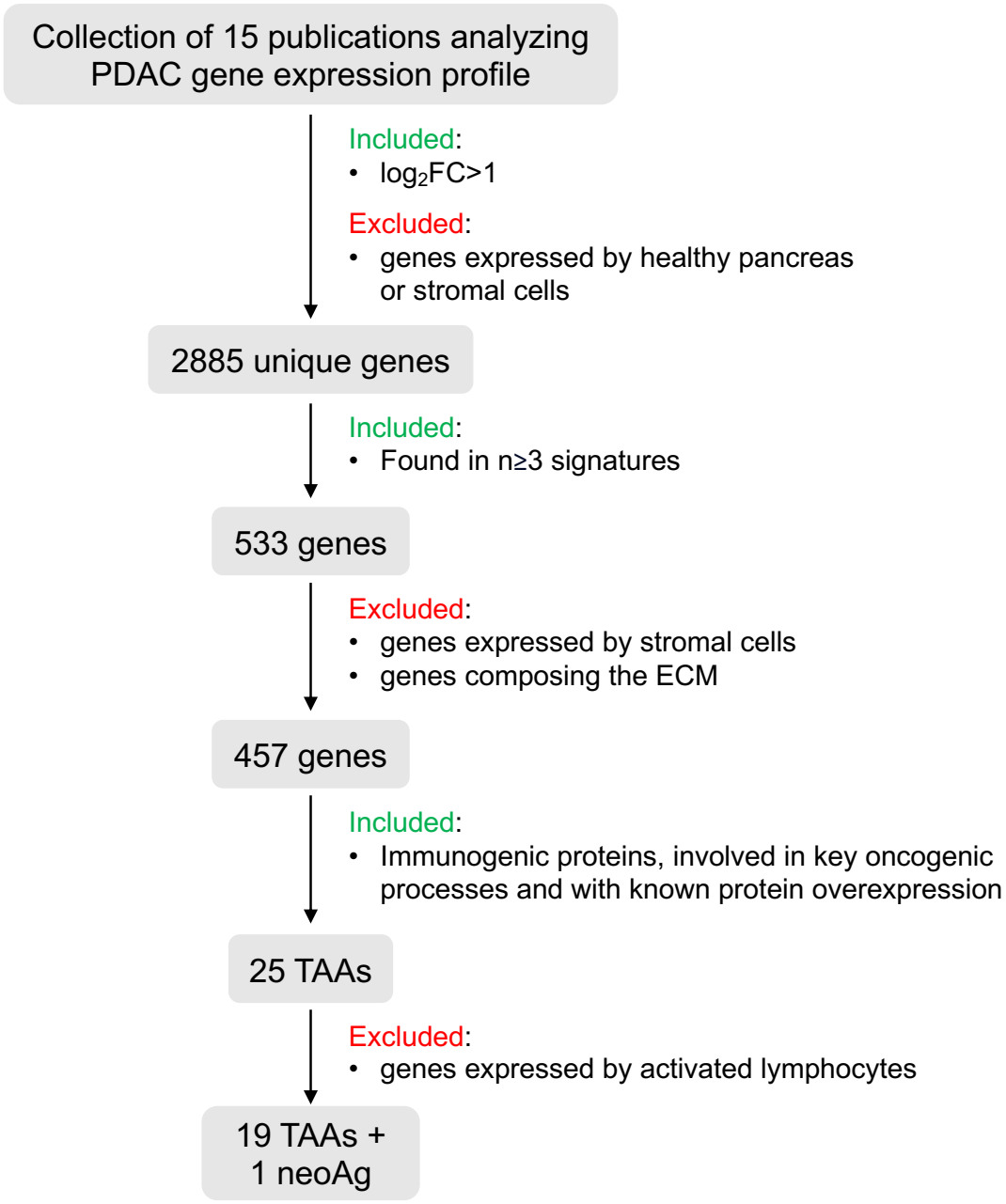

Figure 12. Workflow for the identification of PDAC-associated antigens. In green are highlighted the inclusion criteria, while in red are the exclusion criteria.

Removing genes expressed also by activated lymphocytes, which could potentially cause fratricides phenomena in the ACT product, we obtained 19 tumor associated antigens: AGR2, ANXA2, CEACAM6, DKK1, ECT2, ENO1, EPCAM, HER2, MET, MSLN, MUC1, MUC4, MUC5AC, MUC16, SERPINB3, TMPRSS4, TOP2a, TSPAN1,

TWIST1. In addition, we included mutant KRAS, which is an oncogenic driver in PDAC and overexpressed by 80% of patients (Kleeff et al. 2016). Following this approach, we identified some new potential targets as well as well-known antigens. Indeed, among genes passing all the filtering criteria we found EPCAM, HER2, MET, MSLN, MUC1 and MUC16, which have been already exploited for ACT strategies, corroborating the strength of such an approach.

Finally, we selected 79 peptides derived from these antigens, restricted for HLA class I alleles frequent in the Caucasian population (Gonzalez-Galarza et al. 2020) (**Table 1**), and exploited them to isolate tumor-specific TCRs.

Antigen	Peptide	Restriction	Reference	Name
AGR2	LLVALSYTL	A*0201	Lee 2012	P1
	RIMFVDPSL	A*0201	Lee 2012	P2
	ALYKSKTSNK	A*0301	Bassani-Sternberg 2019	P3
ANXA2	KIRSEFKRK	A*0301	Bassani-Sternberg 2019	P4
	RLYDSMKGK	A*0301	Bassani-Sternberg 2019	P5
	SIRKEVKGDL	A*0201	Antwi 2009	P6
CEACAM6	VLYGPDVPTI	A*0201	Patent EP2547354A2	P7
DKK1	ALGGHPLLGV	A*0201	Qian 2007	P8
	ILYPGGNKV	A*0201	Qian 2007	P9
ENO1	TIAPALVSK	A*0301	Bassani-Sternberg 2019	P10
	RIAKAVNEK	A*0301	Bassani-Sternberg 2019	P11
	QIGSVTESL	A*0201	Antwi 2009	P12
	YISPDQLADL	A*0201	Antwi 2009	P13
EPCAM	VVAGIVVLV	A*0201	Choi 2018	P15
	KPYDSKSLRTAL	B*0702	Bassani-Sternberg 2019	P16
c-MET	YVDPVITSI	A*0201	Schag 2004	P17
	SYREDPIVY	C*0702	Bassani-Sternberg 2019	P18
MSLN	SLLFLLFSL	A*0201	Stromnes 2015	P19
	VLPLTVAEV	A*0201	Stromnes 2015	P20
	AFYPGYLCSL	A*2402	Tsukagoshi 2018	P21
	KLLGPHVEGL	A*0201	Yokokawa 2005	P22
	KLLGPHVLGV	A*0201	Yokokawa 2005	P23
MUC1	APDNRPAL	B*0702	Gatard Scheikl 2017	P28
MUC4	LLGVGTFVV	A*0201	Wu 2009	P29
	APSSHDATL	B*0702	Bassani-Sternberg 2019	P30
	FPSSVTNTL	B*0702	Bassani-Sternberg 2019	P31
	SPSGETATSSL	B*0702	Bassani-Sternberg 2019	P32
	IPRVPSKVSA	B*0702	Bassani-Sternberg 2019	P33
	VPTGHATSL	B*0702	Bassani-Sternberg 2019	P34
MUC5AC	FLNDAGACV	A*0201	Yamazoe 2011	P35
	TCQPTCRSL	A*2402	Yamazoe 2011	P36
SERPINB3	VLHKAFVEV	A*0201	Duan 2013	P37
	VLSGVLHKA	A*0201	Duan 2013	P38
TMPRSS4	SLDVKPLRK	A*0301	Bassani-Sternberg 2019	P39
TSPAN1	VYTTMAEHF	A*2402 C*0702	Bassani-Sternberg 2019	P40
ECT2	SLNTPNSNR	A*0301	Bassani-Sternberg 2019	P41
	RAFSFSKTPK	A*0301	Bassani-Sternberg 2019	P42
	QTRPPASLK	A*0301	Bassani-Sternberg 2019	P43
	RPVQRLPSV	B*0702	Bassani-Sternberg 2019	P44
	IPSPSLVSL	B*0702	Bassani-Sternberg 2019	P45
TOP2A	FLYDDNQRV	A*0201	Berinstein 2012	P46
TWIST1	SLNEAFAAL	A*0201	Poon 2011	P47
	KLAARYIDFL	A*0201	Poon 2001	P48
HER2	ILHNGAYSL	A*0201	Kawashima 1998	P49
	RLLQETELV	A*0201	Rongcun 1999	P50
	VVLGVVFGI	A*0201	Kawashima 1998	P51
	YMIMVKCWMI	A*0201	Rongcun 1999	P52
	HLYQGCQVV	A*0201	Kawashima 1998	P53
	YLVPQQGFFC	A*0201	Scardino 2001	P54
	TYLPANASL	A*2402	Okugawa 2000	P55

HER2	ILHNGAYSL	A*0201	Kawashima 1998	P49
	RLLQETELV	A*0201	Rongcun 1999	P50
	VVLGVVFGI	A*0201	Kawashima 1998	P51
	YMIMVKCWMI	A*0201	Rongcun 1999	P52
	HLYQGCQVV	A*0201	Kawashima 1998	P53
	YLVPQQGFFC	A*0201	Scardino 2001	P54
	TYLPANASL	A*2402	Okugawa 2000	P55
MUC16	SPSPVTSTL	C*0702	Bassani-Sternberg 2019	P56
	SPRLNTQGNTAL	B*0702	Bassani-Sternberg 2019	P57
	SPRFVETTRSSSSL	B*0702	Bassani-Sternberg 2019	P58
	SPVSATSVL	B*0702	Bassani-Sternberg 2019	P59
	SPQSMSNTL	B*0702	Bassani-Sternberg 2019	P60
	TPGNRAISL	B*0702	Bassani-Sternberg 2019	P61
	SPHPVTALL	B*0702	Bassani-Sternberg 2019	P62
	SPTHVTQSL	B*0702	Bassani-Sternberg 2019	P63
	IPRVFTSSI	B*0702	Bassani-Sternberg 2019	P64
	TPGGTRQSL	B*0702	Bassani-Sternberg 2019	P65
	SPQNLRNTL	B*0702	Bassani-Sternberg 2019	P66
	SPAGEAHSL	B*0702	Bassani-Sternberg 2019	P67
	LPSPTTAGPLL	B*0702	Bassani-Sternberg 2019	P68
	RQPNILVHL	C*0702 A*0205	Bassani-Sternberg 2019	P69
	KMISAIPTL	C*0702 A*0205	Bassani-Sternberg 2019	P70
	SPHPVSTTF	C*0702 B*0702 B*1801 C*0701	Bassani-Sternberg 2019	P71
KRAS	YKLVVVGACGVGKSALT	nd	C29331	PR18
	KLVVVGAGDVGKSALTI	nd	Kubuschok 2006	PR54
	KLVVVGADGV	A*02	Schirle 2000	PR6-V10
	KLVVVGAAGV	A*02	Schirle 2000	PR7-V10
	EYKLVVVGAVGVGKS	A*02	Quandt 2018	KRAS1
	KLVVVGAVGV	A*02	Kubuschok 2006	KRAS2
	VVGADGVGK	A*1101	Tran 2015	KRAS3
	GADGVGKSAL	C*0802	Tran 2015	KRAS4

Table 1. Selected immunogenic peptides derived from the identified TAAs and mutant KRAS. 79 peptides were selected to be restricted for common HLA class I alleles in the Caucasian population. Among them, 35 originate from the ligandome of 3 PDAC patients and 3 from the ligandome of a PDAC cell line.

2.2 Isolation of tumor-specific TCRs targeting relevant antigens in gastrointestinal tumors

To isolate novel anti-tumor TCRs we applied the pipeline already developed and employed by the host lab (Ruggiero et al. 2022; Potenza et al. 2023). PBMCs collected from 10 healthy donors have been repetitively stimulated with autologous APCs loaded with pools of different peptides (**Table 2**). Initially, we focused our efforts on the isolation of MUC1- and MSLN-specific TCRs, which are tumor-associated antigens that have been extensively employed as targets for ACT strategies against gastrointestinal tumors. However, all T cells stimulated with MUC1 or MSLN peptides

pools died few weeks after the first stimulation or did not produce a specific activation.

		Stimulation round					
Donor	**Peptide pool**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
#1	Pool MUC1						US
#2	Pool MUC1						
#3	Pool MUC1				US		
#4	Pool MSLN				US		
#5	Pool MSLN						
#6	Pool MSLN						
#7	Pool A*0201						
#8	Pool MSLN			US			
#9	Pool MSLN						
#10	Pool MSLN						

Table 2. Scheme of HDs employed for the isolation of tumor-specific TCRs. PBMCs derived from 10 different healthy donors (#n°) were stimulated with the indicated pools. In green there is the successful maintenance of T cell culture, in orange is reported the loss of T lymphocytes. US represents the culture that have been tested and reported as unspecific. S=stimulation.

Interestingly, we observed T cell response only in one donor (HD7), which was stimulated with a larger pool containing multiple antigens. More in detail, we stimulated T cells derived from one healthy donor (HD7) with a pool of HLA-A*02:01 restricted peptides (pool A*02:01), since it is the most frequent HLA class I allele in the Caucasian population (Gonzalez-Galarza et al. 2020) (**Table 3**). Specifically, PBMCs derived from HD7 were stimulated every 10-12 days with autologous antigen presenting cells (APCs) previously loaded with the pool A*02:01 restricted peptides. Alternatively, immortalized autologous B cells (Ruggiero et al. 2022) have been exploited as APCs. After 3 stimulation rounds, we observed a T cell response by measuring CD107a expression, IFNγ and IL-2 production (**Figure 13A, B**). To identify the epitope that was eliciting T cell activation, we challenged T cells with 8 sub-pools of peptides designed in the way that each peptide is present in only two sub-pools, following a mapping grid (**Figure 13C**). We detected a strong response toward SP2 and SP7, but also a mild response toward SP1 and SP6. By deconvoluting the mapping grid, we defined the recognized epitopes as P9 and P35 (**Figure 13D**).

pool A*0201

Antigen	Name
AGR2	P1
	P2
ANXA2	P6
CEACAM6	P7
DKK1	P8
	P9
ENO1	P12
	P13
c-MET	P17
MUC4	P29
MUC5AC	P35
SERPINB3	P37
	P38
TOP2A	P46
TWIST1	P48

Table 3. List of the 15 peptides forming the pool A*0201. All peptides are restricted for the HLA-A*0201 allele.

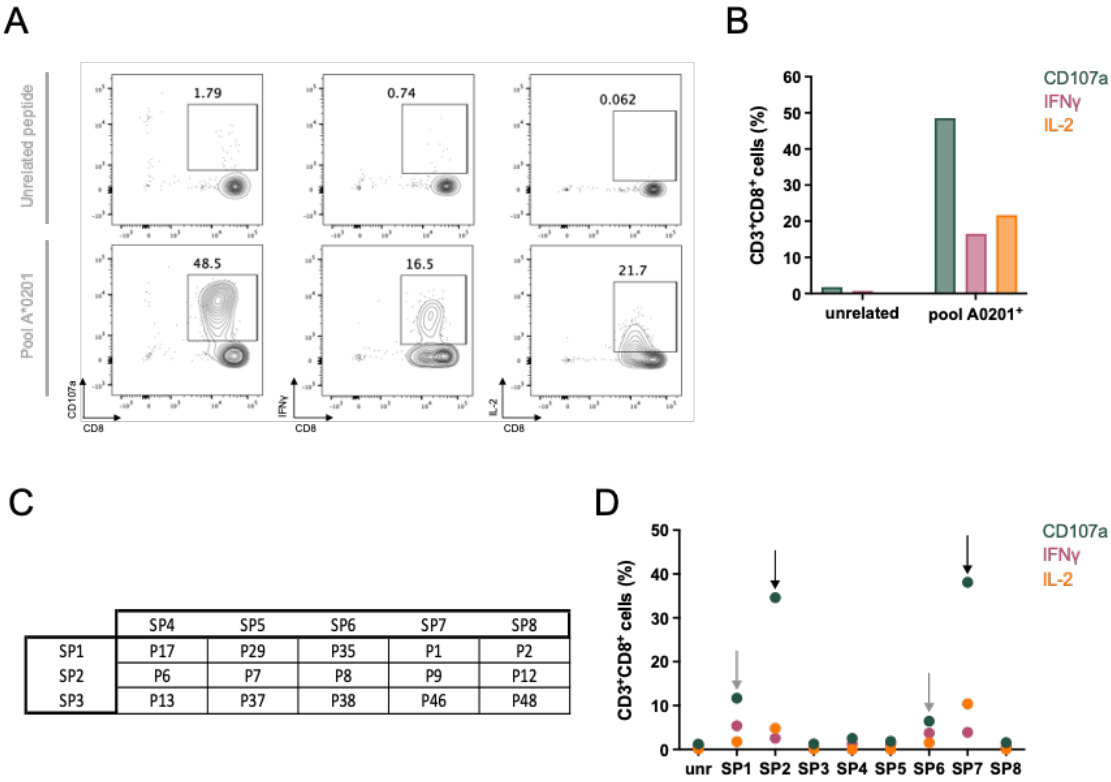

Figure 13. Enrichment of tumor-specific T cells from HD7. (A,B) After 3 rounds of stimulation, expression of CD107a and production of IL-2 and INFγ were measured by flow cytometry after a 6 hour co-culture of T cells with autologous APCs pulsed with the pool A*0201 or an unrelated peptide. (C) Mapping grid of peptides sub-pools. (D) Frequency of CD107a$^+$,

IL-2^{+} and INFγ^{+} CD8 T cells upon 6 hours stimulation with autologous APCs pulsed with peptide subpools. High CD107a expression was detected in response to SP2 and SP7, as indicated by black arrows. Mild CD107a expression was detected in response to SP1 and SP8, as indicated by gray arrows. IFN=interferon; IL=interleukin; SP=subpool; P=peptide; unr=unrelated.

Therefore, we pulsed APCs with P9 or P35 and split T cells into two different cultures to further enrich for these specificities. One additional stimulation step with P9 was sufficient to achieve up to 73% degranulating cells (**Figure 14A**), while for P35 we observed 13% of CD107a positive CD8 T cells after the fifth stimulation (**Figure 14B**).

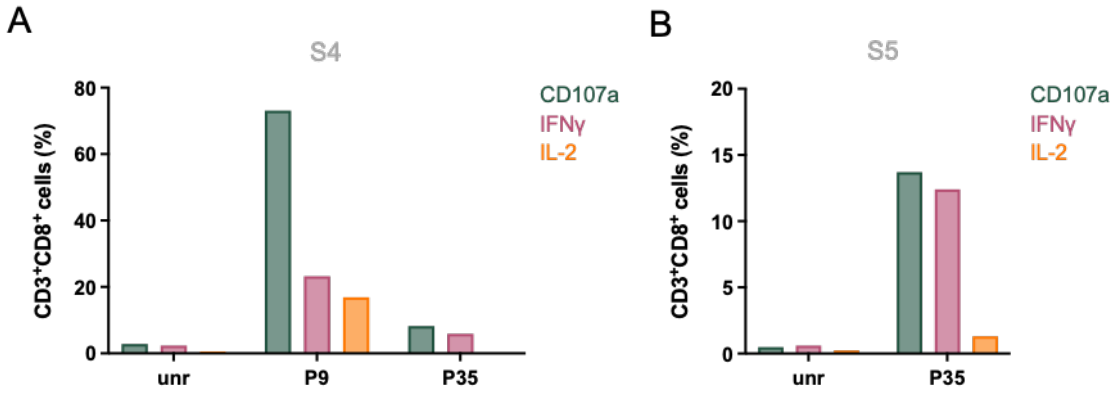

Figure 14. Expansion of P9- and P35-specific T cells. (A) T cell specificity was assessed by measuring CD107a expression, INFγ and IL-2 production upon 6 hours of co-culture with T2 cells (HLA-A*0201 positive) pulsed with either P9, P35 or an unrelated peptide. (B) P35-specific T cell culture was further tested at stimulation round 5 as in (A). IFN=interferon; IL-2=interleukin 2; SP=subpool; P=peptide; unr=unrelated; S=stimulation IFNγ= interferon gamma.

P9 was the immunodominant epitope for this T cell population. However, since we observed a slight activation also with other peptides (**Figure 13D**), we sought to remove P9 (and P35) from the stimulation pool to unmask novel T cell specificities. We thawed HD7 PBMCs and stimulated them with the same protocol described above with the same pool of A*0201 restricted peptides, deprived of P9 and P35. We also added P47 to the peptide pool (**Table 4**).

modified pool A*0201

Antigen	Name
AGR2	P1
	P2
ANXA2	P6
CEACAM6	P7
DKK1	P8
ENO1	P12
	P13
c-MET	P17
MUC4	P29
SERPINB3	P37
	P38
TOP2A	P46
TWIST1	P47
	P48

Table 4. List of the 14 peptides constituting the modified version of pool A*0201.

As before, a response against the peptide pool was detected at the end of the third stimulation by measuring CD107a expression and IFNγ production, despite we did not observe IL-2 secretion (**Figure 15A, B**). To define the peptide recognized by T cells, we tested the activated cells with APCs loaded with sub-pools (**Figure 15C**).

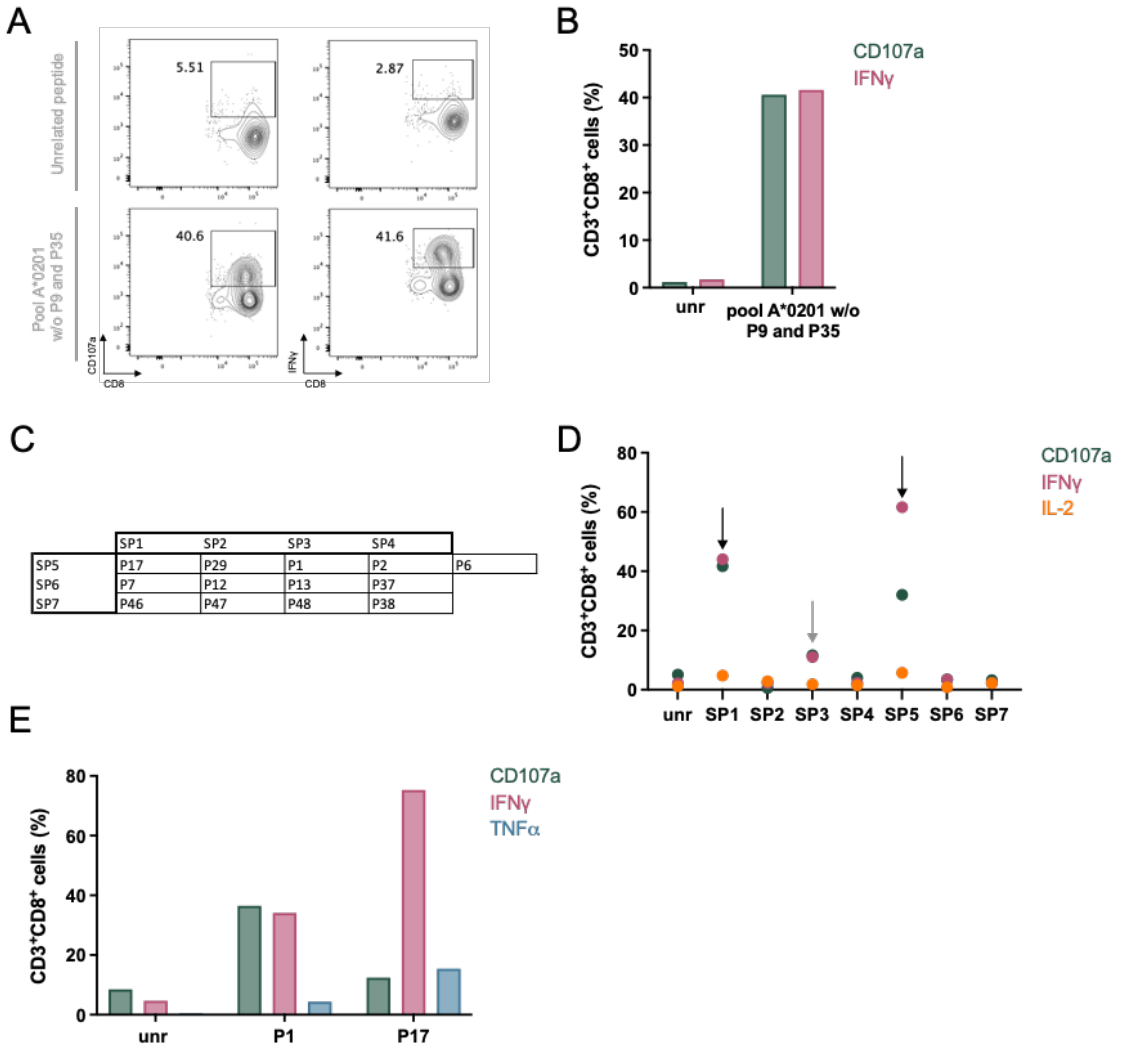

Figure 15. Enrichment of tumor-specific T cells from frozen HD7 PBMCs stimulated with a modified pool. (A,B) Expression of CD107a and INFγ production were measured by flow cytometry after a 6 hour co-culture of T cells with autologous APCs pulsed with the modified pool A*0201 or an unrelated peptide. (C) Mapping grid of peptides sub-pools. (D) Frequency of CD107a$^+$, IL-2$^+$ and INFγ^+ CD8 T cells upon 6 hours stimulation with autologous APCs pulsed with peptide subpools. High CD107a expression was detected in response to SP1 and SP5, as indicated by black arrows. Mild CD107a expression was detected in response to SP3, as indicated by the gray arrow. (E) T cell specificity was confirmed upon co-culture with T2 (HLA-A*0201$^+$) cells pulsed with individual peptides or an unrelated one. IFN=interferon; IL-2=interleukin 2; SP=subpool; P=peptide; unr=unrelated; IFNγ= interferon gamma TNFα=tumor necrosis factor a.

We observed two potent responses toward SP1 and SP5, and a mild one toward SP3. By resolving the mapping grid, we identified P17 and P1 as target peptides. (**Figure 15D**). However, since SP5 contains also P6, which immunogenicity cannot be confirmed by a response toward a second sub-pool, we separated HD7 T cells in two different cultures and stimulated them with APCs pulsed with P17 or P1 and P6. We assessed T cell responses against individual peptides and noted the

recognition of P1 and P17 only, as underlined by the CD107a expression, IFNγ and TNFα production (**Figure 15E**).
Overall, we isolated 4 different T cell anti-tumor specificities from one single healthy donor.

P9 is a peptide derived from DKK1, a soluble inhibitor of the Wnt/β-catenin pathway, involved in promoting tumor cell proliferation and dissemination (Kimura et al. 2021; Qi et al. 2021). In PDAC, DKK1 overexpression has been evaluated as biomarker for an early detection and is generally associated with a poor prognosis (D.-J. Liu et al. 2017).
P35 is an epitope of MUC5AC, which is a mucin that creates a protective barrier underneath the stomach and respiratory tract epithelium. It is found upregulated in different solid tumors, where it fosters invasiveness and migration abilities (Lakshmanan et al. 2016; Manne et al. 2021).
P17 derives from c-MET, a well-known tumor associated antigen already exploited as target of CAR-T cell therapies (Shah et al. 2023). In pancreatic cancer, cMET mediates pro-efficient tumor-stroma interactions and together with its ligand, the hepatocyte growth factor, favors tumorigenesis (Rucki et al. 2017; Xu et al. 2020).
P1 originates from AGR2, a protein resident in the endoplasmic reticulum. In PDAC, AGR2 participates in promoting tumorigenesis resolving ER stress caused by the intense protein synthesis demand and enhancing transcription (Dumartin et al. 2017; Zhiheng Zhang et al. 2021). In addition, it is associated with reduced survival and a metastatic phenotype (Tian et al. 2017; H. Zhang et al. 2021).

In addition to HD7, we wondered if the TCR hunting pipeline could be applied to isolate new anti-tumor TCRs from the peripheral blood of cancer patients. To evaluate this, we stimulated T cells derived from the peripheral blood of one PDAC patient with the pool A*0201 plus mutant KRAS-derived and MSLN-derived peptides (pool cancer, **Table 5**). Indeed, in 6 different attempts we could not isolate MSLN-specific TCRs from healthy donors.

pool cancer

Antigen	Name
AGR2	P1
	P2
ANXA2	P6
CEACAM6	P7
DKK1	P8
ENO1	P12
	P13
c-MET	P17
MSLN	P19
	P20
	P22
	P23
MUC4	P29
SERPINB3	P37
	P38
TOP2A	P46
TWIST1	P47
	P48
KRAS	PR18
	PR6 V10
	PR7 V10
	KRAS1
	KRAS2
	KRAS3
	KRAS4

Table 5. List of 25 peptides composing the cancer pool.

As reported above, PBMCs retrieved from one PDAC patient were repetitively stimulated with autologous APCs loaded with the "cancer pool". At the second stimulation round we already retrieved CD107a expression together with IFNγ and IL-2 production (**Figure 16A, B**). We further stimulated T cells and subsequently identified the epitope inducing T cell activity by co-culturing them with APCs pulsed with sub-pools (**Figure 16C**). Only upon stimulation with SP3 and SP6, we found up to 71.6% CD107a and 85.8% IFNγ producing cells, highlighting P23 as the target peptide (**Figure 16D**). By challenging T cells with T2 pulsed with P23, we confirmed its specific recognition (**Figure 16E**) and thus isolated P23-specific T cells.

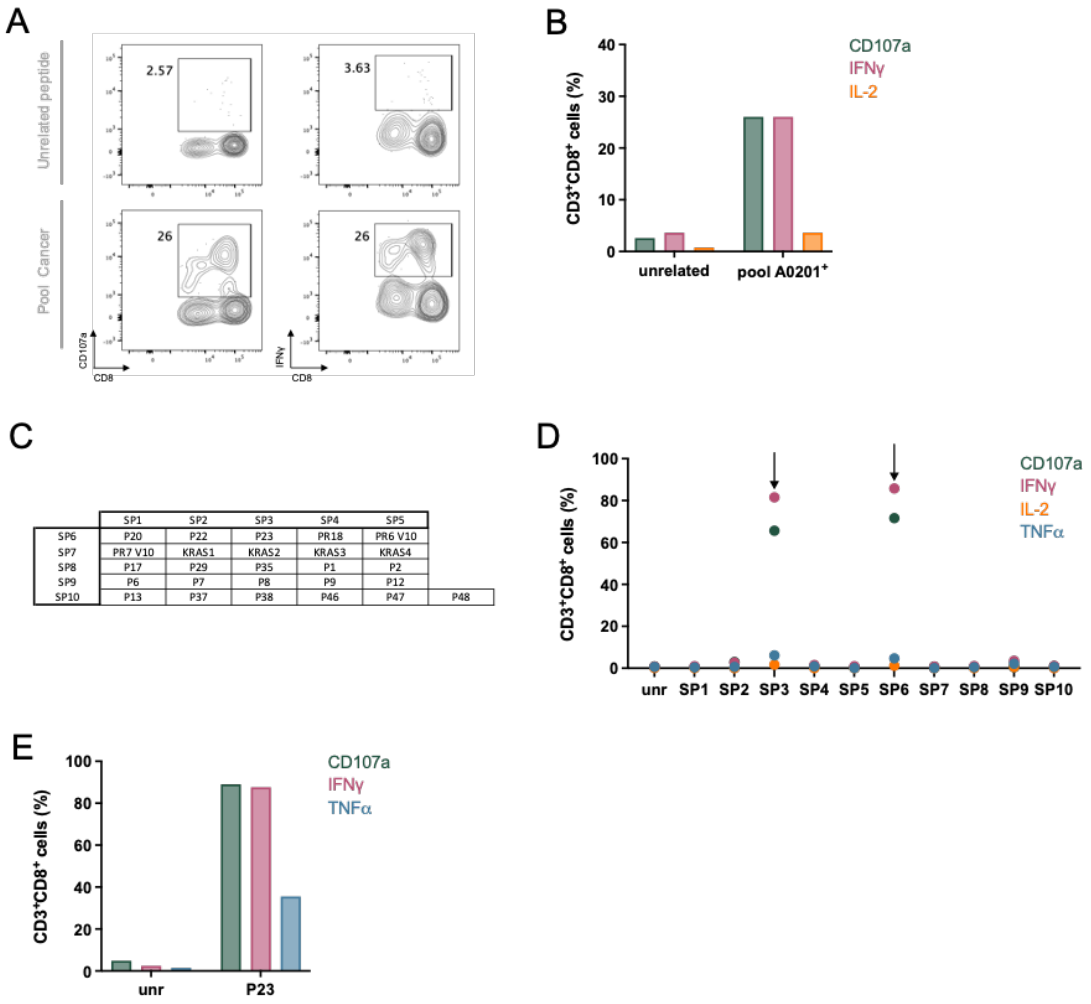

Figure 16. Enrichment of tumor-specific T cells from PBMCs of one PDAC patient. (A,B) T cells were co-cultured for 6 hours with autologous APCs loaded with the cancer pool or an unrelated peptide. Expression of CD107a, INFγ and IL-2 production were measured by flow cytometry. (C) Mapping grid of sub-pools of peptides. (D) Frequency of CD107a+, IL-2+ and INFγ+ and TNFα+ CD8 T cells upon 6 hours stimulation with autologous APCs pulsed with peptide subpools. T cell activation, reported as CD107a and IFNγ expression, was observed for SP3 and SP6, as indicated by black arrows. (E) To validate T cell specificity, T cells were challenged with T2 (HLA-A*0201 positive) cells pulsed with the individual peptide or an unrelated one. IFN=interferon; IL=interleukin; SP=subpool; P=peptide; unr=unrelated; TNF=tumor necrosis factor.

P3 derives from mesothelin (MSLN), a tumor-associated antigen commonly overexpressed in solid tumors. MSLN-specific CAR-T and TCR-T cells have been developed to specifically treat mesothelioma, pancreatic and ovarian cancer (Stromnes et al. 2015; Anderson et al. 2019; Adusumilli et al. 2021). MSLN is overexpressed in PDAC, where it promotes metastatization (Chen et al. 2013; Stromnes et al. 2015).

To better evaluate the relevance of DKK1, MUC5AC, MET, AGR2 and MSLN in patients harboring gastrointestinal tumors, we analyzed the transcriptome of primary and metastatic PDAC and CRC patients enrolled at Ospedale San Raffaele. In a cohort of 10 chemonaïve PDAC primary samples and 35 PDAC liver metastases (Balestrieri et al. *in preparation*), all the antigens were highly expressed, with MUC5AC being the most variable (**Figure 17A, right**). This further validates the robustness of the established antigen selection workflow. Moreover, we observed that AGR2 and MET are upregulated both in primary CRC and liver metastases samples (*dataset* from Potenza et al. 2023) (**Figure 17A, left**). As summarized in **Figure 17B**, the vast majority of PDAC patients display high expression of all these antigens confirming their relevance as therapeutic target for an ACT strategy. Importantly, 2 out of 5 TCRs could be potentially exploited also to treat colorectal cancer.

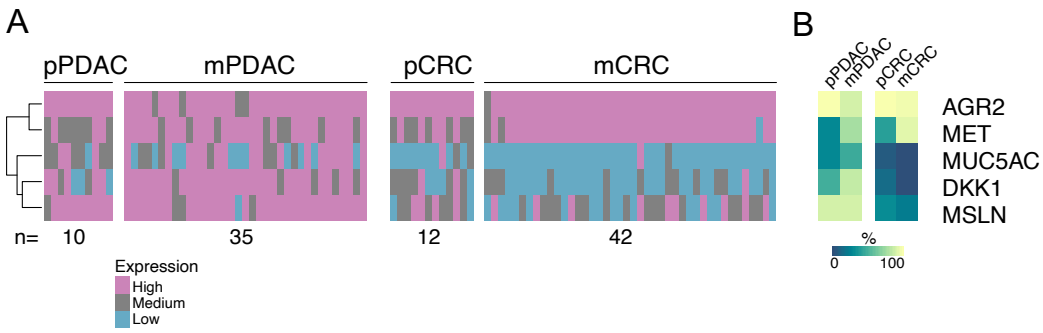

Figure 17. RNAseq analyses of PDAC and CRC patients highlight the therapeutic potential of the isolated T cell specificities. (A) Expression levels of AGR2, MET, MUC5AC, DKK1 and MSLN in primary and metastatic lesions in the liver collected from PDAC and CRC patients. (B) Frequency of PDAC and CRC samples displaying high antigen expression.

2.3 High-throughput TCR sequencing determines the proper TCR a and b pairing to reconstruct the anti-tumor TCR

To determine the TCR sequences of T cell populations responding to the selected TAAs, we collected RNA samples at the end of each stimulation round. RNA was extracted to perform high throughput TCR sequencing and therefore establish the proper TCR αβ pairing (Ruggiero et al. 2015). As expected, TCR clonality progressively narrows overtime, confirming the enrichment of tumor-specific T cells. From the first stimulation of HD7, we expanded two T cell specificities. For DKK1 (P9), we observed 85.1% prevalent CDR3α and a 97.3% prevalent CDR3β

aminoacidic sequences. After we split the T cell culture and separately stimulated it with P35 (MUC5AC), we could identify the second TCR sequences with a frequency of 62.4% and 46.2% for the alpha and the beta chain, respectively (**Figure 18**).

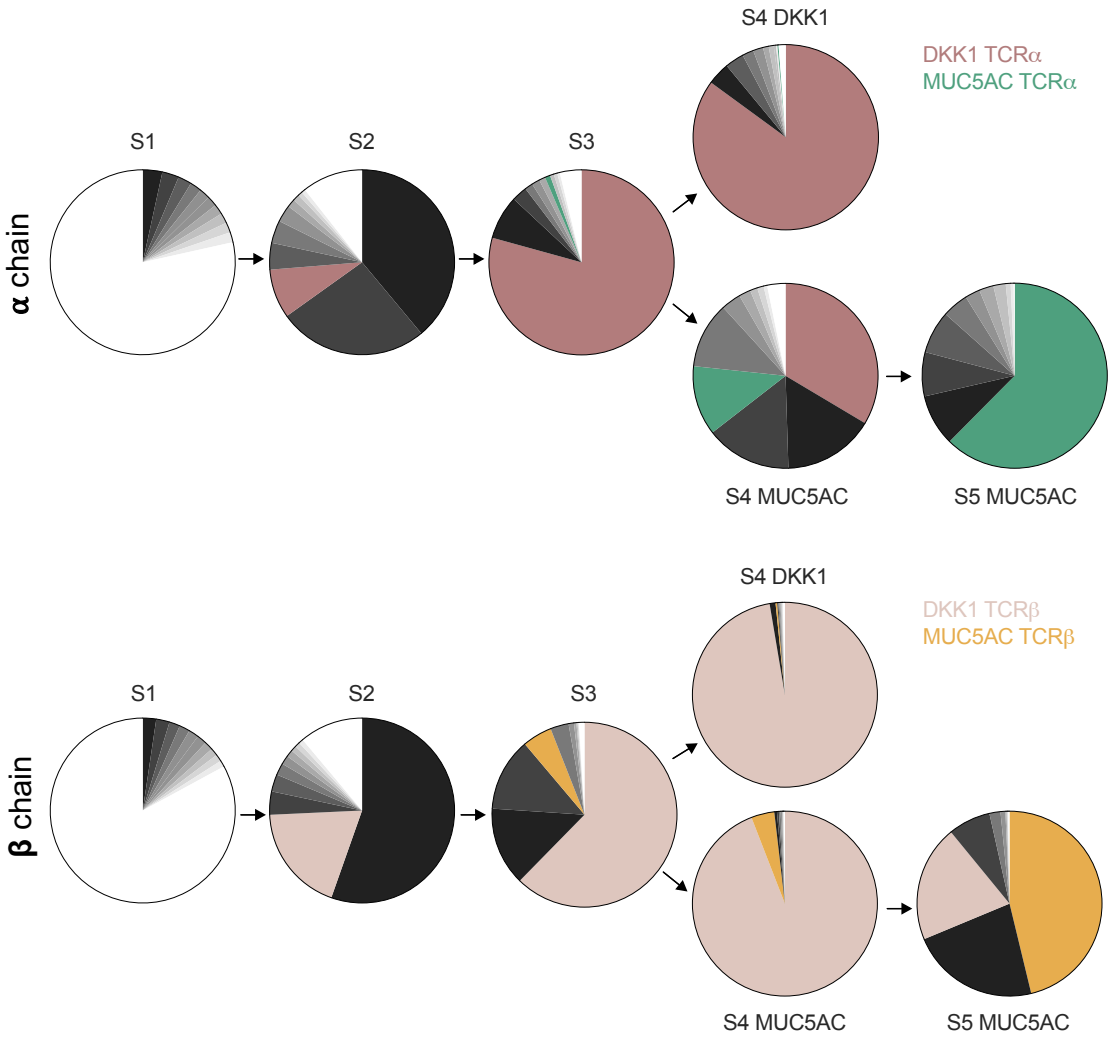

Figure 18. Longitudinal tracking of DKK1- and MUC5AC-specific TCR sequences. The 10 most frequent sequences are plotted in pie charts for the α (top) and β (bottom) CDR3 regions. In different colors are highlighted the sequences belonging to DKK1 and MUC5AC TCRs. S=stimulation.

The second stimulation of HD7 with a pool deprived of P9 and P35 permitted the enrichment of additional anti-tumor T cells. In particular, we observed a 97.9% prevalent CDR3α and a 83.5% prevalent CDR3β specific for MET (P17), and another TCR pairing (frequencies: 57.4% for CDR3α a 39.9% for CDR3β) for AGR2 (P1) (**Figure 19**).

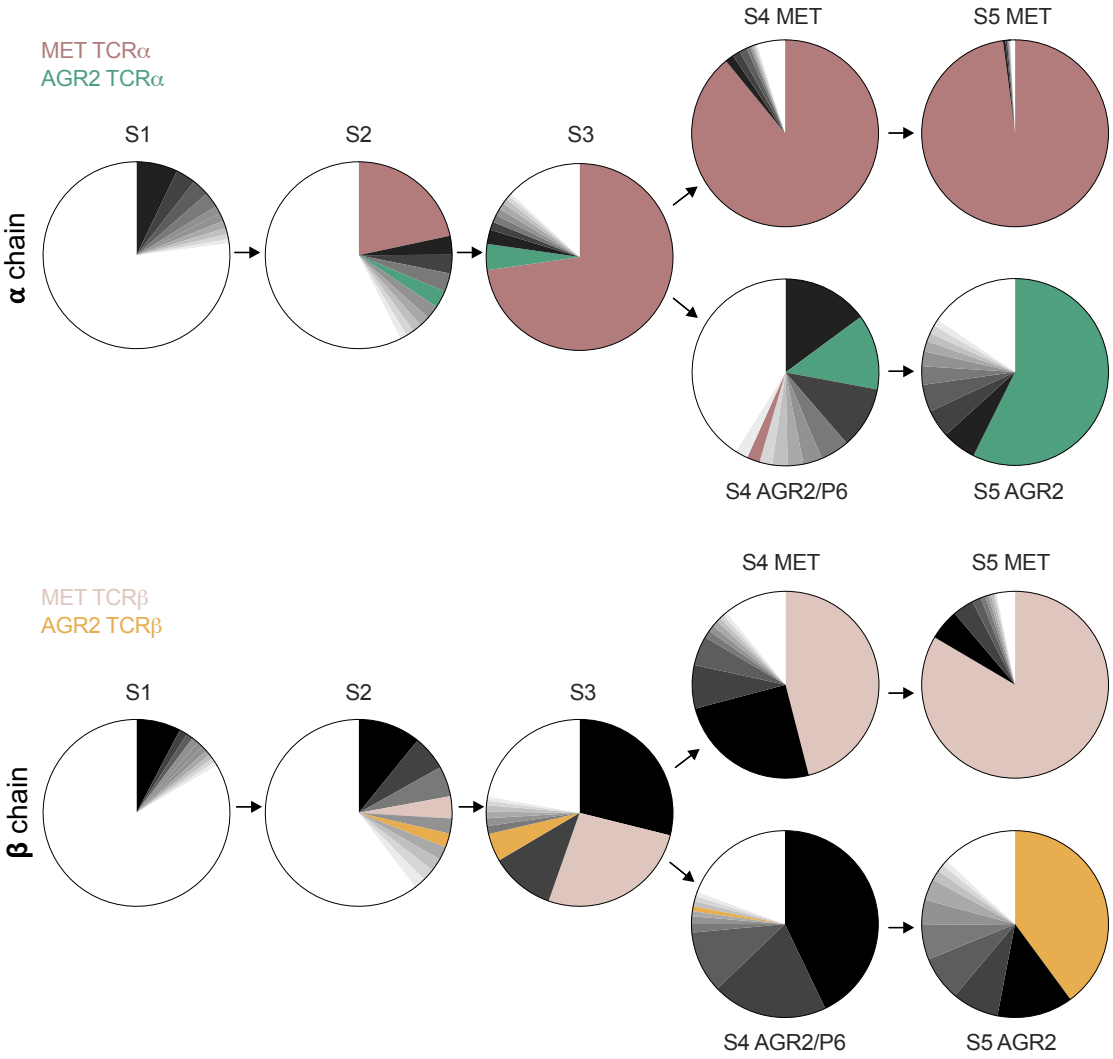

Figure 19. Longitudinal tracking of MET- and AGR2-specific TCRs. The 10 most frequent sequences are plotted in pie charts for the α (top) and β (bottom) CDR3 regions. In different colors are highlighted the sequences belonging to MET and AGR2 TCRs. S=stimulation.

For MSLN (P23), an enrichment of the specific clone was already observed at 2nd stimulation (**Figure 20**). Upon the last stimulation we obtained a monoclonal population. Indeed, we detected prevalent CDR3α and CDR3β sequences with 97.2% and 98.3% frequencies. Interestingly, starting from the initial stimulation we observed the expansion of another TCR (in black) which specificity is not determined.

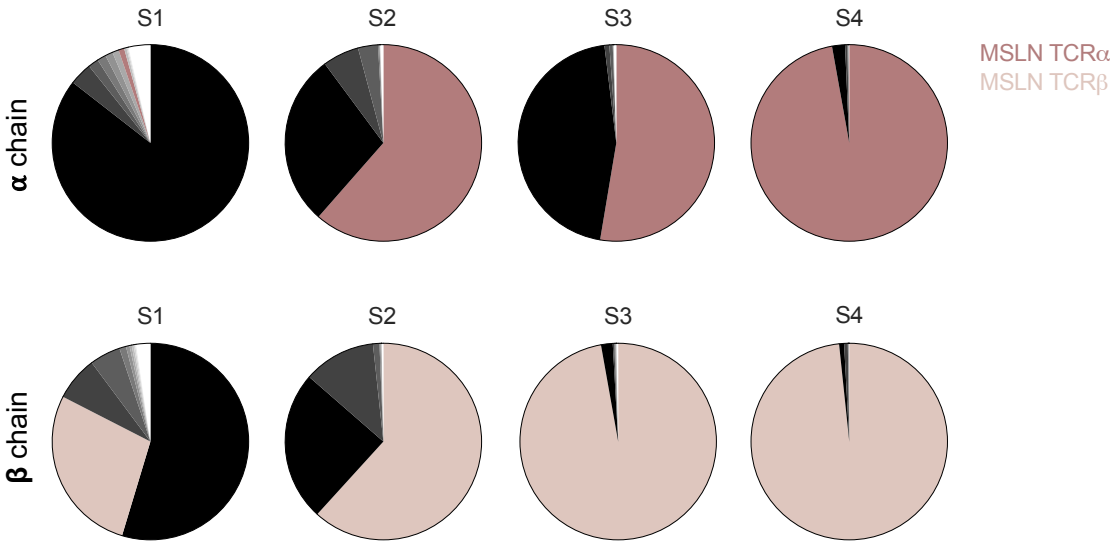

Figure 20. Longitudinal tracking of MSLN-specific TCR expanded from one PDAC patient. The 10 most frequent sequences are plotted in pie charts for the α (top) and β (bottom) CDR3 regions. MSLN CDR3α and CDR3β sequences are outlined in dark and light pink, respectively. S=stimulation.

To summarize, we isolated 5 different anti-tumor TCRs to generate TCR-edited T cells harboring therapeutic potential against gastrointestinal tumors**.**

3. TCR-REDIRECTED T CELLS DISPLAY ANTI-TUMOR ACTIVITY

3.1 TAAs- specific TCRs display differences in cytokine production but comparable tumor killing ability

After the isolation of the novel TCR specificities, we reconstructed the proper TCR alpha and beta chains by employing the IMGT software and cloned wild type sequences into a bidirectional lentiviral vector (**Figure 21A**). To generate TCR-edited T cells, we coupled the genetic disruption of the endogenous TCR repertoire with the lentiviral transduction of tumor-specific TCRs. Briefly, PBMCs collected from healthy donors were stimulated with aCD3/CD28 magnetic beads two days before the editing procedure. At day 2, BE4max was delivered in combination with sgRNAs targeting *TRAC* and *TRBC1/2* by nucleofection. The following day we redirected T cell specificity by lentiviral vector delivery of each anti-tumor TCR (**Figure 21B**). TCR disruption was achieved with a 96.7% mean efficiency as measured by CD3 protein loss (**Figure 21C, right**). Notably, for all tumor-specific TCRs, we obtained a mean transduction

efficiency around 80% confirmed by the restoration of CD3 expression (**Figure 21C, right**) and by the high frequency of specific Vβ-positive cells (**Figure 21C, left**).

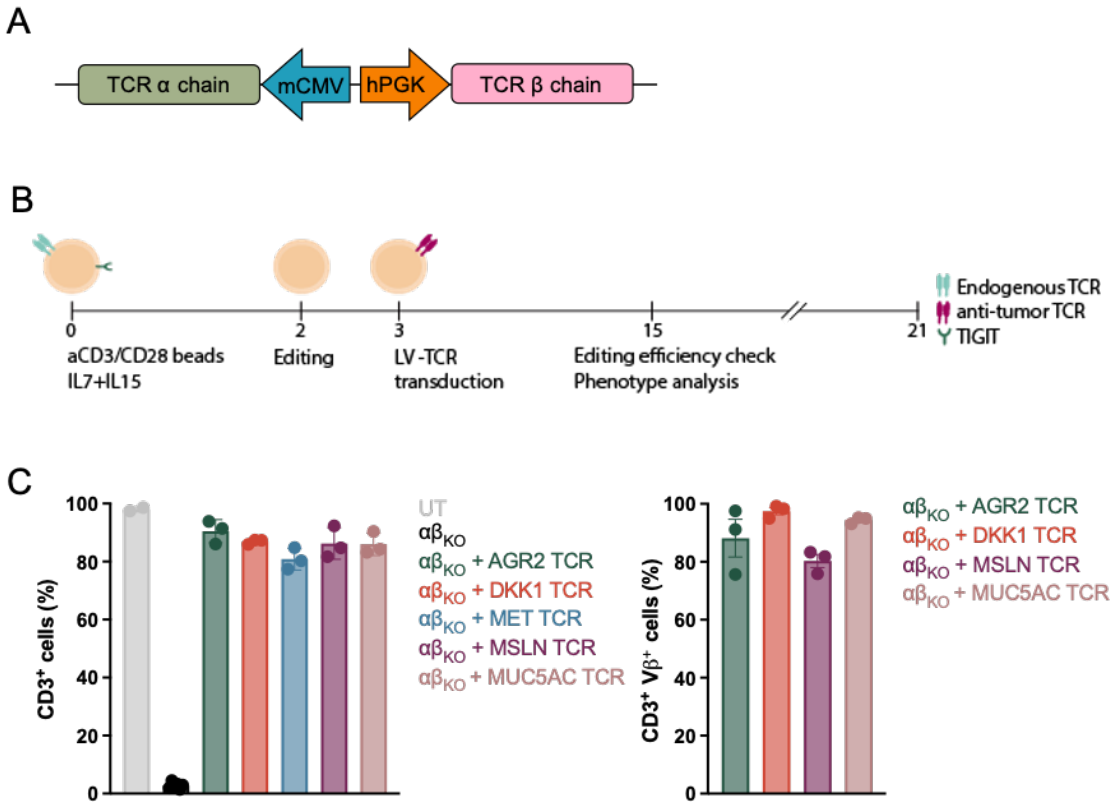

Figure 21. Generation of TCR-edited T cells. (A) Schematic representation of bidirectional lentiviral vector encoding for tumor-specific TCRs. (B) Experimental layout of T cell manipulation procedure. (C) TCR transduction efficiency was measured by evaluating CD3 expression reconstitution and quantifying the positivity for specific TCR Vβ chains. Data are represented as mean ±SEM. n=3 biological replicates.

To assess the anti-tumor activity of the novel cell products, we first tested each TCR specificity in terms of cytokine production. We co-cultured TCR-edited T cells with T2 cell lines pulsed with the specific peptide or an unrelated control. Stimulation with Phorbol myristate acetate (PMA) and Ionomycin (IONO), which bypass the TCR-dependent activation, was added as positive control of T cell fitness. While all TCR-edited T cells responded to PMA/Iono stimulation (**Figure 22**), only DKK1- and MSLN-specific T cells activated the release of conventional pro-inflammatory cytokines and expressed the degranulation marker CD107a upon recognition of the specific peptide (**Figure 22B, Figure 22D**). In addition to DKK1- and MSLN-TCRs, AGR2-TCR triggered the upregulation of CD107a and the production of IFNγ, even though at low frequencies compared to the other two cellular products (**Figure 22A**).

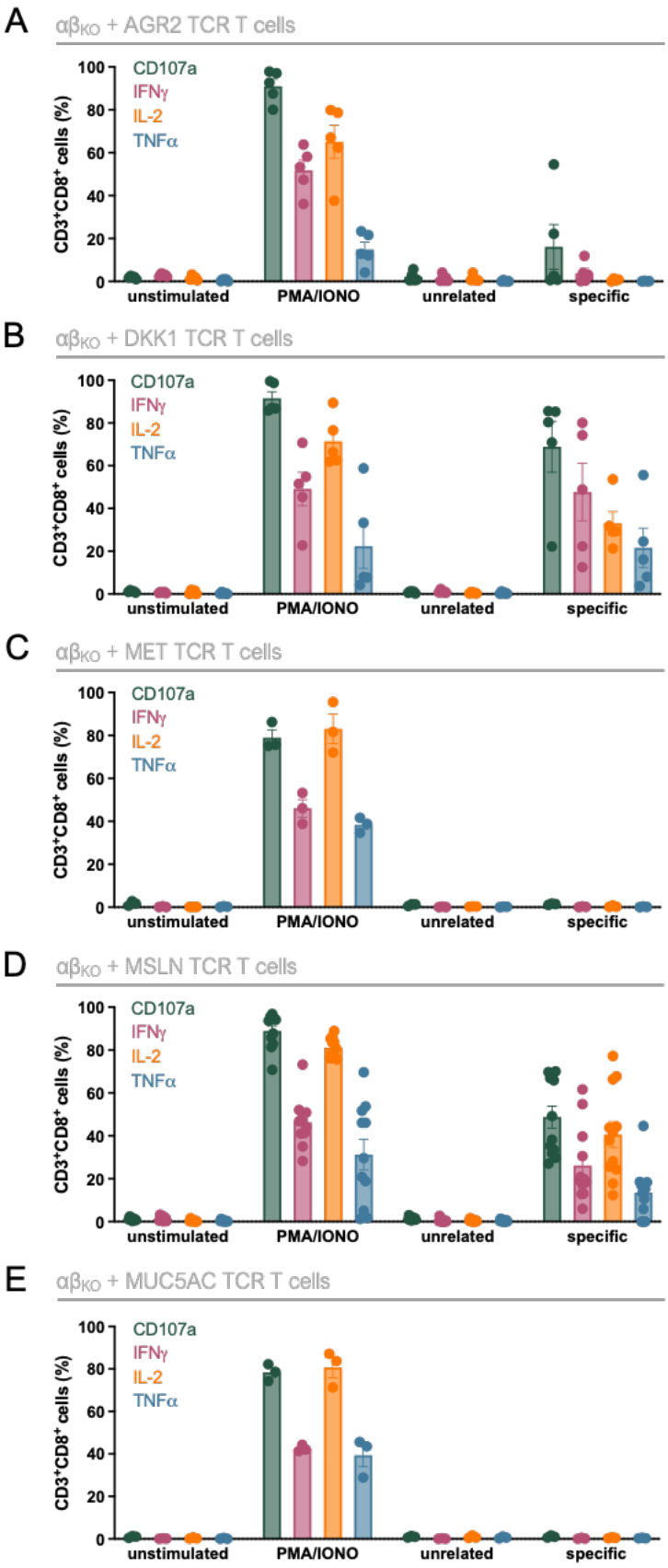

Figure 22. TAAs-specific TCRs differ for cytokine production and CD107a expression. Expression of CD107a and production of IFNγ, IL-2 and TNFα were measured by flow cytometry

in unstimulated AGR2-(**A**), DKK1-(**B**), MET-(**C**), MSLN-(**D**) and MUC5AC-(**E**) specific T cells, T cells stimulated with PMA/Iono for 6 hours and upon 6 hours co-culture of T cells with T2 cell line pulsed with the specific peptide or an unrelated one. Data are represented as mean ±SEM. A minimum of n=3 biological replicates were employed. IFN=interferon; IL=interleukin; TNF=tumor necrosis factor.

Next, to assess the ability of redirected T cells of eliminating tumor cells, we challenged TCR-edited T cells against PDAC cell lines expressing each antigen and harboring the HLA-A*02:01. Surprisingly, all TCR-edited T cell products efficiently killed PDAC cell lines, while sparing solid tumor cell lines selected to be antigen negative or HLA unrelated (**Figure 23**).

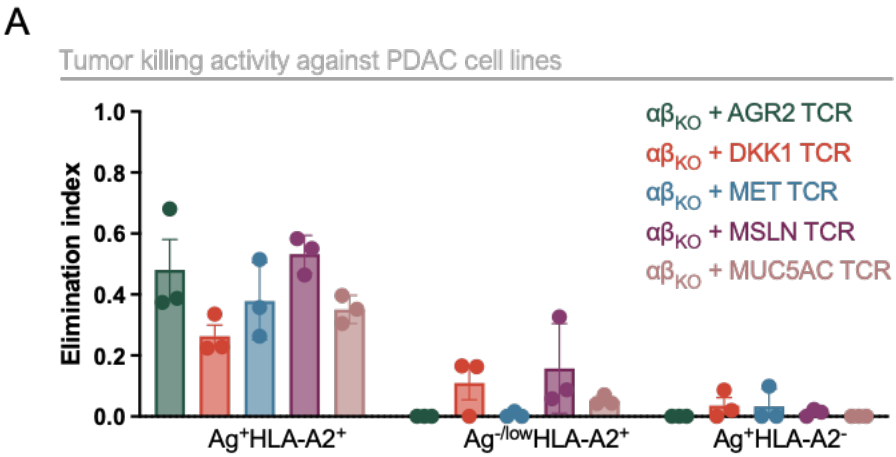

Figure 23. TCR-edited T cells display specific activity against PDAC cell lines. Anti-tumor activity exerted by TCR-edited T cells after a 24 hours co-culture with $Ag^{+}HLA\text{-}A2^{+}$ PDAC cell lines plated in a 5:1 effector-to-target ratio. TCR-edited T cells were co-cultured with antigen negative/low and HLA-unrelated solid tumor cell lines as controls for TCR specificity. Cytotoxicity is reported as elimination index. Data are represented as mean ±SEM. n=3 biological replicates. Ag=antigen.

We next tested the ability of TCR-edited T cells to recognize patient derived organoids expressing HLA-A*02:01 and the antigens. Importantly, all TCR-edited T cell products mediated efficient killing of primary CRC and metastatic CRC generated from liver metastases (mCRC) (**Figure 24A, B**). In addition, DKK1- and MSLN-redirected T cells were tested against primary PDAC PDOs and displayed anti-tumor activity (**Figure 24C**). Overall, these results highlight the ability of even TCRs suboptimal in triggering cytokine production by T cells, i.e AGR2-, MET- and MUC5AC TCRs, in eliminating clinically relevant tumor cells after recognition of naturally processed antigens.

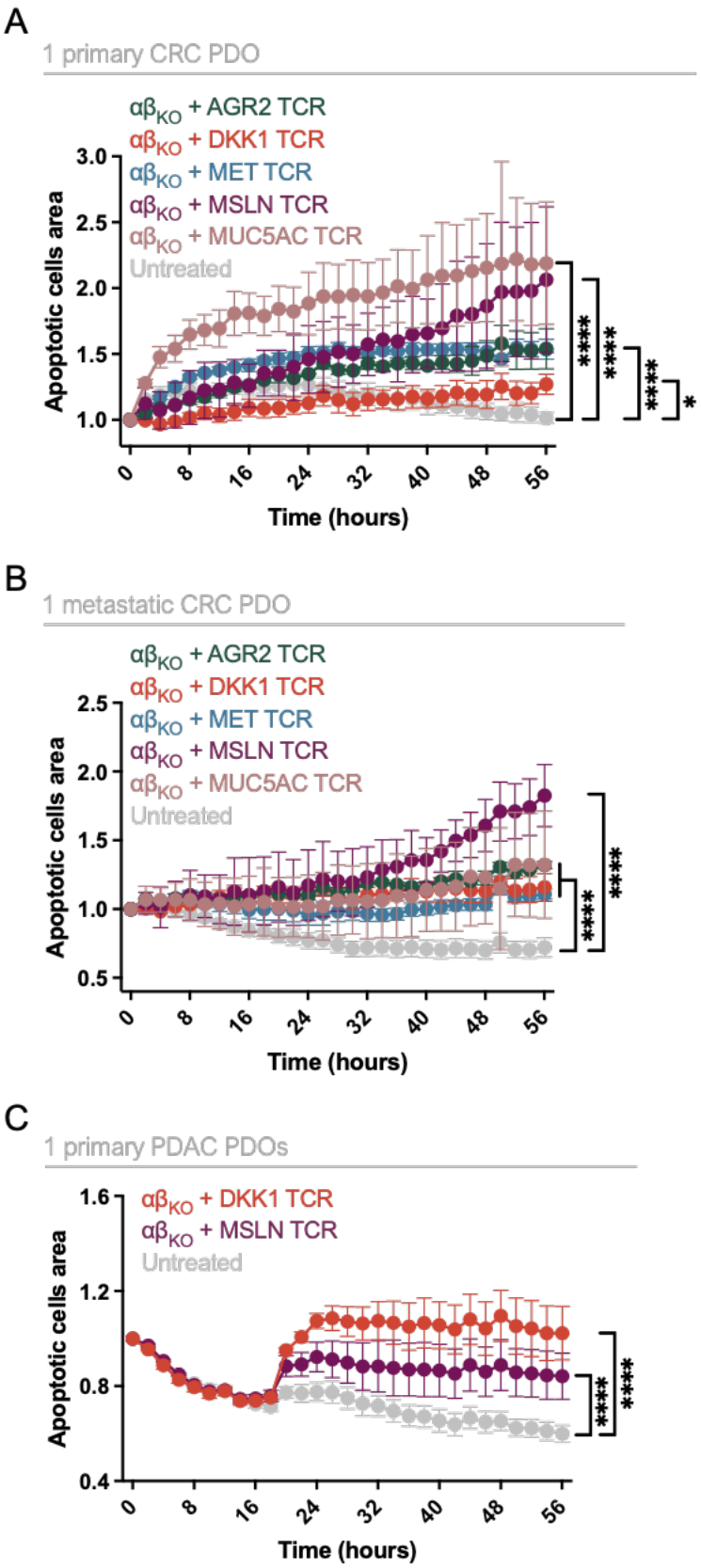
A
1 primary CRC PDO
αβKO + AGR2 TCR
αβKO + DKK1 TCR
αβKO + MET TCR
αβKO + MSLN TCR
αβKO + MUC5AC TCR
Untreated
Apoptotic cells area
Time (hours)
****
****
****
*
B
1 metastatic CRC PDO
αβKO + AGR2 TCR
αβKO + DKK1 TCR
αβKO + MET TCR
αβKO + MSLN TCR
αβKO + MUC5AC TCR
Untreated
Apoptotic cells area
Time (hours)
****
****
C
1 primary PDAC PDOs
αβKO + DKK1 TCR
αβKO + MSLN TCR
Untreated
Apoptotic cells area
Time (hours)
****
****

Figure 24. TCR-edited T cells efficiently eliminate patient-derived organoids from gastrointestinal tumors. TCR-edited T cells were co-cultured with one primary (A) and one metastatic (**B**) CRC PDOs in a 10:1 effector-to-target ratio. (**C**) DKK1- and MSLN-specific T cells were co-cultured with one primary PDAC PDO in a 5:1 effector-to-target ratio. Tumor killing is represented as apoptotic cells area calculated by measuring the caspase 3/7 production in target cells and acquired by Incucyte Live-Cell Analysis System. Data are represented as mean ±SEM. n=3 biological donors for each TCR specificity. *p≤0.05, ****p≤0.0001 by linear regression curve. CRC=colorectal, PDAC=pancreatic ductal adenocarcinoma, PDO=patient-derived organoids.

After acknowledging that MET- and MUC5AC- specific T cells were not able to produce conventional pro-inflammatory cytokines (**Figure 22**), we decided to prioritize the characterization of AGR2-, DKK1- and MSLN-specific TCRs.

3.2 Characterization of AGR2-, DKK1- and MSLN-specific TCRs

First, we assessed DKK1- and MSLN-specific TCRs polyfunctionality in terms of co-expression of conventional cytotoxic cytokines together with the degranulation marker CD107a. TCR-edited T cells were tested in a peptide titration assay using pulsed T2 cells. While MSLN-TCR displayed a larger polyfunctionality, DKK1-TCR showed a more persistent response even to low peptide concentrations (**Figure 25A**).



Figure 25. DKK1- and MSLN-TCRs show differences in the co-expression of pro-inflammatory markers. (**A**) Polyfunctionality of TCR-edited T cells was tested after a 6 hours co-culture with T2 cell line pulsed with different peptides concentration. Pies depict the co-expression of CD107a, IFNγ, IL-2 and TNFα. Data are represented as mean ±SEM. n=3

biological donors for each TCR specificity. ns=not significant, IFN=interferon; IL=interleukin; TNF=tumor necrosis factor.

To evaluate TCRs co-receptor dependency, we sorted $CD8^+$ and $CD4^+$ T cell subsets out of the TCR-edited bulk population. As expected, different TCRs displayed different properties upon co-culture with target cells. Specifically, AGR2- and MSLN-TCR needs the CD8 coreceptor to mediate tumor killing (**Figure 26A, C**), while DKK1 TCR confers autonomous cytotoxic abilities to both CD8 and CD4 T cells (**Figure 26B**).

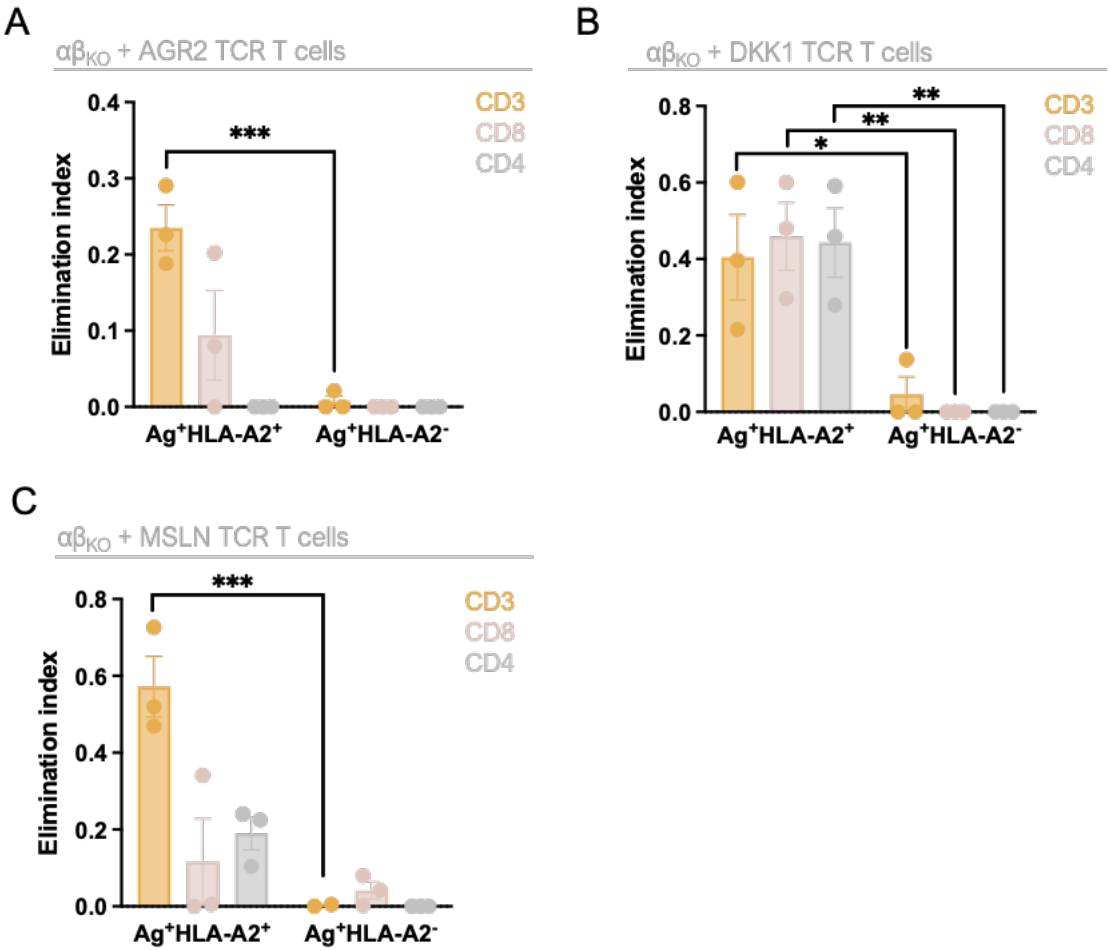

Figure 26. Novel TAA-TCRs display diverse dependency on CD8 and CD4 co-receptor. Bulk TCR-edited T cells were sorted to isolate CD8 and CD4 T cells. CD3, CD8 and CD4 T cells specific for AGR2 (A), specific for DKK1 (B) or for MSLN (C) were co-cultured in a 5:1 effector-to-target ratio for 24 hours with antigen positive HLA-A2 expressing cell line (BxPC3 $A2^+$) or HLA-unrelated cell line (IGROV1), as control. Tumor cell killing is represented as elimination index. Data are represented as mean ±SEM. n=3 biological donors for each TCR specificity. *p≤0.05 **p≤0.01 ***p≤0.001.

4. TIGIT DISRUPTION HAS THE POTENTIAL TO IMPROVE TUMOR KILLING BY TCR-EDITED T CELLS

To boost the activity of tumor-specific T cells we simultaneously disrupted the endogenous TCR repertoire, to achieve full redirection of T cell specificity, and *TIGIT*. T cells were enriched from healthy donors' PBMCs with anti-CD3/CD28 beads and stimulated with a combination of IL-7/IL-15 to maintain a stem cell memory phenotype. To generate TCR-edited (TCR_{ED}) and TCR-edited TIGIT disrupted ($TCR_{ED}TIGIT_{KO}$) T cells, BE4max was co-delivered with sgRNAs targeting the TCR only or together with the sgRNA targeting *TIGIT*. By sanger sequencing, we confirmed that multiple target genes' disruption is highly efficient. Specifically, we observed 90.4% functional knockout at *TRAC locus* and 77% and 64.2% at *TRBC1* and *TRBC2 loci* in TCR disrupted T cells. Importantly, the editing efficiency was comparable in TCR_{ED} and $TCR_{ED}TIGIT_{KO}$ cells (82.4% disruption frequency at *TRAC*, 65.8% disruption frequency at *TRBC1*, 55.2% disruption frequency at *TRBC2*, and 80.8% disruption frequency at *TIGIT*) (**Figure 27A**). Multiplex editing was successfully coupled with LV delivery of anti-tumor TCRs, allowing the development of AGR2-, DKK1- and MSLN-specific T cells wild type or deprived of TIGIT expression (**Figure 27B**) which were comparable in terms of memory phenotype differentiation (**Figure 27C**).

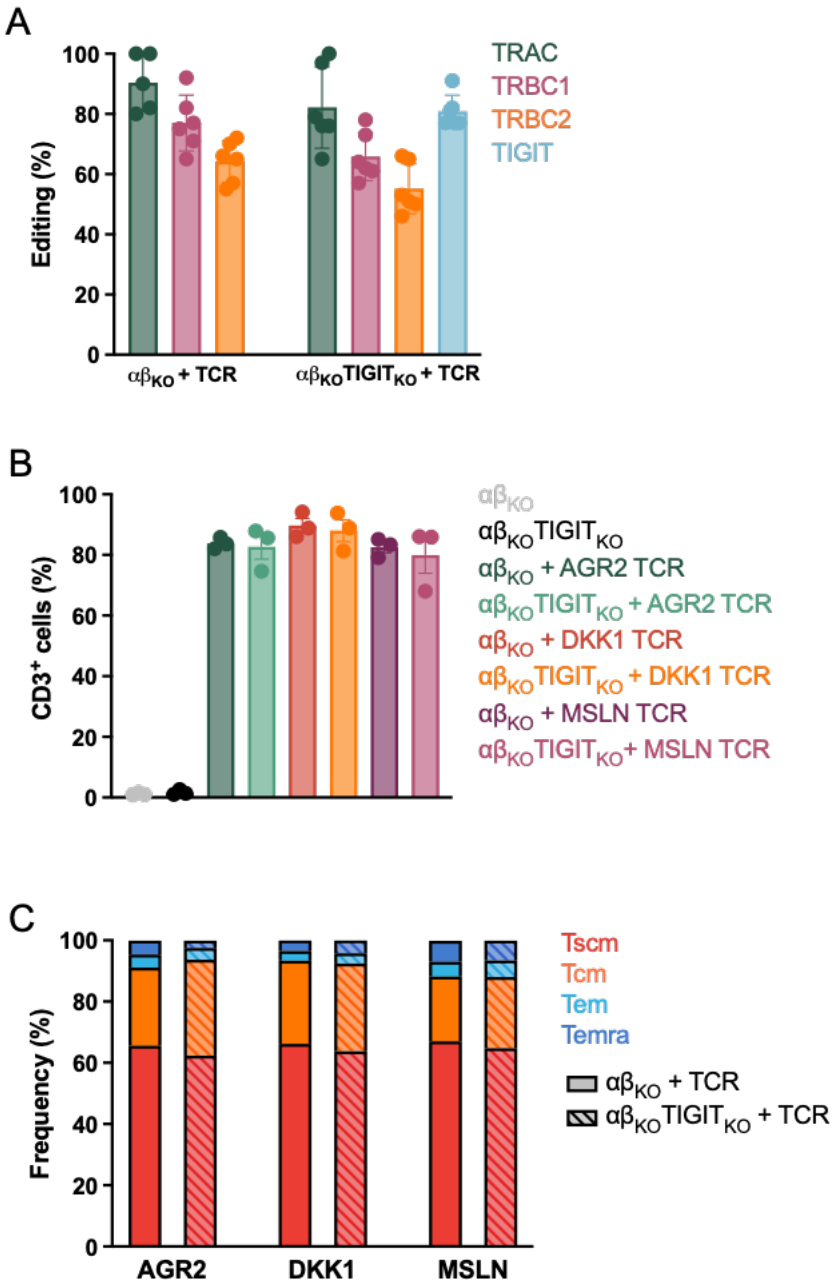

Figure 27. Generation of TCR-edited TIGIT disrupted T cells. (A) Frequency of C•G-to-T•A conversion at on-target sites mediated by BE4max. (B) Frequency of CD3 expression in Untreated (UT), TCR knockout, TCR knockout TIGIT disrupted T cells, as well as T cells with redirected specificity toward tumor cells (right). (C) Distribution of CD3+ T-cell memory phenotypes described according to CD45RA and CD62L expression markers as T_{CM} (CD45RA-, CD62L+), T_{SCM} (CD45RA+CD62L+), T_{EMRA} (CD45RA+CD62L-) and T_{EM} (CD45RA-CD62L-) of untreated vs. genetically engineered T cells. Data are represented as mean ±SEM. n=3 biological donors for each TCR specificity.

To assess if TIGIT disruption confers functional benefits to tumor-specific T cells, we challenged all T cell products with a mCRC patient-derived organoid expressing the HLA-A*02:01 and the three antigens, even though at different levels (**Figure 28A**). Surprisingly, while TIGIT disruption improved the ability of both DKK1- and MSLN-specific T cells to eliminate mCRC PDOs (**Figure 28B, C**), it did not affect the functionality of AGR2-specific T cells (**Figure 28D**).

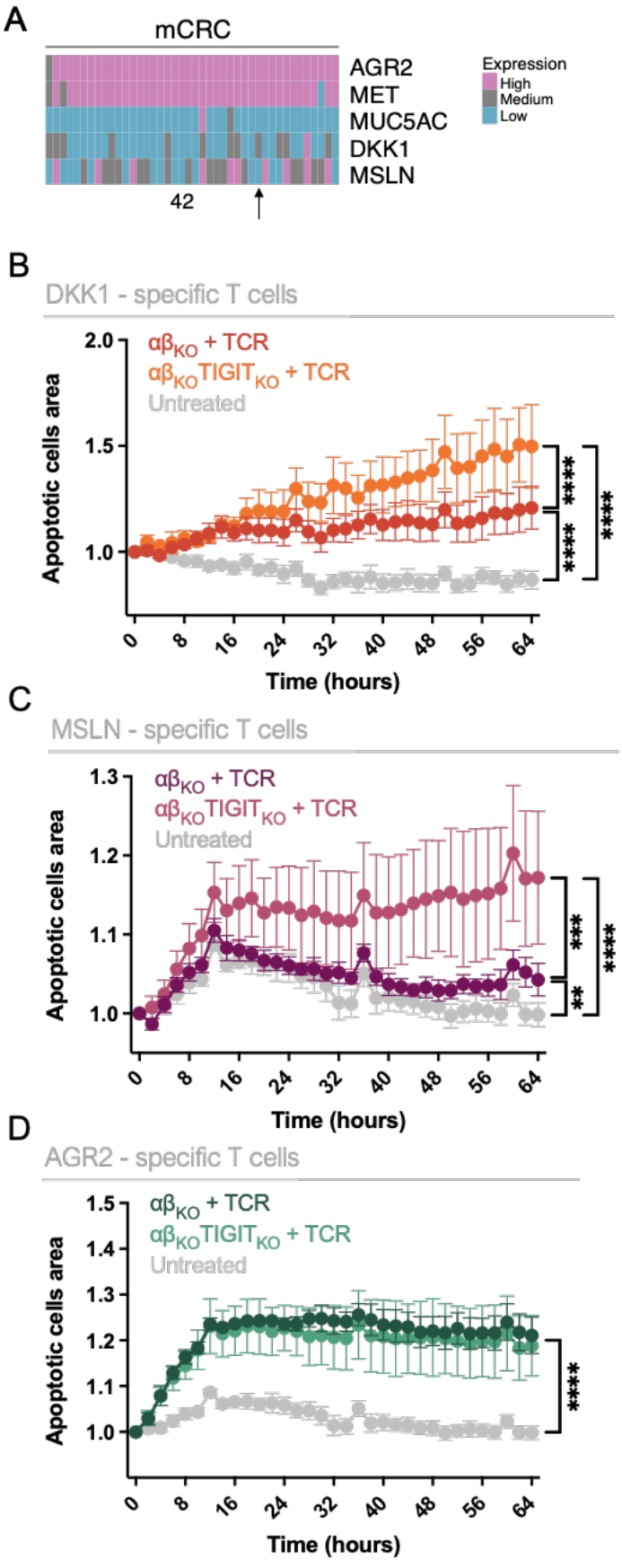
A
mCRC
AGR2
MET
MUC5AC
DKK1
MSLN
42
Expression
High
Medium
Low
B
DKK1 - specific T cells
αβKO + TCR
αβKOTIGITKO + TCR
Untreated
Apoptotic cells area
Time (hours)
C
MSLN - specific T cells
αβKO + TCR
αβKOTIGITKO + TCR
Untreated
Apoptotic cells area
Time (hours)
D
AGR2 - specific T cells
αβKO + TCR
αβKOTIGITKO + TCR
Untreated
Apoptotic cells area
Time (hours)

Figure 28. TIGIT disruption improves the ability of DKK1- and MSLN-specific, but not of AGR2-specific, T cells in eliminating tumor cells. (A) RNA seq data representing the expression levels of AGR2, MET, MUC5AC, DKK1 and MSLN in CRC metastatic lesion. Black arrow indicates the sample from which was derived the metastatic PDO exploited in following experiments. (B) DKK1-specific T cells were co-cultured with one metastatic CRC PDO in a 10:1 effector-to-target ratio. (C, D) MSLN- and AGR2-specific T cells were co-cultured with one metastatic CRC PDO in a 5:1 effector-to-target ratio. Tumor killing is represented as apoptotic cells area calculated by measuring the caspase 3/7 production in target cells and acquired by Incucyte Live-Cell Analysis System. Data are represented as mean ±SEM. n=3 biological donors for each TCR specificity. **p≤0.01, ***p≤0.001, ****p≤0.0001 by linear regression curve. CRC=colorectal, PDO=patient-derived organoids.

To further evaluate the effects of TIGIT disruption in different cellular products, we selected AGR2- and MSLN-specific T cells to be evaluated *in vivo*. To assess engineered T cells anti-tumor activity, we employed an orthotopic mouse model of CRC liver metastases already developed by Potenza, Balestrieri et al. (Potenza et al. 2023). Briefly, mCRC PDO, previously transduced with Firefly luciferase, were injected into the liver of immunocompromised NSG mice to mimic liver metastases onset. After three days, mice were treated with a single dose of AGR2- or MSLN- T cells, either or not disrupted in TIGIT. T cell expansion and tumor burden were analysed every week for 26 days (**Figure 29A**). Similarly to what we observed *in vitro,* both TCR_{ED} and $TCR_{ED}TIGIT_{KO}$ AGR2-specific T cells efficiently slowed tumor growth and TIGIT disruption did not ameliorate T cell tumor killing abilities (**Figure 29B**). On the other hand, only $TCR_{ED}TIGIT_{KO}$ MSLN-specific T cells were endowed with the capacity to control tumor growth (**Figure 29C**).

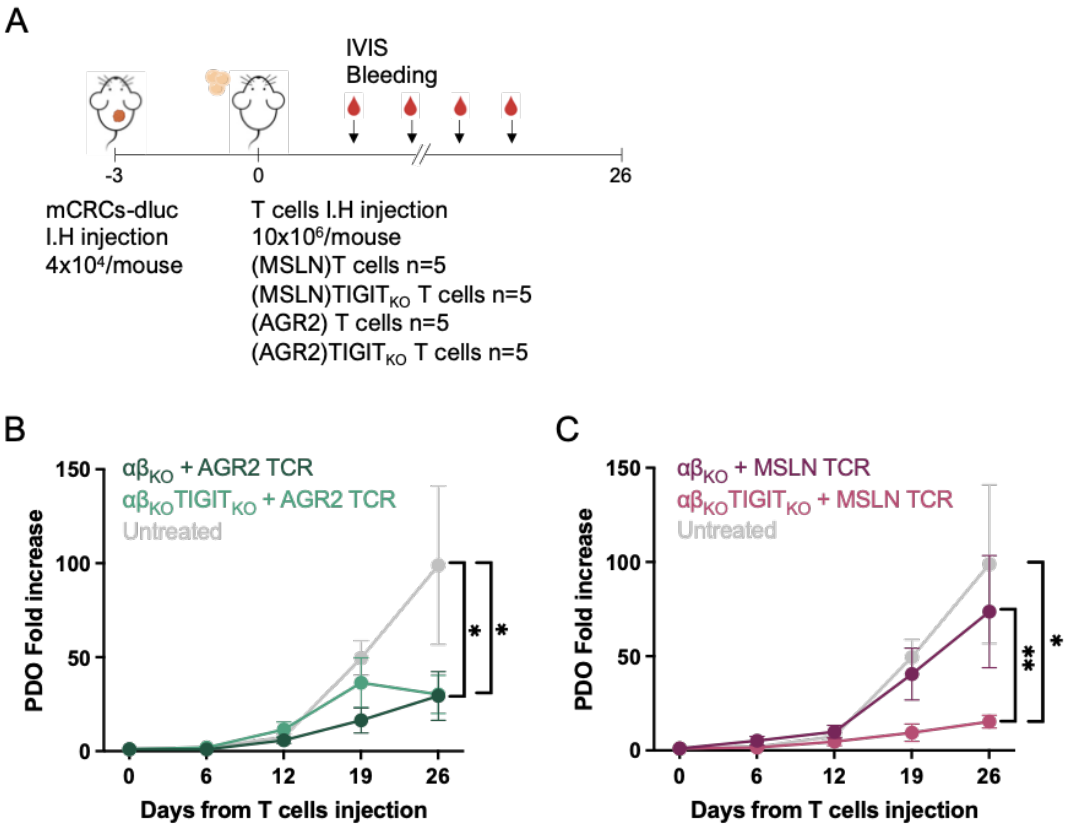

Figure 29. TIGIT disruption ameliorates tumor killing abilities of MSLN-specific T cells in vivo. (A) Experimental layout for the *in vivo* experiments. $4x10^5$ mCRC-dluc PDOs were surgically transplanted into the liver of NSG mice. Three days after, $10x10^6$ T cells were intrahepatically injected. (B, C) Tumor growth kinetic was measured by recording the bioluminescence. Fold increase was calculated RLU at day X/RLU at day 0. Data are represented as mean ±SEM. *p≤0.05, **p≤0.01 by linear regression curve. CRC=colorectal, PDO=patient-derived organoids. I.H=intrahepatic.

Interestingly, we observed a higher expansion of AGR2-specific TCR_{ED} and MSLN-specific $TCR_{ED}TIGIT_{KO}$, each compared to its TCR-specific counterpart (**Figure 30A, B**). However, the enhanced expansion was not dependent on alterations in memory phenotype differentiation or CD8/CD4 frequency overtime (**Figure 30C, D**).

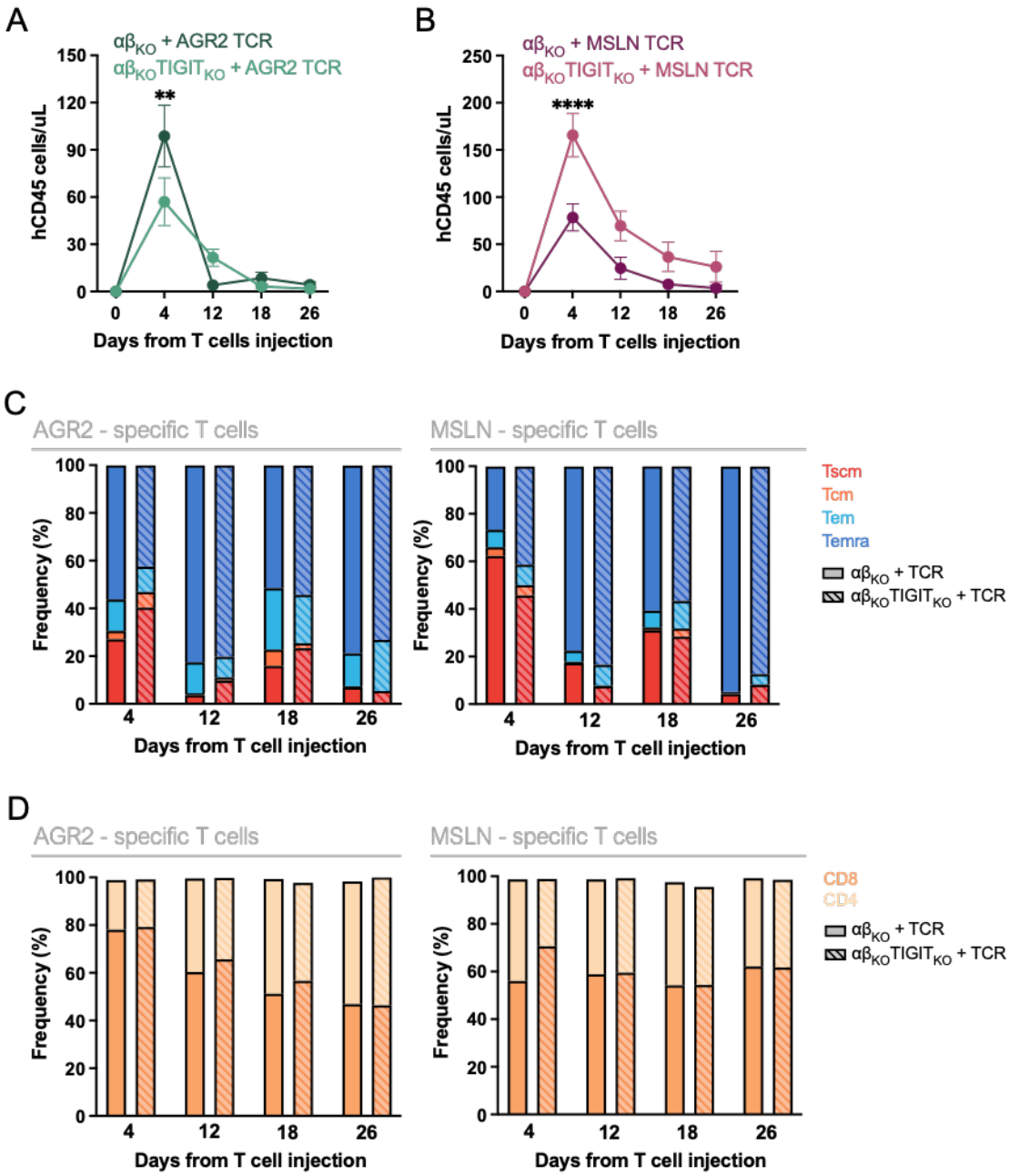

Figure 30. Characterization of TIGIT disruption effects on adoptively transferred AGR2- and MSLN-specific T cells. Immunophenotypic analyses of tumor-specific T cells isolated from mice peripheral blood overtime. Absolute counts (A), memory phenotype differentiation distribution according to CD45RA and CD62L expression markers as T_{CM} (CD45RA-, CD62L+), T_{SCM} (CD45RA+CD62L+), T_{EMRA} (CD45RA+CD62L-) and T_{EM} (CD45RA-CD62L-) (B) and CD8/CD4 population frequencies (C) were determined by flow cytometry. Data are shown as mean±SEM. n=5 mice for each experimental group. **p≤0.01, ****p≤0.0001.

Based on these evidences, we started investigating the possible molecular mechanism underlying the different behavior of TCR_{ED} and $TCR_{ED}TIGIT_{KO}$ cells in the two anti-tumor TCRs contexts. Of notice, the CRC liver metastases organoid we used

to perform both the *in vitro* and *in vivo* experiments show high levels of AGR2 but low levels of MSLN mRNAs expression, respectively (**Figure 28A, black arrow**). To understand if $TIGIT_{KO}$-related functional benefits were linked to an increased cytolytic activity upon recognition of low antigen levels, we performed a peptide titration assay. Specifically, TCR_{ED} and $TCR_{ED}TIGIT_{KO}$ T cells transduced with AGR2 or MSLN TCRs were cocultured with T2 cells loaded with diluting amount of the proper immunogenic epitope. We did not observe differences in the proportion of degranulating cells or in the production of pro-inflammatory cytokines between TCR_{ED} and $TCR_{ED}TIGIT_{KO}$ T cells, independently from the TCR analysed (**Figure 31A, 32A**).

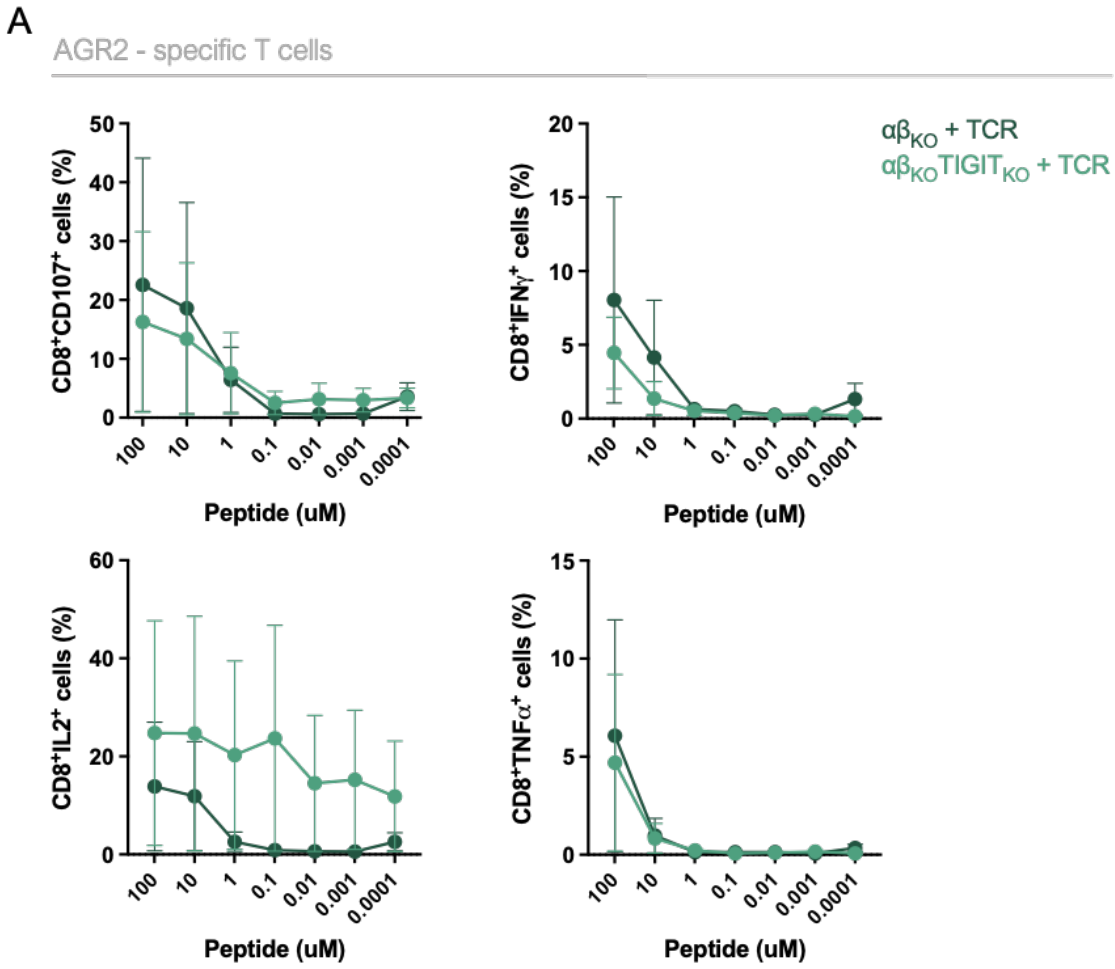

Figure 31. The expression of conventional inflammatory cytokines and degranulation marker in AGR2-specific T cells is not affected by TIGIT disruption. TCR-edited T cells wild type or disrupted in TIGIT were tested by 6 hours co-culture with T2 cell line pulsed with different peptides concentration. By flow cytometry the expression of CD107a and production of IFNγ, IL-2 and TNFα was measured. Data are shown as mean ± s.e.m; n=3 independent donors.

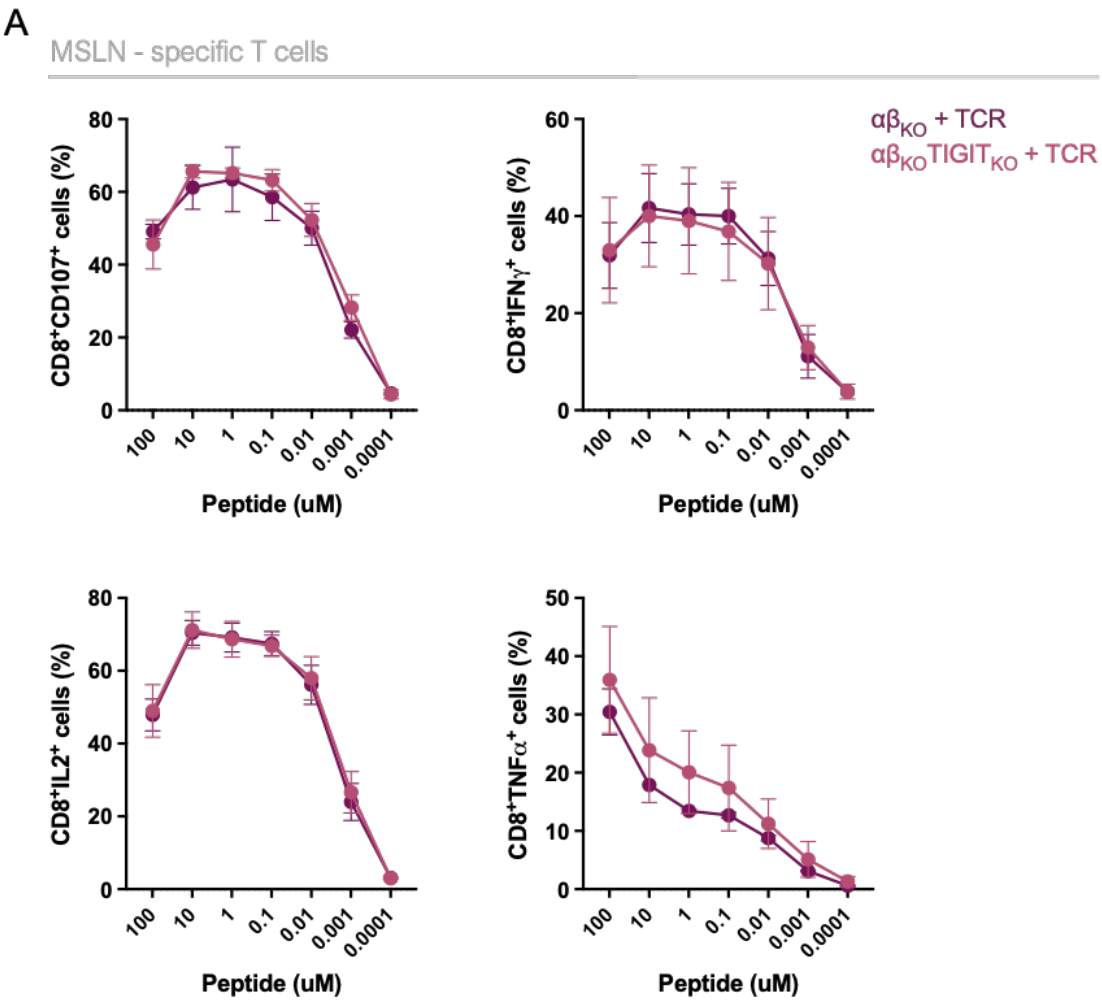

Figure 32. The expression of conventional inflammatory cytokines and degranulation marker in MSLN-specific T cells is not affected by TIGIT disruption. TCR-edited T cells wild type or disrupted in TIGIT were tested by 6 hours co-culture with T2 cell line pulsed with different peptides concentration. By flow cytometry the expression of CD107a and production of IFNγ, IL-2 and TNFα was measured. Data are shown as mean ± s.e.m; n=3 independent donors.

To confirm that the effect of TIGIT disruption on TCR-edited T cells is not related to the antigen amount, we challenged AGR2- and MSLN-specific T cells with a different mCRC PDOs. Once again, TIGIT disruption improved the ability of MSLN-specific T cells to eliminate mCRC PDOs (**Figure 33A**) and it did not affect the functionality of AGR2-specific T cells (**Figure 33B**).

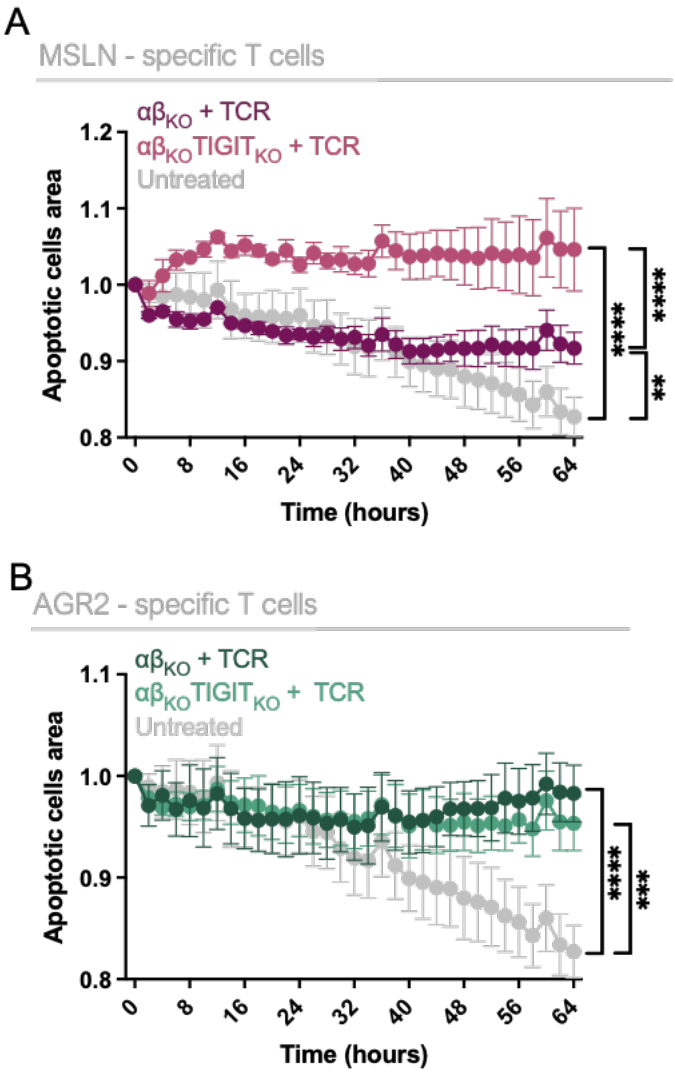

Figure 33. TIGIT disruption improves the ability of MSLN-specific, but not of AGR2-specific, T cells when challenged with a different PDO. MSLN- (**A**) and AGR2-specific (**B**) T cells were co-cultured with a different metastatic CRC PDO in a 5:1 effector-to-target ratio. Tumor killing is represented as apoptotic cells area calculated by measuring the caspase 3/7 production in target cells and acquired by Incucyte Live-Cell Analysis System. Data are represented as mean ±SEM. n=3 biological donors for each TCR specificity. **p≤0.01, ***p≤0.001, ****p≤0.0001 by linear regression curve. CRC=colorectal, PDO=patient-derived organoids.

DISCUSSION

Adoptive T cell therapy represents a promising therapeutic strategy for cancer patients and proved successful in treating hematological malignancies. Yet, the adoptive transfer of tumor-specific T cells for the treatment of solid tumors (Louis et al. 2011; Parkhurst et al. 2011; Ahmed et al. 2015; C. H. J. Lamers et al. 2016; Heczey et al. 2017b; Shi et al. 2020), accounting for more than 80% of new cancer diagnoses each year, has shown inconsistent results in clinical studies (Sung et al. 2021). This is mainly due to the lack of TCRs and CARs targeting relevant tumor antigens, as well as to the multitude of inhibitory mechanisms exerted by the cooperation of cancer and stromal cells, blunting T cell effector functions. In the era of genome editing, T cell engineering has been largely exploited in the attempt to ameliorate tumor recognition and overcome immunosuppression (Bonini et al. 2023). Depleting the endogenous TCR repertoire from T cells is essential to generate universal off-the-shelf CAR-T cells (McGuirk et al. 2021; Ottaviano et al. 2022; Chiesa et al. 2023) as well as for TCR-based products to achieve a full redirection of specificity (Provasi et al. 2012; Mastaglio et al. 2017; Stadtmauer et al. 2020; Ruggiero et al. 2022; Potenza et al. 2023). Indeed, TCR-editing prevents TCR chain mispairing, avoids the dilution of the anti-tumor effect due to the competition with the endogenous repertoire for the signaling machine, and circumvent the use of modified or murine TCR constant regions, which are potentially immunogenic (Manfredi et al. 2020).

Besides the redirection of TCR specificity, T cells have been genetically manipulated to improve their persistence and survival (Gurusamy et al. 2020; Kumar et al. 2021; Prinzing et al. 2021; Belk et al. 2022) and avoid T cell exhaustion (Y. Zhang et al. 2017; Stadtmauer et al. 2020; Jafarzadeh et al. 2021; Potenza et al. 2023; Agarwal et al. 2023), demonstrating superior anti-tumor functions in pre-clinical models of solid tumors. However, the first clinical trials employing T cells engineered by TALENs and CRISPR/Cas9 raised concerns about the safety of manipulated cellular products, since these tools produce a double strand brake leading to off-target events with potential pathological effects. Anti-CD19 CAR-T cells for the treatment of pediatric B-ALL and TCR-T cells used for refractory multiple myeloma and liposarcoma were multiplex engineered by TALEN and CRISPR/Cas9, respectively (Qasim et al. 2017; Stadtmauer et al. 2020). Notably, translocations were observed in both products at different frequencies, and were still detectable up to 170 days after T cell infusion.

To overcome this limitation, base editors have been developed as tools to produce single base substitutions in the genome, avoiding the double strand brake. By employing a Cytosine Base Editor (CBE), we simultaneously knocked out TRAC, TRBC1, TRBC2 and TIGIT to concurrently disrupt the endogenous TCR repertoire and harness the immune suppressive tumor microenvironment, and we assessed the safety profile of our manipulation procedure. We observed that the CBE precisely edited all target genes, with a comparable efficiency to CRISPR/Cas9. Nevertheless, we detected translocations only in CRISPR/Cas9 treated cells (up to 0.44% frequency), as also reported by other groups (Webber et al. 2019; Stadtmauer et al. 2020; Turchiano et al. 2021). To have a complete blueprint of the off-target events installed by CBE in our products, we checked for sgRNA-dependent and -independent off-target editing. First, we performed targeted NGS of the top predicted off-target sites within coding regions for each sgRNA. As expected, we observed large variability between different T cell donors and detected only one off-target mutation with a frequency around 20%. To uncover the global mutational effects of the CBE, we used ultra-deep whole exome sequencing. One out of four T cell donors displayed a missense mutation with unknown significance in a senescence-related gene. Importantly, CBE-mediated editing did not affect T cell fitness, expansion, or functionality. Altogether, these results show that in primary T cells base editing represents a safer option compared to CRISPR/Cas9 also in the multiplex context, allowing for high on-target efficiency and minimal introduction of off-target events. Accordingly, sgRNA-dependent off-target deamination has been rarely detected in T cells (Preece et al. 2020; Georgiadis et al. 2021). Conversely to what we observed, CBE activity was associated with spurious base conversion at a genome-wide level. However, it is important to notice that casual genome deamination by CBE was measured using cell-free models (Y. Yu et al. 2020; Doman et al. 2020) or HEK293T (Gaudelli et al. 2020). In a recent work, the authors characterized the activity of base editors (and other tools) in clinically relevant cells (i.e. hSPCs) and outlined how intrinsic features of target sites, optimization of BE constructs, and culture conditions participate in determining the genotoxic impact of base editing (Fiumara et al. 2023). Thus, it is conceivable that what has been reported in the literature could not reflect the safety profile of our therapeutic T cells, which present minimal genome-wide deamination. In addition, the differences we observed could be explained by the amount of CBE mRNA we introduced in our system, which is 11 times and 5 times lower compared to Gaudelli et al. and Fiumara, Ferrari et al, respectively. Despite concerns regarding the safety profile of genetic manipulations, engineered T cells developed by programmable DNA nucleases have been implemented in 47 clinical

trials (Bonini et al. 2023). Our results suggest that alternative platforms, such as base editors, should be preferred when multiplex editing is necessary, to limit the possibility of translocations and complex chromosomal aberrations observed in T cells upon CRISPR/Cas9 treatment (Kosicki, Tomberg, e Bradley 2018; Webber et al. 2019a; Stadtmauer et al. 2020; Leibowitz et al. 2021; Turchiano et al. 2021; Tsuchida et al. 2023). By further optimizing the delivery of the CBE we may be able to diminish the incidence of off-target events, even though inherent features of each T cell donor can influence the efficiency and the precision of gene editing. Still, the development of a consensus strategy to assess the pathogenic risk associated with these technologies represents a major obstacle for clinical translation of engineered cellular products.

The selection of relevant tumor antigens is a key issue in the development of an ACT strategy targeting solid tumors. By setting up a funnel approach, we started from the investigation of PDAC gene expression data and ended up identifying 19 promising tumor-associated antigens and 1 neoantigen to be targeted by T cells. We collected all overexpressed genes and then filtered them by different criteria to exclude (i) antigens expressed by stromal cells and activated lymphocytes, (ii) antigens with marginal roles in proliferation, survival, and invasiveness of cancer cells and (iii) non-immunogenic proteins. In the past few years, it was shown that genes upregulated by activated lymphocytes, which we excluded from our pool of antigens, could also represent potential targets for ACT (Mamonkin et al. 2015; Li et al. 2021; Freiwan et al. 2022; Lu et al. 2022; Chiesa et al. 2023; N. Watanabe et al. 2023). Accordingly, several antigens characterized by a high expression in malignant and normal hematopoietic cells, have been successfully explored as CAR-T cells targets. Different strategies have been developed to prevent undesired recognition of the healthy T cell compartment and fratricides events, including the use of genome editing to mask healthy hSPCs from CAR-T cell recognition in the context of allo-bone marrow transplantation (Casirati et al. 2023; Wellhausen et al. 2023) or to disrupt the target antigen in the manufactured CAR-T cell product (Gomes-Silva et al. 2017; Li et al. 2021; Cooper et al. 2018; Georgiadis et al. 2021; Chiesa et al. 2023). In an orthotopic mouse model of AML, CAR-T cells targeting FLT3, KIT and IL-3RA effectively eradicated leukemic cells while sparing transplanted hSPCs that were previously engineered for epitope editing (Casirati et al. 2023). The same strategy proved efficient for CD45-specific CAR-T cells, which controlled tumor growth in xenografts models of AML, B cell lymphoma and T-ALL reconstituted with epitope-edited hSPCs (Wellhausen et al. 2023). Another example is represented by CD7,

which have been depleted in CD7-specific CAR-T cells by CRISPR/Cas9 (Gomes-Silva et al. 2017; Li et al. 2021; Cooper et al. 2018) or base editors(Georgiadis et al. 2021; Chiesa et al. 2023). The disruption of CD7 in the therapeutic product allowed tumor cell killing and avoided fratricide events, that can also occur during the manufacturing procedure. This further widens the range of possible antigens to be investigated as potential targets for the treatment of gastrointestinal tumors.

In the context of solid tumors, the high intra-tumoral heterogeneity and the rapid cancer cell evolution is associated with antigen variability, that can affect the efficacy of T cell-based therapies. The potential to target virtually every antigen, combined with the high avidity, counterbalances the risk of HLA loss, possibly favoring the use of TCRs compared to CARs in this setting. Based on these premises, we selected peptides restricted for the most frequent HLA class I alleles and by employing a pipeline set up in our lab (Ruggiero et al. 2022; Potenza et al. 2023) we isolated 5 tumor-specific HLA-A*0201 restricted TCRs. Four TCRs, recognizing AGR2, DKK1, MET and MUC5AC were retrieved from the same healthy donor, while a MSLN-specific TCR was isolated from a PDAC patient. We used the peripheral blood of healthy donors as starting material, even if they harbor a low frequency of self-antigens specific TCRs, because tumor-specific T cells in patients display reduced cellular fitness due to exhaustion and are usually difficult to expand (Noviello et al. 2019; Oliveira et al. 2021; Potenza et al. 2023). On the other hand, the isolation of a MSLN-specific TCR was successful only when a PDAC patient was employed as the source of T cells, probably due to the low expression of MSLN in normal tissues. MET (Shah et al. 2023) and MSLN (Stromnes et al. 2015; Anderson et al. 2019; Adusumilli et al. 2019; 2021) are common tumor-associated antigens, which have been already targeted by ACT. In a pilot phase 1/2 clinical trial, 7 patients affected by metastatic melanoma or metastatic triple-negative breast cancer have been treated with (MET)CAR-T cells and 4/7 showed stable disease (Shah et al. 2023). MSLN-specific CAR-T cell products have been investigated in clinical settings for multiple solid tumors, including PDAC and CRC, and displayed limited efficacy probably due to transient CAR expression (Beatty et al. 2018; Adusumilli et al. 2021) and the use of murine portions in the construct, potentially immunogenic in humans (Maus et al. 2013; Haas et al. 2019). Clinical trials employing fully human MSLN CAR constructs are now ongoing. Recently, T cells engineered to express a MSLN-specific T cell receptor fusion construct (TRuC) were evaluated in a phase 1/2 clinical trial for the treatment of refractory solid tumors. Compared to a traditional CAR, a TRuC consists of a single chain that determines the specificity fused with the CD3ε subunit of the T

cell receptor to activate the signaling pathways downstream of the TCR complex. Importantly, the overall survival rate was 70% among 30 patients treated and grade 3-4 CRS was observed in 25% (Hassan et al. 2023). Based on these results, MET and MSLN represent promising antigens expressed by a variety of solid tumors.

DKK1 also represents a well-recognized antigen, toward which a dendritic cell-based vaccine (Qian et al. 2012) and a neutralizing monoclonal antibody (Klempner et al. 2022) have been developed and employed for the treatment of multiple myeloma, and gastroesophageal and prostate cancer, respectively. On the other hand, AGR2 and MUC5AC have been extensively used as biomarkers in gastrointestinal and breast cancer. By analysing the transcriptome of primary and metastatic PDAC and CRC patients enrolled at San Raffaele, we observed a high expression of all antigens by PDAC patients, while a greater variability was found in the CRC cohort. Overall, these data corroborate the strength of our workflow for the identification of relevant new antigens to target by ACT.

We built bidirectional lentiviral vectors to deliver the newly isolated anti-tumor TCRs in TCR base edited T cells, thus generating T cell products with a fully redirected specificity. We cultured TCR-edited lymphocytes in the presence of IL-7 and IL-15, to preserve a T cell stem memory phenotype, which have been associated with a better expansion and long-term persistence in patients (Cieri et al. 2013; Biasco et al. 2015; Oliveira et al. 2015). When challenged with cancer cell lines, all TCR-edited T cell products proved effective in eliminating PDAC antigen positive HLA matched cell lines while sparing negative controls. Moreover, these T cells displayed killing abilities also when tested against patient-derived organoids, generated from CRC primary lesions and liver metastases or PDAC primary tumors. Noticeably, upon recognition of the target peptide pulsed on T2 cells, only 2 out of 5 T cell products produced classical pro-inflammatory cytokines (IFNγ, IL-2 and TNFα) and upregulated the degranulation marker CD107a, uncoupling tumor killing abilities from cytokine production. Indeed, T lymphocytes can exploit two different cytotoxic mechanisms, consisting in the exocytosis of granules containing perforin and granzyme B or based on the FasL-Fas interaction (Golstein e Griffiths 2018). The activation of these two cytolytic pathways, and the resulting killing of the target cell, was negatively associated with cytokine synthesis. Indeed, the strength of the cytokine secretory response relies on the level of TCR engagement, which is interrupted with the apoptosis of the target cell (Fortier, Nacy, e Sitkovsky 1989; Mosmann, Li, e Sad 1997). To evaluate whether cytokine release is associated with the strength of TCR-pMHC binding or to specific signaling programs activated by the different TCRs, we

are currently determining the affinity and the avidity of our anti-tumor TCRs (by multimers and Lumicks technologies) and investigating T cell transcriptome upon stimulation with target cells.

The use of checkpoint inhibitors has revolutionized the immune-oncology field. Indeed, the identification of inhibitory pathways, able to promote T cell exhaustion, and the validation of therapeutic approaches aimed at interfering with these pathways to restore a cancer immunotherapy effect, have been highly successful in selected diseases. However, ICB administration bears some limitations, among which a limited penetration into the tumor bed and the induction of adverse events triggered by the loss of peripheral tolerance (D. B. Johnson et al. 2016; Heczey et al. 2017b; Adusumilli et al. 2019; Durgin et al. 2021). In addition, ICB therapeutic efficacy is associated with the presence of an endogenous repertoire of tumor-specific T cells infiltrating the tumor mass, which is nearly absent in PDAC (Karamitopoulou 2019) and not abundant in CRC tumors (Overman et al. 2017; 2018). To prevent the exhaustion of adoptively transferred T cells and boost the therapeutic efficacy of tumor-specific T cells, we envisaged to combine TCR editing with the genetic disruption of relevant checkpoint inhibitors. Among them, TIGIT is a leading IR in gastrointestinal tumors (Steele et al. 2020; Liang et al. 2021; Cui Zhou et al. 2022) and exert its inhibitory function by binding to CD155 and CD112, which have been found upregulated on tumor cells and antigen-presenting cells in PDAC microenvironment (Cui Zhou et al. 2022). TIGIT is highly expressed on T cells infiltrating primary and metastatic colorectal cancer (Liang et al. 2021) and PDAC (Heiduk et al. 2023), and its presence predicts poor T cell functionality in terms of cytokines production (Liang et al. 2021) and other exhaustion features (Steele et al. 2020). In mouse models of metastatic melanoma, NSCLC and cervical cancer, it was observed that TIGIT blockade prevents T cell exhaustion and improves CD8 T cell tumor infiltration (Qing Zhang et al. 2018; L. Liu et al. 2022).

The observations made in preclinical results led to the development of anti-TIGIT IgG1 and monoclonal antibodies that are currently under clinical testing (Rousseau, Parisi, e Barlesi 2023).

Based on these premises, we generated TCR-edited, TIGIT-disrupted T cells. First, we assessed that TIGIT disruption does not impair T cell expansion or memory phenotype differentiation. To evaluate if TIGIT knockout confers a functional improvement to T cells, we cocultured AGR2-, DKK1- and MSLN-specific T cells with a metastatic CRC PDO. We observed that TIGIT disruption improved tumor killing abilities of both DKK1- and MSLN-specific T cells while having no impact on the

efficacy of AGR2-specific cells. This result was fully recapitulated *in vivo*: MSLN- and AGR2-T cells were challenged with PDOs in an orthotopic model of CRC liver metastases. We detected a different behavior between MSLN- and AGR2-T cells in terms of *in vivo* expansion: TIGIT knockout conferred an expansion advantage and boosted the anti-tumor activity of MSLN-specific T cells only. RNAseq analysis on the liver metastases tested in these experiments indicate high AGR2 and low MSLN expression. Based on these findings we reasoned that high antigen concentration on target cells might overcome the detrimental effects of TIGIT expression on engineered T cells. However, we could not observe any difference between $TIGIT_{COMP}$ and $TIGIT_{KO}$ cells, in terms of frequency of degranulating cells, in peptide titration assays.

While the action of TIGIT on the anti-tumor activity of NK cells has been extensively studied, its role on the function of $CD8^+$ T cells is still unclear. TIGIT can act by different mechanisms, which can be categorized as cell-extrinsic and cell-intrinsic (Manieri, Chiang, e Grogan 2017). By competing with the co-stimulatory molecule CD226 (also called DNAM-1) for the binding of CD155 and CD112 on APCs, TIGIT engagement stimulates the activation of tolerogenic pathways, resulting in the secretion of IL-10 and consequent suppression of proper T cell priming, proliferation and production of IFNγ. Upon antigen rechallenge, TIGIT blockade can rescue T cell function (X. Yu et al. 2009). In addition, TIGIT interaction with its ligands shut down multiple signaling pathways implicated in cytotoxic functions, among which PI3K and MAPK cascade (S. Liu et al. 2013; Johnston et al. 2014). In an autoimmune context, it was observed that TIGIT directly inhibit T cell function and activation by downregulating the surface expression of the TCR and CD3ε, thus acting upstream the TCR signaling (Joller et al. 2011). On the other hand, independently from the presence of its ligands, TIGIT can suppress cytotoxic functions by interfering with CD226 dimerization (Shibuya et al. 1996; Johnston et al. 2014; Banta et al. 2022). With this study we observed that TIGIT signaling may differentially affect T cell function according to the TCR affinity and/or antigen concentration on target cells.

Overall, our results provide insights into the generation of safer advanced therapeutic medicinal products to treat gastrointestinal tumors. Base editing demonstrated to be a valuable alternative to CRISPR/Cas9 to circumvent unwanted genome rearrangements in multiplex genome editing protocols for T cells. Once prioritized the most promising TCRs also based on their safety profile, and once established the role of TIGIT in the functionality of TCR-edited T cells, we will select

the optimal candidate for a first-in-man clinical trial of adoptive T cell therapy for high risk gastrointestinal tumors.

MATHERIALS AND METHODS

Cell lines and cell culture

BxPC3, PANC1 and AsPC1 human PDAC cell lines, SW480 colorectal cancer cell line and IGROV1 ovarian cancer cell line were cultured in RPMI 1640 (Lonza/Euroclone) supplemented with 10% fetal bovine serum (FBS, Carlo Erba), 1% penicillin-streptomycin (Lonza) and 2 mM glutamine (Lonza). BxPC3 stably expressing HLA-A*02 MHC molecule were cultured in RPMI 1640 (Lonza/Euroclone) supplemented with 10% fetal bovine serum (FBS, Carlo Erba), 1% penicillin- streptomycin (Lonza) and 2 mM glutamine (Lonza). T2 cell line was cultured in IMDM (Lonza/Euroclone) supplemented with 10% fetal bovine serum (FBS, Carlo Erba), 1% penicillin-streptomycin (Lonza) and 2 mM glutamine (Lonza). HEK293T cells were maintained in IMDM (Lonza/Euroclone) supplemented with 10% fetal bovine serum (FBS, Carlo Erba), 1% penicillin- streptomycin (Lonza) and 2 mM glutamine (Lonza).

Organoids generation and maintenance

Patient-derived organoids originated from CRC liver metastases were provided by Oronzina Botrugno (Functional Genomics of Cancer Unit, San Raffaele Scientific Institute), as described previously (Vlachogiannis et al. 2018). Briefly, freshly isolated samples were minced and incubated two times with 5 mL PBS/EDTA 5mM at room temperature for 15'. Tumor tissue was enzymatically digested with a solution of TrypLE (Gibco) 2X in PBS/EDTA 1mM for 30-60 minutes. Cancer cells released in the supernatant were collected and washed with Basal Medium composed of Advanced DMEM/F12 (Thermo Fisher) supplemented with 1% penicillin- streptomycin (Lonza), 2 mM glutamine (Lonza), 10 mM HEPES (Thermo Fisher) and 50ug/mL Primocin (Invivogen). Cell pellets were resuspended in Matrigel (Corning) and seeded in 24-well plates. CRC specific culture medium was added after Matrigel polymerization consisting in Basal Medium supplemented with 1X B27 (Thermo Fisher), 1X N2 (Thermo Fisher), 50 ng/mL recombinant EGF (PeproTech), 100 ng/mL recombinant Noggin (PeproTech), 500 ng/mL R-spondin 1 (PeproTech), 10 nM Gastrin (Sigma-Aldrich), 10 ng/mL FGF-10 (PeproTech), 10 ng/mL FGF-b (PeproTech), 100 ng/mL Wnt-3A (R&D Systems), 1 uM Protaglandin E2 (Tocris Bioscience), 10 uM Y27-632 (Sigma-Aldrich), 4 mM Nicotinamide (Sigma-Aldrich), 2.5 mM N-acetyl cysteine (Sigma-Aldrich), 0.5 uM A83-01 (Tocris Bioscience), 5 uM SB202190 (Sigma-Aldrich).

PDAC patient-derived organoids were kindly provided by Maximilian Reichert (Department of Medicine II, Klinikum rechts der Isar, Technische Universität

München) and generated as described previously (Driehuis et al. 2020). Briefly, PDAC tissues were minced and digested with 5 mg/mL collagenase II solution in Advanced DMEM/F12 (Thermo Fisher) for 2 hours at 37°C. Cells were washed, resuspended with Matrigel (Corning) and plated in 24-wells. PDAC specific culture medium was added after Matrigel polymerization consisting in Basal Medium supplemented with 1X B27 (Thermo Fisher), 1X N2 (Thermo Fisher), 50 ng/mL recombinant EGF (PeproTech), 100 ng/mL recombinant Noggin (PeproTech), 250 ng/mL R-spondin 3 (PeproTech), 10 nM Gastrin (Sigma-Aldrich), 100 ng/mL FGF-10 (PeproTech), 100 ng/mL Wnt-3A (R&D Systems), 10 uM Y27-632 (Sigma-Aldrich), 4 mM Nicotinamide (Sigma-Aldrich), 2.5 mM N-acetyl cysteine (Sigma-Aldrich), 0.5 uM A83-01 (Tocris Bioscience). To split organoids, growth medium was removed and Matrigel domes were incubated with 500 uL of 1U/mL Dispase II (Sigma-Aldrich) in Basal Medium at 37°C for 1 hour. Following Matrigel digestion, organoids were collected and washed with PBS at 1200 rpm for 5 minutes. To expand them cell pellets were resuspended in TrypLE 1X and incubated up to 15' at 37°C in order to obtain a single cell suspension. After that, organoids were washed 2 times with PBS and centrifuged 1500 rpm for 5 minutes. Following the removal of supernatant, cell pellets are mixed with new Matrigel and plated as described above.

To generate PDOs-luc organoids, PDOs were transduced with a lentiviral vector containing luciferase under the control of a constitutive promoter and a neomycin resistance cassette (Addgene # 21471). Briefly, PDOs were digested with TrypLE 1X to obtain a single cell suspension and resuspended at a concentration of $0.5x10^6$ cells/mL in Advanced DMEM/F12 containing the lentiviral vector and 8 µg/ml of Polybrene (Sigma-Aldrich). After spin infection, cells were allowed to recover for 2 hours at 37°C and then plated as usual. PDOs were maintained with antibiotic selection for at least 3 weeks and then checked for luciferase expression.

PDOs with stable expression of HLA-A*02 were generated by employing the same strategy.

T cells isolation from PBMCs

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from the peripheral blood of healthy donors or PDAC patients by density gradient centrifugation using Ficoll-Hypaque (Lymphoprep™, Fresenius). The PBMCs isolated were washed 3 times to remove platelets and counted by trypan-blue exclusion. Cells were either used for tumor-specific T cell isolation or T cell manufacturing.

Peptides

Peptides were synthesized to specifications of validated sequence, sterility, greater than 70% purity and absence of endotoxin. Peptides used for stimulation and isolation of TAA-specific T cells were selected from published works (Table 1). Peptides were mixed in equal amounts in all pools at a concentration of 1 μg/ml per peptide. 25 subpools, each one containing up to 6 peptides (10 μg/ml per peptide), were generated according to specific mapping matrix, with each peptide being included in only two intersecting subpools.

Generation of immortalized B cells

Immortalized B cells were generated as previously described by Ruggiero et al. (Ruggiero et al. 2022). Autologous B cells were sorted from HD7 and the PDAC patient PBMCs by using the CD19 microbeads (Miltenyi Biotec). By spin infection, B cells were transduced with a lentiviral vector encoding for the BCL6/BCL2L1 transgenes and cultured on a γ-irradiated (100 Gy) mouse fibroblast cell line stably expressing CD40L (3T3-CD40L) in a 10:1 ratio. B cells were maintained in IMDM (Euroclone/Lonza) supplemented with 10% fetal bovine serum (FBS; BioWhittaker), 1% penicillin-streptomycin (Euroclone/Lonza), 2 mM glutamine and 50 ng/mL of IL-21 (Miltenyi Biotec) and added with fresh 3T3-CD40L two times a week.

Isolation and expansion of tumor-specific T cells

TAA-specific T cells were isolated as previously described (Ruggiero et al. 2022). Briefly, PBMCs derived from HD7 or the PDAC patient were isolated, plated at $5x10^6$ cells/mL in X- VIVO supplemented with 5% FBS, 1% penicillin-streptomycin, 2mM glutamine, 5 ng/mL IL-7, 5 ng/mL IL-15 and 10 ng/mL of IL-21. These cells were stimulated with one pool of peptides in the presence of 1μg/mL of anti-CD28 antibody (BD Biosciences) and sorted for CD137 after 26-30 hours (CD137 MicroBead Kit, human; Miltenyi Biotec). From the negative fraction, CD3 negative cells were isolated by depletion of $CD3^+$ and exploited as source of APCs. CD3 negative cells were irradiated at 30Gy, pulsed with the same pool of peptides and co-cultured with $CD137^+$ cells at 20:1-100:1 ratio in in X- VIVO supplemented with 5% FBS, 1% penicillin-streptomycin, 2mM glutamine, 5 ng/mL IL-7, 5 ng/mL IL-15 and 10 ng/mL of IL-21. Cells were stimulated every 2 weeks with APCs (CD3 negative cells or immortalized B cells) loaded with the pool of peptides. From stimulation round 2, T cell cultures were tested for the recognition of the entire pool and individual peptides, by measuring CD107 expression, IFNγ, IL-2 and TNFα production.

TCR repertoire sequencing

RNA samples of TAAs-specific T cells were collected at each stimulation round to track the TCR repertoire overtime. RNA was extracted employing the Arcturus Pico Pure RNA extraction kit (Life Technology) and CDR3 regions amplified by a modified rapid amplification of cDNA ends (RACE) approach (Dmitry A. Bolotin et al. 2012; Ruggiero et al. 2015). Samples were sequenced by using an Illumina MiSeq Sequencer and CDR3 clonotypes identified with the MiXCR software (Dmitriy A. Bolotin et al. 2015). Based on the CDR3 selection, *TRAC* and *TRBC* alignments were analyzed by IMGT software to define the proper TCR α and β chains sequences. The sequences were codon optimized and synthetized by Twist Bioscience.

Lentiviral vectors

Bidirectional lentiviral vectors encoding all TAAs specific TCR (AGR2-, DKK1-, MET-, MUC5AC-, MSLN-) chains were employed to redirect T cell specificity. Under the minimal CMV promoter with antisense orientation were cloned TCR α chains, while under the PGK (human PhosphoGlycerate Kinase) with sense orientation were cloned TCR β chains (Amendola et al. 2005). Bidirectional lentiviral vector encoding human HLA-A*02 were used to stably express HLA-A*02 allele in HLA-A*02 negative PDOs and cell lines. Under the minimal CMV promoter with antisense orientation was cloned a puromycin resistance cassette, and under the PGK with sense orientation was cloned the HLA-A*02 allele. Sanger sequencing of cloned products was performed to validate the cloning procedure. Lentiviral vectors were packaged by an integrase competent third-generation construct and pseudotyped by the Vescicular Stomatitis Virsus (VSV) envelope (Lévy et al. 2012).

Editing of T cells

PBMCs were isolated form healthy donors and enriched for $CD3^+$ cells using anti-CD3/CD28 magnetic beads (Dynabeads; Invitrogen) and seeded at a concentration of $2x10^6$ cells/mL in RPMI 1640 (Lonza/Euroclone) supplemented with 10% fetal bovine serum (FBS, Carlo Erba), 1% penicillin- streptomycin (Lonza), 2 mM glutamine (Lonza), 5 ng/mL IL-7 (Peprotech), 5 ng/mL IL-15 (Peprotech). Two days after stimulation T cells were washed 3 times with PBS and cell number determined by trypan-blue exclusion. For singleplex experiments, $1x10^6$ T cells were electroporated with 1μg of sgRNA (Synthego) and 1.5μg of BE4max or ABE8.20m mRNA. BE4max and ABE8.20m mRNA were kindly provided by Martina Fiumara (Gene transfer technologies and new gene therapy strategies Unit, San Raffaele

Scientific Institute). For multiplex experiments, $1x10^6$ T cells were electroporated with 0.33μg of each sgRNA (Synthego) together with 1.5μg of BE4max. For CRISPR/Cas9 comparison experiments, $1x10^6$ T cells were electroporated with ribonucleoprotein complexes formed by SpCas9 (Synthego) with each sgRNA (Synthego) in a 1:9 ratio. The 4D-nucleofector (Lonza, Basel, Switzerland) and P3 kit was used for with $1x10^6$ T cells per 30 μL strip, and up to $5x10^6$ T cells per 110μL, and the Nucleofector program EO-115. T cells were allowed to recover for 10 min at 37°C following electroporation and then seeded in X- VIVO supplemented with 5% FBS, 1% penicillin-streptomycin, 2mM glutamine, 5 ng/mL IL-7, 5 ng/mL IL-15 at a concentration of $2x10^6$ cells/mL. The day after, edited T cells were transduced with a lentiviral vector containing the tumor-specific TCR. Six days after T cell isolation, Dynabeads were magnetically removed, cells washed and seeded at $0.5x10^6$ cells/mL.

Assessment of gene disruption efficiency

Genomic DNA was isolated (DNeasy blood and tissue Kit, Qiagen) 12 days after the editing procedure. By PCR the Cas9-targeted loci were amplified and submitted for Sanger sequencing. PCR amplicons of base edited cells were analyzed using the web app EditR (Kluesner et al. 2018). In CRISPR/Cas9 comparison experiments, PCR amplicons were analyzed using Inference of CRISPR Editing (ICE) algorithm (Synthego).

Droplet Digital PCR for translocation quantification

Translocation assays were designed with two primers, amplifying the translocation product, plus one probe designed to bind near the cut site. 3 independent donors of untreated, base edited and CRISPR/Cas9 edited cells were run as a duplexed assay formed by an experimental primer+probe combination (FAM) specific for a translocation outcome (BioRad) and the internal reference prime+probe combination (VIC) amplifying the human GAPDH (Thermofisher Scientific). Reactions were made with 50 ng of genomic DNA per each assay and the ddPCR Supermix for Probes (no dUTP) (BioRad) following the manufacturer's instructions. Droplets were generated and analyzed using QX200 Droplet-digital PCR system (BioRad). Frequency was calculated as fractional abundance adjusted for two-copies of reference sequence per genome using the QuantaSoft ver 14.0 software (BioRad).

sgRNA-dependent off-target analysis

Off-target sites for each sgRNA were predicted using the web app CRISPOR and filtered to be located into exons. Top off-target sites were selected (8 total) and genomic DNA extracted 14 days after the editing procedure (DNeasy blood and tissue Kit, Qiagen). Primers (NGS grade, Eurofins) were designed to amplify a 300-500 bp region centered on the off-target sequence. Genomic DNA was amplified using Platinum™SuperFi™ DNA polymerase (Thermofisher Scientific), high fidelity according to the manufacturer's protocol, using the cycle [98°C-30'']-[98°C-5']-24x[gradient 65°-55°C-30'', 72°C-30'']-[72°C-5']-[4°C-hold]. For OT8 the number of cycles was increased to 35. Amplicons were purified from 2% agarose gel (QIAquick Gel Extraction Kit, Qiagen) and submitted to Eurofins for amplification with indexing adaptors and sequencing on an Illumina MiSeq2 x 300bp run. Sequencing data were analysed with CRISPResso2 (Clement 2019) to detect and quantify of insertions, mutations and deletions. First, input NGS sequences were trimmed using the Trimmomatic software (http://www.usadellab.org/cms/?page=trimmomatic) based on the phred33 score to remove low-quality positions (score < 30) and Illumina adapters, keeping only trimmed reads having length greater than 100 bp to ensure a good coverage of the reference region (CRISPResso2 options: –trim_sequences –trimmomatic_command trimmomatic –trimmomatic_options_string 'ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10 MINLEN:100'). The cleaned sequences were mapped to the corresponding input reference locus region (i.e., on/off-target depending on the sample) and RNA guides were provided to focus the analysis on that specific region. Specifically, the computed alleles were quantified within the sgRNA sequences (that represent the editing window of the base editor) by measuring the number of reads and their relative abundances based on total read counts. Moreover, both the targeted and the produced nucleotide were provided (CRISPResso2 options: --base_editor_output --conversion_nuc_from C/G --conversion_nuc_to T/A), to measure the frequency of substitutions. Finally, CRISPResso2 output alleles were post-processed by correcting all the mismatch positions outside the quantification window and re-quantifying the total read counts (and consequently the corresponding relative abundances). Alleles showing a relative abundance lower than the false positive threshold (set at 0.2% based on untreated samples) were filtered out. Comparing untreated to base edited samples, the final results were obtained by classifying and quantifying the (expected) SNVs of the base editor, other SNVs, insertions, and deletions, as well as their combinations.

sgRNA-independent off-target analysis

Base edited cells disrupted in *TRAC*, *TRBC1, TRBC2,* and *TIGIT,* and electroporated cells, as untreated controls, were generated from 4 healthy donors. Genomic DNA was isolated in sterile conditions (DNeasy blood and tissue Kit, Qiagen) at the end of the manipulation procedure (17 days of *in vitro* culture). Whole Exome Sequencing was performed by Genewiz (Azenta) using the Agilent SureSelect Human All Exon V7 kit and running an Illumina NovaSeq 2x150bp with an estimated coverage of 500X per sample, and input data were analyzed following the GATK "Best Practice Workflows" to identify variants in each sample. In details, FastQC (v0.11.9) was used to check the quality of the sequencing reads, trim-galore (v0.6.6) was employed to trim low-quality bases, and then the Seqtk toolkit (v1.3) was used to randomly down-sample abundant samples to 190M reads to avoid sample unbalance. After that, input sequences were aligned to the human genome assembly (GRCh38) using BWA (v0.7.17). The computed alignments were processed with Picard (v2.25.6) MarkDuplicates to mark duplicates and GATK (v4.2.0) BaseRecalibrator + ApplyBQSR to recalibrate base quality scores on dbSNP known sites. HaplotypeCaller was used to call variants in each sample by emitting condensed non-variant blocks (i.e., -ERC GVCF), while CombineGVCFs and GenotypeGVCFs were employed to combine and genotype variants, respectively. VariantFiltration was then applied to filter results based on their 'QualityByDepth' (i.e., --filter-expression 'QD < 2.0') and overall coverage 'DP' (i.e., --filter-expression 'DP < 500'). Moreover, additional per-sample filters were applied on variants belonging to each sample to identify the private ones, that is, variants having low genotype quality (i.e., GQ < 80) and low coverage (i.e., DP < 50) were removed. The untreated sample of each donor was used as germline reference, and its variants were filtered out from the corresponding treated sample, as such variants were considered as already present in the initial cell population and not induced by the base editor. A final step was performed to get rid of multi-allelic variants (mainly involving repetitive sequences). SnpEff (v5.0) was used to annotate the resulting variants on the canonical isoform from the GRCh38.p13.RefSeq reference database, and the downstream analyses involved their classification according to the type (insertion, deletion, or SNV), with a specific focus on all SNVs to classify the specific mutation events. Assessment of variants on a panel of cancer related genes and on T cell related gene lists was performed based on variant annotations.

Immunophenotyping by flow cytometry

For surface staining, cells were incubated with the appropriate cocktail of antibodies in FACS buffer consisting of 1X phosphate-buffered saline (PBS) plus 2% FBS. Staining was performed at RT for 10 minutes. For the analysis of the AGR2- and MSLN-specific T cells, phycoerythrin (PE) fluorescently-labelled Vβ14 antibody (Beckman coulter) was used following the manufacturer's instructions. For DKK1-specific T cells phycoerythrin (PE) fluorescently-labelled Vβ8 antibody (Beckman coulter) was used following the manufacturer's instructions. For MET-specific T cells, fluorescein (FITC) fluorescently-labelled Vβ13.1 antibody (Beckman coulter) was used following the manufacturer's instructions. For MUC5AC-specific T cells, phycoerythrin (PE) fluorescently-labelled Vβ12 antibody (Beckman coulter) was used following the manufacturer's instructions.

For intracellular staining assays, cells were collected after 6 hours of co-culture and stained for their vitality with Zombie viability kit (BioLegend) for 5 minutes followed by 10 minutes incubation at RT with surface antibodies (anti-human CD3, anti-human CD4, anti-human CD8). Cells were fixed and permeabilized with Fix/Perm buffer set (BioLegend). Intracellular staining was performed with antibodies specific for human cytokines (anti-human IFN-γ, anti-human TNF-α, anti-human IL-2).

Flow cytometry data were acquired using one of the following instruments: BD FACS Canto II (BD Biosciences). Data analysis was performed using FlowJo software (Tree star Inc).

Fluorochrome-conjugated monoclonal antibody	Vendor	Catalog number
CD3-FITC	BD Biosciences	555339
CD3-BV510	BioLegend	317332
CD3-PerCP	BioLegend	300326
CD4-APC	BioLegend	357408
CD4-PE	BioLegend	344606
CD4-PB	BioLegend	317429
CD4-PerCP	BD Biosciences	345770
CD4-APC-Cy7	BioLegend	300517
CD8-APC	BioLegend	301014
CD8-PE	BioLegend	344706
CD8-PE-Cy7	BioLegend	344712
7AAD-PerCP	BD Biosciences	7081885
Zombie Violet fixable viability kit	BioLegend	423114
Zombie Aqua fixable viability kit	BioLegend	423102
TNFα- PE-Cy7	BioLegend	502930
IFNγ-APC-Cy7	BioLegend	502530
HLA-A2-FITC	BioLegend	343304
HLA-A2-PE	BioLegend	343306
HLA-A2-APC	BioLegend	343307
IL2-PE	BioLegend	500307
IL2-APC	BioLegend	500310
Rat anti mouse CD45-PerCP	BioLegend	103130
CD45-BV510	BioLegend	304036
CD45RA-PE-Cy7	BioLegend	304126
CD45RA-PB	BioLegend	304123
CD62L-APC-Cy7	BioLegend	304814
CD62L-FITC	BioLegend	305606
TCR Vβ8-PE	Beckman Coulter	IM2289
TCR Vβ12-PE	Beckman Coulter	IM2291
TCR Vβ13.1- FITC	Beckman Coulter	IM1554
TCR Vβ14-PE	Beckman Coulter	IM2047
TIGIT-PE-Cy7	BioLegend	372713

Functional assays for T cell recognition of target cells

To evaluate cytokine production TAAs-specific or TCR-edited T cells were co-cultured with HLA-A*0201 target cells, that were either autologous APCs or immortalized B cells or T2 cell line. Target cells were pulsed for 16 hours with peptide pools and subpools (assembled as described above), or with individual peptides and an unrelated one (final concentration 10 ug/mL). As positive control of T cell functionality, TCR-edited T cells were stimulated with 50 ng/ml phytohemagglutinin (PMA)(Sigma-Aldrich) and 1µg/ml ionomycin (Sigma-Aldrich). The co-culture was plated in IMDM supplemented with 10% FBS, 1% pen-trep, 1% glutamine plus 1 µg/ml anti-CD28 monoclonal antibody (BD Biosciences) and 1 µg/ml Golgi Stop protein transport inhibitor (BD Biosciences). Immediately after seeding we added anti-human CD107a antibody (7.5 µg/ml). 6 hours later we stained the co-culture for intracellular cytokine detection.

We assess the ability of TCR-edited T cells to recognize naturally processed antigens and eliminate cancer cells by different approaches. TCR-edited T cells were co-cultured with antigen positive HLA-A*02 PDAC cell line (BxPC3-A2$^+$), antigen negative (PANC1 or SW480) and HLA unrelated controls (IGROV1) for 24 hours at 5:1 effector-to-target ratio. Prior to co-culture target cells were counted, seeded and stimulated overnight with 200ng/mL human recombinant IFNγ (Peprotech). The co-culture was plated in IMDM supplemented with 10% FBS, 1% pen-trep, 1% glutamine plus 1 µg/ml anti-CD28 monoclonal antibody. The number of residual target cells was determined by live/dead staining and flow cytometry to calculate the elimination index (1-(number of residual target cells in the presence of TCR-edited T cells/number of residual target cells when seeded alone).

To evaluate the killing of patient-derived organoids we used the Incucyte Live Imaging system. PDOs were released from the Matrigel and plated overnight at a concentration of 30-50x10^4/mL in Basal medium supplemented with 200ng/mL human recombinant IFNγ (Peprotech). The following day, PDOs were labelled with 1μM Cytolight Rapid Red Dye (Sartorius) following the manufacturer's instruction. Cells were seeded at different effector-to-target ratio as indicated in IMDM IMDM supplemented with 10% FBS, 1% pen-trep, 1% glutamine plus 1 µg/ml anti-CD28 monoclonal antibody and 5μM of Caspase-3/7 Green (Sartorius). Images were collected every 2 hours and apoptotic cell area was quantified by measuring the green signal in red PDOs.

To test TCRs co-receptor dependency we isolated CD8 and CD4 T cells and co-cultured at 5:1 E:T ratio for up to 48 hours with antigen positive HLA-A*02 PDAC cell line (BxPC3-A2$^+$) and one HLA unrelated control (IGROV1, AsPC1). By performing magnetic cell separation, we isolated CD8 and CD4 T cells. Briefly, to select for CD4 T cells, we stained CD3 T cell populations with PE-conjugated anti-CD4 antibody, added anti-PE magnetic beads (Milteny) according to manufacturer's instruction and proceeded to column-based sorting. Prior to co-culture target cells were counted, seeded, and stimulated overnight with 200ng/mL human recombinant IFNγ (Peprotech).

In vivo experiments

For the in vivo assessment of anti-tumor efficacy in mCRC setting, we employed 7-10-week-old male non-irradiated immuno-deficient NSG mice from Jackson Labs. Each mouse was intra-hepatically injected with 4x10^4 HLA-A2$^+$ Antigen$^+$ LUC-mPDOs (Potenza et al. 2023). After three days, a single dose of TCR-edited TIGIT$_{COMP}$ or TCR-

edited $TIGIT_{KO}$ T cells were delivered by intrahepatic injection ($10x10^6$ $CD3^+$ cells). Tumor control was evaluated using small animal bioluminescence imaging (BLI) with the IVIS SpectrumCT System. Each mouse received an intraperitoneal injection of 150 mg luciferin/kg body weight 10 min before BLI. Mice were kept at RT and anaesthetized (2-3% isofluorane and 1L/min oxygen) for image acquisition. Images were analysed using Living Image 4.5 (Perkin Elmer) by measuring the total flux (photons/s) within the selected region of interest. To monitor T cell expansion overtime bleeding were performed once a week and T cells analyzed by flow cytometry. Mice were euthanized when tumor burden reached $1x10^9$ BLI.

Statistical analyses

Sample size and statistical tests are described in the figure. Data are expressed as mean±SEM unless otherwise. One-way ANOVA and two-way ANOVA with Tukey's multiple test, linear regression, were performed using and Prism V.9.3.1 (GraphPad Software, San Diego, California, USA). P value <0.05 was taken to indicate statistical significance unless otherwise stated.

REFERENCES

Adusumilli, Prasad S., Marjorie G. Zauderer, Isabelle Rivière, Stephen B. Solomon, Valerie W. Rusch, Roisin E. O'Cearbhaill, Amy Zhu, et al. 2021. «A Phase I Trial of Regional Mesothelin-Targeted CAR T-Cell Therapy in Patients with Malignant Pleural Disease, in Combination with the Anti-PD-1 Agent Pembrolizumab». *Cancer Discovery* 11 (11): 2748–63. https://doi.org/10.1158/2159-8290.CD-21-0407.

Adusumilli, Prasad S., Marjorie Glass Zauderer, Valerie W. Rusch, Roisin O'Cearbhaill, Amy Zhu, Daniel Ngai, Erin McGee, et al. 2019. «Regional delivery of mesothelin-targeted CAR T cells for pleural cancers: Safety and preliminary efficacy in combination with anti-PD-1 agent.» *Journal of Clinical Oncology* 37 (15_suppl): 2511–2511. https://doi.org/10.1200/JCO.2019.37.15_suppl.2511.

Agarwal, Sangya, M. Angela Aznar, Andrew J. Rech, Charly R. Good, Shunichiro Kuramitsu, Tong Da, Mercy Gohil, et al. 2023. «Deletion of the Inhibitory Co-Receptor CTLA-4 Enhances and Invigorates Chimeric Antigen Receptor T Cells». *Immunity* 56 (10): 2388-2407.e9. https://doi.org/10.1016/j.immuni.2023.09.001.

Ahmadzadeh, Mojgan, Anna Pasetto, Li Jia, Drew C. Deniger, Sanja Stevanović, Paul F. Robbins, e Steven A. Rosenberg. 2019. «Tumor-Infiltrating Human CD4+ Regulatory T Cells Display a Distinct TCR Repertoire and Exhibit Tumor and Neoantigen Reactivity». *Science Immunology* 4 (31): eaao4310. https://doi.org/10.1126/sciimmunol.aao4310.

Ahmed, Nabil, Vita S. Brawley, Meenakshi Hegde, Catherine Robertson, Alexia Ghazi, Claudia Gerken, Enli Liu, et al. 2015. «Human Epidermal Growth Factor Receptor 2 (HER2) -Specific Chimeric Antigen Receptor-Modified T Cells for the Immunotherapy of HER2-Positive Sarcoma». *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 33 (15): 1688–96. https://doi.org/10.1200/JCO.2014.58.0225.

Allard, Bertrand, Maria Serena Longhi, Simon C. Robson, e John Stagg. 2017. «The Ectonucleotidases CD39 and CD73: Novel Checkpoint Inhibitor Targets». *Immunological Reviews* 276 (1): 121–44. https://doi.org/10.1111/imr.12528.

Alnefaie, Alaa, Sarah Albogami, Yousif Asiri, Tanveer Ahmad, Saqer S. Alotaibi, Mohammad M. Al-Sanea, e Hisham Althobaiti. 2022. «Chimeric Antigen Receptor T-Cells: An Overview of Concepts, Applications, Limitations, and Proposed Solutions». *Frontiers in Bioengineering and Biotechnology* 10. https://www.frontiersin.org/articles/10.3389/fbioe.2022.797440.

Alvisi, Giorgia, Alberto Termanini, Cristiana Soldani, Federica Portale, Roberta Carriero, Karolina Pilipow, Guido Costa, et al. 2022. «Multimodal Single-Cell Profiling of Intrahepatic Cholangiocarcinoma Defines Hyperactivated Tregs as a Potential Therapeutic Target». *Journal of Hepatology* 77 (5): 1359–72. https://doi.org/10.1016/j.jhep.2022.05.043.

Amendola, Mario, Mary Anna Venneri, Alessandra Biffi, Elisa Vigna, e Luigi Naldini. 2005. «Coordinate Dual-Gene Transgenesis by Lentiviral Vectors Carrying Synthetic Bidirectional Promoters». *Nature Biotechnology* 23 (1): 108–16. https://doi.org/10.1038/nbt1049.

Anderson, Kristin G., Valentin Voillet, Breanna M. Bates, Edison Y. Chiu, Madison G. Burnett, Nicolas M. Garcia, Shannon K. Oda, et al. 2019. «Engineered Adoptive T-Cell Therapy Prolongs Survival in a Preclinical Model of Advanced-Stage Ovarian Cancer». *Cancer Immunology Research* 7 (9): 1412–25. https://doi.org/10.1158/2326-6066.CIR-19-0258.

Anzalone, Andrew V., Luke W. Koblan, e David R. Liu. 2020. «Genome Editing with CRISPR–Cas Nucleases, Base Editors, Transposases and Prime Editors».

Nature Biotechnology 38 (7): 824–44. https://doi.org/10.1038/s41587-020-0561-9.

Arcangeli, Silvia, Camilla Bove, Claudia Mezzanotte, Barbara Camisa, Laura Falcone, Francesco Manfredi, Eugenia Bezzecchi, et al. 2022. «CAR T Cell Manufacturing from Naive/Stem Memory T Lymphocytes Enhances Antitumor Responses While Curtailing Cytokine Release Syndrome». American Society for Clinical Investigation. 15 giugno 2022. https://doi.org/10.1172/JCI150807.

Badea, Liviu, Vlad Herlea, Simona Olimpia Dima, Traian Dumitrascu, e Irinel Popescu. 2008. «Combined Gene Expression Analysis of Whole-Tissue and Microdissected Pancreatic Ductal Adenocarcinoma Identifies Genes Specifically Overexpressed in Tumor Epithelia». *Hepato-Gastroenterology* 55 (88): 2016–27.

Bailey, Peter, David K. Chang, Katia Nones, Amber L. Johns, Ann-Marie Patch, Marie-Claude Gingras, David K. Miller, et al. 2016. «Genomic Analyses Identify Molecular Subtypes of Pancreatic Cancer». *Nature* 531 (7592): 47–52. https://doi.org/10.1038/nature16965.

Banta, Karl L., Xiaozheng Xu, Avantika S. Chitre, Amelia Au-Yeung, Chikara Takahashi, William E. O'Gorman, Thomas D. Wu, et al. 2022. «Mechanistic convergence of the TIGIT and PD-1 inhibitory pathways necessitates co-blockade to optimize anti-tumor CD8+ T cell responses». *Immunity* 55 (3): 512-526.e9. https://doi.org/10.1016/j.immuni.2022.02.005.

Beatty, Gregory L., Mark H. O'Hara, Simon F. Lacey, Drew A. Torigian, Farzana Nazimuddin, Fang Chen, Irina M. Kulikovskaya, et al. 2018. «Activity of Mesothelin-Specific Chimeric Antigen Receptor T Cells Against Pancreatic Carcinoma Metastases in a Phase 1 Trial». *Gastroenterology* 155 (1): 29–32. https://doi.org/10.1053/j.gastro.2018.03.029.

Belk, Julia A., Winnie Yao, Nghi Ly, Katherine A. Freitas, Yan-Ting Chen, Quanming Shi, Alfredo M. Valencia, et al. 2022. «Genome-Wide CRISPR Screens of T Cell Exhaustion Identify Chromatin Remodeling Factors That Limit T Cell Persistence». *Cancer Cell* 40 (7): 768-786.e7. https://doi.org/10.1016/j.ccell.2022.06.001.

Bendle, Gavin M., Carsten Linnemann, Anna I. Hooijkaas, Laura Bies, Moniek A. de Witte, Annelies Jorritsma, Andrew D. M. Kaiser, et al. 2010. «Lethal Graft-versus-Host Disease in Mouse Models of T Cell Receptor Gene Therapy». *Nature Medicine* 16 (5): 565–70, 1p following 570. https://doi.org/10.1038/nm.2128.

Berdien, B., U. Mock, D. Atanackovic, e B. Fehse. 2014. «TALEN-Mediated Editing of Endogenous T-Cell Receptors Facilitates Efficient Reprogramming of T Lymphocytes by Lentiviral Gene Transfer». *Gene Therapy* 21 (6): 539–48. https://doi.org/10.1038/gt.2014.26.

Biasco, Luca, Serena Scala, Luca Basso Ricci, Francesca Dionisio, Cristina Baricordi, Andrea Calabria, Stefania Giannelli, et al. 2015. «In Vivo Tracking of T Cells in Humans Unveils Decade-Long Survival and Activity of Genetically Modified T Memory Stem Cells». *Science Translational Medicine* 7 (273): 273ra13. https://doi.org/10.1126/scitranslmed.3010314.

Biller, Leah H., e Deborah Schrag. 2021. «Diagnosis and Treatment of Metastatic Colorectal Cancer: A Review». *JAMA* 325 (7): 669–85. https://doi.org/10.1001/jama.2021.0106.

Binnewies, Mikhail, Edward W. Roberts, Kelly Kersten, Vincent Chan, Douglas F. Fearon, Miriam Merad, Lisa M. Coussens, et al. 2018. «Understanding the Tumor Immune Microenvironment (TIME) for Effective Therapy». *Nature Medicine* 24 (5): 541–50. https://doi.org/10.1038/s41591-018-0014-x.

Bolotin, Dmitriy A., Stanislav Poslavsky, Igor Mitrophanov, Mikhail Shugay, Ilgar Z. Mamedov, Ekaterina V. Putintseva, e Dmitriy M. Chudakov. 2015. «MiXCR:

Software for Comprehensive Adaptive Immunity Profiling». *Nature Methods* 12 (5): 380–81. https://doi.org/10.1038/nmeth.3364.
Bolotin, Dmitry A., Ilgar Z. Mamedov, Olga V. Britanova, Ivan V. Zvyagin, Dmitriy Shagin, Svetlana V. Ustyugova, Maria A. Turchaninova, Sergey Lukyanov, Yury B. Lebedev, e Dmitriy M. Chudakov. 2012. «Next Generation Sequencing for TCR Repertoire Profiling: Platform-Specific Features and Correction Algorithms». *European Journal of Immunology* 42 (11): 3073–83. https://doi.org/10.1002/eji.201242517.
Bonini, Chiara, Aude G. Chapuis, Michael Hudecek, Sonia Guedan, Chiara Magnani, e Waseem Qasim. 2023. «Genome Editing in Engineered T Cells for Cancer Immunotherapy». *Human Gene Therapy* 34 (17–18): 853–69. https://doi.org/10.1089/hum.2023.128.
Bove, Camilla, Silvia Arcangeli, Laura Falcone, Barbara Camisa, Rita El Khoury, Beatrice Greco, Anna De Lucia, et al. 2023. «CD4 CAR-T Cells Targeting CD19 Play a Key Role in Exacerbating Cytokine Release Syndrome, While Maintaining Long-Term Responses». *Journal for Immunotherapy of Cancer* 11 (1): e005878. https://doi.org/10.1136/jitc-2022-005878.
Brahmer, Julie R., Scott S. Tykodi, Laura Q. M. Chow, Wen-Jen Hwu, Suzanne L. Topalian, Patrick Hwu, Charles G. Drake, et al. 2012. «Safety and Activity of Anti-PD-L1 Antibody in Patients with Advanced Cancer». *The New England Journal of Medicine* 366 (26): 2455–65. https://doi.org/10.1056/NEJMoa1200694.
Brichard, V., A. Van Pel, T. Wölfel, C. Wölfel, E. De Plaen, B. Lethé, P. Coulie, e T. Boon. 1993. «The Tyrosinase Gene Codes for an Antigen Recognized by Autologous Cytolytic T Lymphocytes on HLA-A2 Melanomas». *The Journal of Experimental Medicine* 178 (2): 489–95. https://doi.org/10.1084/jem.178.2.489.
Brown, Christine E., Darya Alizadeh, Renate Starr, Lihong Weng, Jamie R. Wagner, Araceli Naranjo, Julie R. Ostberg, et al. 2016. «Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy». *New England Journal of Medicine* 375 (26): 2561–69. https://doi.org/10.1056/NEJMoa1610497.
Buchholz, Malte, Mike Braun, Anna Heidenblut, Hans A. Kestler, Günter Klöppel, Wolff Schmiegel, Stephan A. Hahn, Jutta Lüttges, e Thomas M. Gress. 2005. «Transcriptome Analysis of Microdissected Pancreatic Intraepithelial Neoplastic Lesions». *Oncogene* 24 (44): 6626–36. https://doi.org/10.1038/sj.onc.1208804.
Camus, Matthieu, Marie Tosolini, Bernhard Mlecnik, Franck Pagès, Amos Kirilovsky, Anne Berger, Anne Costes, et al. 2009. «Coordination of Intratumoral Immune Reaction and Human Colorectal Cancer Recurrence». *Cancer Research* 69 (6): 2685–93. https://doi.org/10.1158/0008-5472.CAN-08-2654.
Casirati, Gabriele, Andrea Cosentino, Adele Mucci, Mohammed Salah Mahmoud, Iratxe Ugarte Zabala, Jing Zeng, Scott B. Ficarro, et al. 2023. «Epitope Editing Enables Targeted Immunotherapy of Acute Myeloid Leukaemia». *Nature* 621 (7978): 404–14. https://doi.org/10.1038/s41586-023-06496-5.
Chen, Shih-Hsun, Wei-Chien Hung, Pu Wang, Colin Paul, e Konstantinos Konstantopoulos. 2013. «Mesothelin Binding to CA125/MUC16 Promotes Pancreatic Cancer Cell Motility and Invasion via MMP-7 Activation». *Scientific Reports* 3 (1): 1870. https://doi.org/10.1038/srep01870.
Chiesa, Robert, Christos Georgiadis, Farhatullah Syed, Hong Zhan, Annie Etuk, Soragia Athina Gkazi, Roland Preece, et al. 2023. «Base-Edited CAR7 T Cells for Relapsed T-Cell Acute Lymphoblastic Leukemia». *New England Journal of Medicine*, giugno. https://doi.org/10.1056/NEJMoa2300709.
Chow, Andrew, Karlo Perica, Christopher A. Klebanoff, e Jedd D. Wolchok. 2022. «Clinical Implications of T Cell Exhaustion for Cancer Immunotherapy».

Nature Reviews Clinical Oncology 19 (12): 775–90. https://doi.org/10.1038/s41571-022-00689-z.

Cieri, Nicoletta, Barbara Camisa, Fabienne Cocchiarella, Mattia Forcato, Giacomo Oliveira, Elena Provasi, Attilio Bondanza, et al. 2013. «IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors». *Blood* 121 (4): 573–84. https://doi.org/10.1182/blood-2012-05-431718.

Cohen, Cyrille J., Zhili Zheng, Regina Bray, Yangbing Zhao, Linda A. Sherman, Steven A. Rosenberg, e Richard A. Morgan. 2005. «Recognition of Fresh Human Tumor by Human Peripheral Blood Lymphocytes Transduced with a Bicistronic Retroviral Vector Encoding a Murine Anti-P53 TCR». *Journal of Immunology (Baltimore, Md.: 1950)* 175 (9): 5799–5808. https://doi.org/10.4049/jimmunol.175.9.5799.

Collisson, Eric A., Anguraj Sadanandam, Peter Olson, William J. Gibb, Morgan Truitt, Shenda Gu, Janine Cooc, et al. 2011. «Subtypes of Pancreatic Ductal Adenocarcinoma and Their Differing Responses to Therapy». *Nature Medicine* 17 (4): 500–503. https://doi.org/10.1038/nm.2344.

Cooper, Matthew L., Jaebok Choi, Karl Staser, Julie K. Ritchey, Jessica M. Devenport, Kayla Eckardt, Michael P. Rettig, et al. 2018. «An "off-the-Shelf" Fratricide-Resistant CAR-T for the Treatment of T Cell Hematologic Malignancies». *Leukemia* 32 (9): 1970–83. https://doi.org/10.1038/s41375-018-0065-5.

Costa-Silva, Bruno, Nicole M. Aiello, Allyson J. Ocean, Swarnima Singh, Haiying Zhang, Basant Kumar Thakur, Annette Becker, et al. 2015. «Pancreatic Cancer Exosomes Initiate Pre-Metastatic Niche Formation in the Liver». *Nature Cell Biology* 17 (6): 816–26. https://doi.org/10.1038/ncb3169.

Couzin-Frankel, Jennifer. 2013. «Breakthrough of the Year 2013. Cancer Immunotherapy». *Science (New York, N.Y.)* 342 (6165): 1432–33. https://doi.org/10.1126/science.342.6165.1432.

Cui Zhou, Daniel, Reyka G. Jayasinghe, Siqi Chen, John M. Herndon, Michael D. Iglesia, Pooja Navale, Michael C. Wendl, et al. 2022. «Spatially Restricted Drivers and Transitional Cell Populations Cooperate with the Microenvironment in Untreated and Chemo-Resistant Pancreatic Cancer». *Nature Genetics* 54 (9): 1390–1405. https://doi.org/10.1038/s41588-022-01157-1.

Cullot, Grégoire, Julian Boutin, Jérôme Toutain, Florence Prat, Perrine Pennamen, Caroline Rooryck, Martin Teichmann, et al. 2019. «CRISPR-Cas9 Genome Editing Induces Megabase-Scale Chromosomal Truncations». *Nature Communications* 10 (1): 1136. https://doi.org/10.1038/s41467-019-09006-2.

Dafni, U., O. Michielin, S. Martin Lluesma, Z. Tsourti, V. Polydoropoulou, D. Karlis, M. J. Besser, et al. 2019. «Efficacy of Adoptive Therapy with Tumor-Infiltrating Lymphocytes and Recombinant Interleukin-2 in Advanced Cutaneous Melanoma: A Systematic Review and Meta-Analysis». *Annals of Oncology* 30 (12): 1902–13. https://doi.org/10.1093/annonc/mdz398.

Deaglio, Silvia, Karen M. Dwyer, Wenda Gao, David Friedman, Anny Usheva, Anna Erat, Jiang-Fan Chen, et al. 2007. «Adenosine Generation Catalyzed by CD39 and CD73 Expressed on Regulatory T Cells Mediates Immune Suppression». *The Journal of Experimental Medicine* 204 (6): 1257–65. https://doi.org/10.1084/jem.20062512.

Dekker, Evelien, Pieter J Tanis, Jasper L A Vleugels, Pashtoon M Kasi, e Michael B Wallace. 2019. «Colorectal Cancer». *The Lancet* 394 (10207): 1467–80. https://doi.org/10.1016/S0140-6736(19)32319-0.

Delorme, E. 1964. «TREATMENT OF PRIMARY FIBROSARCOMA IN THE RAT WITH IMMUNE LYMPHOCYTES». *The Lancet* 284 (7351): 117–20. https://doi.org/10.1016/S0140-6736(64)90126-6.

DembiĆ, Zlatko, Werner Haas, Siegfried Weiss, James McCubrey, Hansruedi Kiefer, Harald von Boehmer, e Michael Steinmetz. 1986. «Transfer of Specificity by Murine α and β T-Cell Receptor Genes». *Nature* 320 (6059): 232–38. https://doi.org/10.1038/320232a0.

Dolton, Garry, Cristina Rius, Aaron Wall, Barbara Szomolay, Valentina Bianchi, Sarah A. E. Galloway, Md Samiul Hasan, et al. 2023. «Targeting of Multiple Tumor-Associated Antigens by Individual T Cell Receptors during Successful Cancer Immunotherapy». *Cell* 0 (0). https://doi.org/10.1016/j.cell.2023.06.020.

Doman, Jordan L., Aditya Raguram, Gregory A. Newby, e David R. Liu. 2020. «Evaluation and Minimization of Cas9-Independent off-Target DNA Editing by Cytosine Base Editors». *Nature Biotechnology* 38 (5): 620–28. https://doi.org/10.1038/s41587-020-0414-6.

Donnadieu, Emmanuel, Maik Luu, Miriam Alb, Brigitte Anliker, Silvia Arcangeli, Chiara Bonini, Biagio De Angelis, et al. 2022. «Time to Evolve: Predicting Engineered T Cell-Associated Toxicity with next-Generation Models». *Journal for ImmunoTherapy of Cancer* 10 (5): e003486. https://doi.org/10.1136/jitc-2021-003486.

Doudna, Jennifer A. 2020. «The Promise and Challenge of Therapeutic Genome Editing». *Nature* 578 (7794): 229–36. https://doi.org/10.1038/s41586-020-1978-5.

Driehuis, Else, Ana Gracanin, Robert Gerhardus Jacob Vries, Hans Clevers, e Sylvia Fernández Boj. 2020. «Establishment of Pancreatic Organoids from Normal Tissue and Tumors». *STAR Protocols* 1 (3): 100192. https://doi.org/10.1016/j.xpro.2020.100192.

Dumartin, L, W Alrawashdeh, S M Trabulo, T P Radon, K Steiger, R M Feakins, M P di Magliano, et al. 2017. «ER stress protein AGR2 precedes and is involved in the regulation of pancreatic cancer initiation». *Oncogene* 36 (22): 3094–3103. https://doi.org/10.1038/onc.2016.459.

Durgin, Joseph S., Fraser Henderson, MacLean P. Nasrallah, Suyash Mohan, Sumei Wang, Simon F. Lacey, Jan Joseph Melenhorst, et al. 2021. «Case Report: Prolonged Survival Following EGFRvIII CAR T Cell Treatment for Recurrent Glioblastoma». *Frontiers in Oncology* 11. https://www.frontiersin.org/articles/10.3389/fonc.2021.669071.

Enache, Oana M., Veronica Rendo, Mai Abdusamad, Daniel Lam, Desiree Davison, Sangita Pal, Naomi Currimjee, et al. 2020. «Cas9 Activates the P53 Pathway and Selects for P53-Inactivating Mutations». *Nature Genetics* 52 (7): 662–68. https://doi.org/10.1038/s41588-020-0623-4.

Engstrand, Jennie, Henrik Nilsson, Cecilia Strömberg, Eduard Jonas, e Jacob Freedman. 2018. «Colorectal cancer liver metastases – a population-based study on incidence, management and survival». *BMC Cancer* 18 (1): 78. https://doi.org/10.1186/s12885-017-3925-x.

Fiumara, Martina, Samuele Ferrari, Attya Omer-Javed, Stefano Beretta, Luisa Albano, Daniele Canarutto, Angelica Varesi, et al. 2023. «Genotoxic Effects of Base and Prime Editing in Human Hematopoietic Stem Cells». *Nature Biotechnology*, settembre, 1–15. https://doi.org/10.1038/s41587-023-01915-4.

Fortier, A. H., C. A. Nacy, e M. V. Sitkovsky. 1989. «Similar Molecular Requirements for Antigen Receptor-Triggered Secretion of Interferon and Granule Enzymes by Cytolytic T Lymphocytes». *Cellular Immunology* 124 (1): 64–76. https://doi.org/10.1016/0008-8749(89)90112-3.

Freiwan, Abdullah, Jaquelyn T. Zoine, Jeremy Chase Crawford, Abishek Vaidya, Stefan A. Schattgen, Jacquelyn A. Myers, Sagar L. Patil, et al. 2022. «Engineering Naturally Occurring CD7- T Cells for the Immunotherapy of Hematological Malignancies». *Blood* 140 (25): 2684–96. https://doi.org/10.1182/blood.2021015020.

Fry, Terry J., Nirali N. Shah, Rimas J. Orentas, Maryalice Stetler-Stevenson, Constance M. Yuan, Sneha Ramakrishna, Pamela Wolters, et al. 2018. «CD22-Targeted CAR T Cells Induce Remission in B-ALL That Is Naive or Resistant to CD19-Targeted CAR Immunotherapy». *Nature Medicine* 24 (1): 20–28. https://doi.org/10.1038/nm.4441.

Gabrilovich, Dmitry I., Suzanne Ostrand-Rosenberg, e Vincenzo Bronte. 2012. «Coordinated Regulation of Myeloid Cells by Tumours». *Nature Reviews. Immunology* 12 (4): 253–68. https://doi.org/10.1038/nri3175.

Gapinske, Michael, Alan Luu, Jackson Winter, Wendy S. Woods, Kurt A. Kostan, Nikhil Shiva, Jun S. Song, e Pablo Perez-Pinera. 2018. «CRISPR-SKIP: programmable gene splicing with single base editors». *Genome Biology* 19 (1): 107. https://doi.org/10.1186/s13059-018-1482-5.

Gaudelli, Nicole M., Alexis C. Komor, Holly A. Rees, Michael S. Packer, Ahmed H. Badran, David I. Bryson, e David R. Liu. 2017. «Programmable Base Editing of A•T to G•C in Genomic DNA without DNA Cleavage». *Nature* 551 (7681): 464–71. https://doi.org/10.1038/nature24644.

Gaudelli, Nicole M., Dieter K. Lam, Holly A. Rees, Noris M. Solá-Esteves, Luis A. Barrera, David A. Born, Aaron Edwards, et al. 2020. «Directed Evolution of Adenine Base Editors with Increased Activity and Therapeutic Application». *Nature Biotechnology* 38 (7): 892–900. https://doi.org/10.1038/s41587-020-0491-6.

Georgiadis, Christos, Jane Rasaiyaah, Soragia Athina Gkazi, Roland Preece, Aniekan Etuk, Abraham Christi, e Waseem Qasim. 2021. «Base-edited CAR T cells for combinational therapy against T cell malignancies». *Leukemia* 35 (12): 3466–81. https://doi.org/10.1038/s41375-021-01282-6.

Gillis, Steven, e Kendall A. Smith. 1977. «Long Term Culture of Tumour-Specific Cytotoxic T Cells». *Nature* 268 (5616): 154–56. https://doi.org/10.1038/268154a0.

Golstein, Pierre, e Gillian M. Griffiths. 2018. «An Early History of T Cell-Mediated Cytotoxicity». *Nature Reviews. Immunology* 18 (8): 527–35. https://doi.org/10.1038/s41577-018-0009-3.

Gomes-Silva, Diogo, Madhuwanti Srinivasan, Sandhya Sharma, Ciaran M. Lee, Dimitrios L. Wagner, Timothy H. Davis, Rayne H. Rouce, Gang Bao, Malcolm K. Brenner, e Maksim Mamonkin. 2017. «CD7-Edited T Cells Expressing a CD7-Specific CAR for the Therapy of T-Cell Malignancies». *Blood* 130 (3): 285–96. https://doi.org/10.1182/blood-2017-01-761320.

Gonzalez-Galarza, Faviel F., Antony McCabe, Eduardo J. Melo Dos Santos, James Jones, Louise Takeshita, Nestor D. Ortega-Rivera, Glenda M. Del Cid-Pavon, et al. 2020. «Allele Frequency Net Database (AFND) 2020 Update: Gold-Standard Data Classification, Open Access Genotype Data and New Query Tools». *Nucleic Acids Research* 48 (D1): D783–88. https://doi.org/10.1093/nar/gkz1029.

Goonesekere, Nalin C. W., Xiaosheng Wang, Lindsey Ludwig, e Chittibabu Guda. 2014. «A Meta Analysis of Pancreatic Microarray Datasets Yields New Targets as Cancer Genes and Biomarkers». *PLOS ONE* 9 (4): e93046. https://doi.org/10.1371/journal.pone.0093046.

Greco, Beatrice, Valeria Malacarne, Federica De Girardi, Giulia Maria Scotti, Francesco Manfredi, Elia Angelino, Camilla Sirini, et al. 2022. «Disrupting N-Glycan Expression on Tumor Cells Boosts Chimeric Antigen Receptor T Cell Efficacy against Solid Malignancies». *Science Translational Medicine* 14 (628): eabg3072. https://doi.org/10.1126/scitranslmed.abg3072.

Grünewald, Julian, Ronghao Zhou, Sara P. Garcia, Sowmya Iyer, Caleb A. Lareau, Martin J. Aryee, e J. Keith Joung. 2019. «Transcriptome-Wide off-Target RNA Editing Induced by CRISPR-Guided DNA Base Editors». *Nature* 569 (7756): 433–37. https://doi.org/10.1038/s41586-019-1161-z.

Grünewald, Julian, Ronghao Zhou, Sowmya Iyer, Caleb A. Lareau, Sara P. Garcia, Martin J. Aryee, e J. Keith Joung. 2019. «CRISPR DNA Base Editors with Reduced RNA Off-Target and Self-Editing Activities». *Nature Biotechnology* 37 (9): 1041–48. https://doi.org/10.1038/s41587-019-0236-6.

Guo, Congting, Xiaoteng Ma, Fei Gao, e Yuxuan Guo. 2023. «Off-Target Effects in CRISPR/Cas9 Gene Editing». *Frontiers in Bioengineering and Biotechnology* 11: 1143157. https://doi.org/10.3389/fbioe.2023.1143157.

Gurusamy, Devikala, Amanda N. Henning, Tori N. Yamamoto, Zhiya Yu, Nikolaos Zacharakis, Sri Krishna, Rigel J. Kishton, et al. 2020. «Multi-Phenotype CRISPR-Cas9 Screen Identifies P38 Kinase as a Target for Adoptive Immunotherapies». *Cancer Cell* 37 (6): 818-833.e9. https://doi.org/10.1016/j.ccell.2020.05.004.

Haapaniemi, Emma, Sandeep Botla, Jenna Persson, Bernhard Schmierer, e Jussi Taipale. 2018. «CRISPR-Cas9 Genome Editing Induces a P53-Mediated DNA Damage Response». *Nature Medicine* 24 (7): 927–30. https://doi.org/10.1038/s41591-018-0049-z.

Haas, Andrew R., Ryan J. Golden, Leslie A. Litzky, Boris Engels, Linlin Zhao, Fangmin Xu, John A. Taraszka, et al. 2023. «Two Cases of Severe Pulmonary Toxicity from Highly Active Mesothelin-Directed CAR T Cells». *Molecular Therapy: The Journal of the American Society of Gene Therapy*, giugno, S1525-0016(23)00316-7. https://doi.org/10.1016/j.ymthe.2023.06.006.

Haas, Andrew R., Janos L. Tanyi, Mark H. O'Hara, Whitney L. Gladney, Simon F. Lacey, Drew A. Torigian, Michael C. Soulen, et al. 2019. «Phase I Study of Lentiviral-Transduced Chimeric Antigen Receptor-Modified T Cells Recognizing Mesothelin in Advanced Solid Cancers». *Molecular Therapy: The Journal of the American Society of Gene Therapy* 27 (11): 1919–29. https://doi.org/10.1016/j.ymthe.2019.07.015.

Halbrook, Christopher J., Costas A. Lyssiotis, Marina Pasca di Magliano, e Anirban Maitra. 2023. «Pancreatic Cancer: Advances and Challenges». *Cell* 186 (8): 1729–54. https://doi.org/10.1016/j.cell.2023.02.014.

Hassan, Raffit, Marcus Butler, Roisin E. O'Cearbhaill, David Y. Oh, Melissa Johnson, Kevin Zikaras, Munisha Smalley, et al. 2023. «Mesothelin-Targeting T Cell Receptor Fusion Construct Cell Therapy in Refractory Solid Tumors: Phase 1/2 Trial Interim Results». *Nature Medicine* 29 (8): 2099–2109. https://doi.org/10.1038/s41591-023-02452-y.

Heczey, Andras, Chrystal U. Louis, Barbara Savoldo, Olga Dakhova, April Durett, Bambi Grilley, Hao Liu, et al. 2017a. «CAR T Cells Administered in Combination with Lymphodepletion and PD-1 Inhibition to Patients with Neuroblastoma». *Molecular Therapy: The Journal of the American Society of Gene Therapy* 25 (9): 2214–24. https://doi.org/10.1016/j.ymthe.2017.05.012.

———. 2017b. «CAR T Cells Administered in Combination with Lymphodepletion and PD-1 Inhibition to Patients with Neuroblastoma». *Molecular Therapy* 25 (9): 2214–24. https://doi.org/10.1016/j.ymthe.2017.05.012.

Heiduk, Max, Anna Klimova, Charlotte Reiche, David Digomann, Carolin Beer, Daniela E. Aust, Marius Distler, Jürgen Weitz, Adrian M. Seifert, e Lena Seifert. 2023. «TIGIT Expression Delineates T-cell Populations with Distinct Functional and Prognostic Impact in Pancreatic Cancer». *Clinical Cancer Research* 29 (14): 2638–50. https://doi.org/10.1158/1078-0432.CCR-23-0258.

Ho, William Y., Hieu N. Nguyen, Matthias Wolfl, Juergen Kuball, e Philip D. Greenberg. 2006. «In Vitro Methods for Generating CD8+ T-Cell Clones for Immunotherapy from the Naïve Repertoire». *Journal of Immunological Methods* 310 (1–2): 40–52. https://doi.org/10.1016/j.jim.2005.11.023.

Hossain, Nasheed, Bita Sahaf, Matthew Abramian, Jay Y. Spiegel, Katie Kong, Stephen Kim, Sharon Mavroukakis, et al. 2018. «Phase I Experience with a

Bi-Specific CAR Targeting CD19 and CD22 in Adults with B-Cell Malignancies». *Blood* 132 (Supplement 1): 490. https://doi.org/10.1182/blood-2018-99-110142.

Hou, Andrew J., Laurence C. Chen, e Yvonne Y. Chen. 2021. «Navigating CAR-T Cells through the Solid-Tumour Microenvironment». *Nature Reviews Drug Discovery* 20 (7): 531–50. https://doi.org/10.1038/s41573-021-00189-2.

Houg, Demi S., e Maarten F. Bijlsma. 2018. «The hepatic pre-metastatic niche in pancreatic ductal adenocarcinoma». *Molecular Cancer* 17 (giugno): 95. https://doi.org/10.1186/s12943-018-0842-9.

Hughes, Marybeth S., Yik Y. L. Yu, Mark E. Dudley, Zhili Zheng, Paul F. Robbins, Yong Li, John Wunderlich, et al. 2005. «Transfer of a TCR Gene Derived from a Patient with a Marked Antitumor Response Conveys Highly Active T-Cell Effector Functions». *Human Gene Therapy* 16 (4): 457–72. https://doi.org/10.1089/hum.2005.16.457.

Ihry, Robert J., Kathleen A. Worringer, Max R. Salick, Elizabeth Frias, Daniel Ho, Kraig Theriault, Sravya Kommineni, et al. 2018. «P53 Inhibits CRISPR-Cas9 Engineering in Human Pluripotent Stem Cells». *Nature Medicine* 24 (7): 939–46. https://doi.org/10.1038/s41591-018-0050-6.

Jafarzadeh, Leila, Elham Masoumi, Hamid Reza Mirzaei, Khadijeh Alishah, Keyvan Fallah-Mehrjardi, Mohammad Khakpoor-Koosheh, Hosein Rostamian, Farshid Noorbakhsh, e Jamshid Hadjati. 2021. «Targeted Knockdown of Tim3 by Short Hairpin RNAs Improves the Function of Anti-Mesothelin CAR T Cells». *Molecular Immunology* 139 (novembre): 1–9. https://doi.org/10.1016/j.molimm.2021.06.007.

Jin, Shuai, Yuan Zong, Qiang Gao, Zixu Zhu, Yanpeng Wang, Peng Qin, Chengzhi Liang, et al. 2019. «Cytosine, but Not Adenine, Base Editors Induce Genome-Wide off-Target Mutations in Rice». *Science (New York, N.Y.)* 364 (6437): 292–95. https://doi.org/10.1126/science.aaw7166.

Jinek, Martin, Krzysztof Chylinski, Ines Fonfara, Michael Hauer, Jennifer A. Doudna, e Emmanuelle Charpentier. 2012. «A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity». *Science* 337 (6096): 816–21. https://doi.org/10.1126/science.1225829.

Johnson, Douglas B., Justin M. Balko, Margaret L. Compton, Spyridon Chalkias, Joshua Gorham, Yaomin Xu, Mellissa Hicks, et al. 2016. «Fulminant Myocarditis with Combination Immune Checkpoint Blockade». *The New England Journal of Medicine* 375 (18): 1749–55. https://doi.org/10.1056/NEJMoa1609214.

Johnson, Laura A., Richard A. Morgan, Mark E. Dudley, Lydie Cassard, James C. Yang, Marybeth S. Hughes, Udai S. Kammula, et al. 2009. «Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen». *Blood* 114 (3): 535–46. https://doi.org/10.1182/blood-2009-03-211714.

Johnston, Robert J., Laetitia Comps-Agrar, Jason Hackney, Xin Yu, Mahrukh Huseni, Yagai Yang, Summer Park, et al. 2014. «The Immunoreceptor TIGIT Regulates Antitumor and Antiviral CD8(+) T Cell Effector Function». *Cancer Cell* 26 (6): 923–37. https://doi.org/10.1016/j.ccell.2014.10.018.

Joller, Nicole, Jason P. Hafler, Boel Brynedal, Nasim Kassam, Silvia Spoerl, Steven D. Levin, Arlene H. Sharpe, e Vijay K. Kuchroo. 2011. «Cutting Edge: TIGIT Has T Cell-Intrinsic Inhibitory Functions». *Journal of Immunology (Baltimore, Md.: 1950)* 186 (3): 1338–42. https://doi.org/10.4049/jimmunol.1003081.

Jung, Kyong Yeun, Sun Wook Cho, Young A. Kim, Daein Kim, Byung-Chul Oh, Do Joon Park, e Young Joo Park. 2015. «Cancers with Higher Density of Tumor-Associated Macrophages Were Associated with Poor Survival Rates». *Journal of Pathology and Translational Medicine* 49 (4): 318–24. https://doi.org/10.4132/jptm.2015.06.01.

Karamitopoulou, Eva. 2019. «Tumour Microenvironment of Pancreatic Cancer: Immune Landscape Is Dictated by Molecular and Histopathological Features». *British Journal of Cancer* 121 (1): 5–14. https://doi.org/10.1038/s41416-019-0479-5.

Kawakami, Y, S Eliyahu, C H Delgado, P F Robbins, L Rivoltini, S L Topalian, T Miki, e S A Rosenberg. 1994. «Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor.» *Proceedings of the National Academy of Sciences of the United States of America* 91 (9): 3515–19.

Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, K. Sakaguchi, E. Appella, J. R. Yannelli, G. J. Adema, T. Miki, e S. A. Rosenberg. 1994. «Identification of a Human Melanoma Antigen Recognized by Tumor-Infiltrating Lymphocytes Associated with in Vivo Tumor Rejection». *Proceedings of the National Academy of Sciences of the United States of America* 91 (14): 6458–62. https://doi.org/10.1073/pnas.91.14.6458.

Kim, Kyoungmi, Seuk-Min Ryu, Sang-Tae Kim, Gayoung Baek, Daesik Kim, Kayeong Lim, Eugene Chung, Sunghyun Kim, e Jin-Soo Kim. 2017. «Highly Efficient RNA-Guided Base Editing in Mouse Embryos». *Nature Biotechnology* 35 (5): 435–37. https://doi.org/10.1038/nbt.3816.

Kimura, Hirokazu, Ryota Sada, Naoki Takada, Akikazu Harada, Yuichiro Doki, Hidetoshi Eguchi, Hideki Yamamoto, e Akira Kikuchi. 2021. «The Dickkopf1 and FOXM1 Positive Feedback Loop Promotes Tumor Growth in Pancreatic and Esophageal Cancers». *Oncogene* 40 (26): 4486–4502. https://doi.org/10.1038/s41388-021-01860-z.

Kleeff, Jorg, Murray Korc, Minoti Apte, Carlo La Vecchia, Colin D. Johnson, Andrew V. Biankin, Rachel E. Neale, et al. 2016. «Pancreatic Cancer». *Nature Reviews. Disease Primers* 2 (aprile): 16022. https://doi.org/10.1038/nrdp.2016.22.

Klempner, Samuel, Jaffer Ajani, Joseph Chao, Hope Uronis, Cynthia Sirard, Michael Kagey, Jason Baum, et al. 2022. «553 DKN-01 and Tislelizumab as a Second-Line (2L) Investigational Therapy in Advanced DKK1 High Gastroesophageal Adenocarcinoma (GEA): DisTinGuish Trial». *Journal for ImmunoTherapy of Cancer* 10 (Suppl 2). https://doi.org/10.1136/jitc-2022-SITC2022.0553.

Klett, Hagen, Hannah Fuellgraf, Ella Levit-Zerdoun, Saskia Hussung, Silke Kowar, Simon Küsters, Peter Bronsert, et al. 2018. «Identification and Validation of a Diagnostic and Prognostic Multi-Gene Biomarker Panel for Pancreatic Ductal Adenocarcinoma». *Frontiers in Genetics* 9: 108. https://doi.org/10.3389/fgene.2018.00108.

Kluesner, Mitchell G., Derek A. Nedveck, Walker S. Lahr, John R. Garbe, Juan E. Abrahante, Beau R. Webber, e Branden S. Moriarity. 2018. «EditR: A Method to Quantify Base Editing from Sanger Sequencing». *The CRISPR Journal* 1 (3): 239–50. https://doi.org/10.1089/crispr.2018.0014.

Komor, Alexis C., Yongjoo B. Kim, Michael S. Packer, John A. Zuris, e David R. Liu. 2016. «Programmable Editing of a Target Base in Genomic DNA without Double-Stranded DNA Cleavage». *Nature* 533 (7603): 420–24. https://doi.org/10.1038/nature17946.

Kosicki, Michael, Kärt Tomberg, e Allan Bradley. 2018. «Repair of Double-Strand Breaks Induced by CRISPR–Cas9 Leads to Large Deletions and Complex Rearrangements». *Nature Biotechnology* 36 (8): 765–71. https://doi.org/10.1038/nbt.4192.

Kumar, Jitendra, Ritesh Kumar, Amir Kumar Singh, Elviche L. Tsakem, Mahesh Kathania, Matthew J. Riese, Arianne L. Theiss, Marco L. Davila, e K. Venuprasad. 2021. «Deletion of Cbl-b Inhibits CD8+ T-Cell Exhaustion and Promotes CAR T-Cell Function». *Journal for Immunotherapy of Cancer* 9 (1): e001688. https://doi.org/10.1136/jitc-2020-001688.

Labani-Motlagh, Alireza, Mehrnoush Ashja-Mahdavi, e Angelica Loskog. 2020. «The Tumor Microenvironment: A Milieu Hindering and Obstructing Antitumor Immune Responses». *Frontiers in Immunology* 11. https://www.frontiersin.org/articles/10.3389/fimmu.2020.00940.

Lahman, Miranda C., Thomas M. Schmitt, Kelly G. Paulson, Nathalie Vigneron, Denise Buenrostro, Felecia D. Wagener, Valentin Voillet, et al. 2022. «Targeting an Alternate Wilms' Tumor Antigen 1 Peptide Bypasses Immunoproteasome Dependency». *Science Translational Medicine* 14 (631): eabg8070. https://doi.org/10.1126/scitranslmed.abg8070.

Lakshmanan, I., S. Rachagani, R. Hauke, S. R. Krishn, S. Paknikar, P. Seshacharyulu, S. Karmakar, et al. 2016. «MUC5AC Interactions with Integrin B4 Enhances the Migration of Lung Cancer Cells through FAK Signaling». *Oncogene* 35 (31): 4112–21. https://doi.org/10.1038/onc.2015.478.

Lamers, Cor Hj, Stefan Sleijfer, Sabine van Steenbergen, Pascal van Elzakker, Brigitte van Krimpen, Corrien Groot, Arnold Vulto, et al. 2013. «Treatment of Metastatic Renal Cell Carcinoma with CAIX CAR-Engineered T Cells: Clinical Evaluation and Management of on-Target Toxicity». *Molecular Therapy: The Journal of the American Society of Gene Therapy* 21 (4): 904–12. https://doi.org/10.1038/mt.2013.17.

Lamers, Cor H.J., Yarne Klaver, Jan W. Gratama, Stefan Sleijfer, e Reno Debets. 2016. «Treatment of metastatic renal cell carcinoma (mRCC) with CAIX CAR-engineered T-cells–a completed study overview». *Biochemical Society Transactions* 44 (3): 951–59. https://doi.org/10.1042/BST20160037.

Lanitis, E., D. Dangaj, M. Irving, e G. Coukos. 2017. «Mechanisms Regulating T-Cell Infiltration and Activity in Solid Tumors». *Annals of Oncology* 28 (dicembre): xii18–32. https://doi.org/10.1093/annonc/mdx238.

Lee, Hye Kyung, Harold E. Smith, Chengyu Liu, Michaela Willi, e Lothar Hennighausen. 2020. «Cytosine Base Editor 4 but Not Adenine Base Editor Generates Off-Target Mutations in Mouse Embryos». *Communications Biology* 3 (1): 1–6. https://doi.org/10.1038/s42003-019-0745-3.

Lee, Hyun Ju, Cheol Yi Hong, Chun-Ji Jin, Mi-Hyun Kim, Youn-Kyung Lee, Thanh-Nhan Nguyen-Pham, Hyunah Lee, et al. 2012. «Identification of Novel HLA-A*0201-Restricted Epitopes from Anterior Gradient-2 as a Tumor-Associated Antigen against Colorectal Cancer». *Cellular & Molecular Immunology* 9 (2): 175–83. https://doi.org/10.1038/cmi.2011.52.

Lee, James C., Sadaf Mehdizadeh, Jennifer Smith, Arabella Young, Ilgiz A. Mufazalov, Cody T. Mowery, Adil Daud, e Jeffrey A. Bluestone. 2020. «Regulatory T cell control of systemic immunity and immunotherapy response in liver metastasis». *Science immunology* 5 (52): eaba0759. https://doi.org/10.1126/sciimmunol.aba0759.

Leibowitz, Mitchell L., Stamatis Papathanasiou, Phillip A. Doerfler, Logan J. Blaine, Lili Sun, Yu Yao, Cheng-Zhong Zhang, Mitchell J. Weiss, e David Pellman. 2021. «Chromothripsis as an On-Target Consequence of CRISPR-Cas9 Genome Editing». *Nature Genetics* 53 (6): 895–905. https://doi.org/10.1038/s41588-021-00838-7.

Levine, Andrew G., Aaron Arvey, Wei Jin, e Alexander Y. Rudensky. 2014. «Continuous Requirement for the TCR in Regulatory T Cell Function». *Nature Immunology* 15 (11): 1070–78. https://doi.org/10.1038/ni.3004.

Lévy, Camille, Fouzia Amirache, Caroline Costa, Cecilia Frecha, Claude P. Muller, Hasan Kweder, Robin Buckland, François-Loïc Cosset, e Els Verhoeyen. 2012. «Lentiviral Vectors Displaying Modified Measles Virus Gp Overcome Pre-Existing Immunity in in Vivo-like Transduction of Human T and B Cells». *Molecular Therapy: The Journal of the American Society of Gene Therapy* 20 (9): 1699–1712. https://doi.org/10.1038/mt.2012.96.

Li, Shiqi, Xinxin Wang, Zhongtao Yuan, Lin Liu, Le Luo, Yu Li, Kun Wu, et al. 2021. «Eradication of T-ALL Cells by CD7-Targeted Universal CAR-T Cells and Initial Test of Ruxolitinib-Based CRS Management». *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 27 (5): 1242–46. https://doi.org/10.1158/1078-0432.CCR-20-1271.

Liang, Rongpu, Xudong Zhu, Tianyun Lan, Dongbing Ding, Zongheng Zheng, Tufeng Chen, Yong Huang, et al. 2021. «TIGIT Promotes CD8+T Cells Exhaustion and Predicts Poor Prognosis of Colorectal Cancer». *Cancer Immunology, Immunotherapy* 70 (10): 2781–93. https://doi.org/10.1007/s00262-021-02886-8.

Lin, Wei, Pawan Noel, Erkut H. Borazanci, Jeeyun Lee, Albert Amini, In Woong Han, Jin Seok Heo, et al. 2020. «Single-cell transcriptome analysis of tumor and stromal compartments of pancreatic ductal adenocarcinoma primary tumors and metastatic lesions». *Genome Medicine* 12 (1): 80. https://doi.org/10.1186/s13073-020-00776-9.

Linette, Gerald P., Edward A. Stadtmauer, Marcela V. Maus, Aaron P. Rapoport, Bruce L. Levine, Lyndsey Emery, Leslie Litzky, et al. 2013. «Cardiovascular Toxicity and Titin Cross-Reactivity of Affinity-Enhanced T Cells in Myeloma and Melanoma». *Blood* 122 (6): 863–71. https://doi.org/10.1182/blood-2013-03-490565.

Liu, De-Jun, Yue-Xia Xie, Xiao-Xing Liu, Yan-Miao Huo, Min-Wei Yang, Xue-Liang Fu, Wei Liu, et al. 2017. «The Role of Dickkopf-1 as a Potential Prognostic Marker in Pancreatic Ductal Adenocarcinoma». *Cell Cycle (Georgetown, Tex.)* 16 (17): 1622–29. https://doi.org/10.1080/15384101.2017.1356510.

Liu, Lu, Aihong Wang, Xiaoli Liu, Sai Han, Yu Sun, Junhua Zhang, Lingyu Guo, e Youzhong Zhang. 2022. «Blocking TIGIT/CD155 signalling reverses CD8+ T cell exhaustion and enhances the antitumor activity in cervical cancer». *Journal of Translational Medicine* 20 (1): 280. https://doi.org/10.1186/s12967-022-03480-x.

Liu, S., H. Zhang, M. Li, D. Hu, C. Li, B. Ge, B. Jin, e Z. Fan. 2013. «Recruitment of Grb2 and SHIP1 by the ITT-like Motif of TIGIT Suppresses Granule Polarization and Cytotoxicity of NK Cells». *Cell Death and Differentiation* 20 (3): 456–64. https://doi.org/10.1038/cdd.2012.141.

Lo, Winifred, Maria Parkhurst, Paul F. Robbins, Eric Tran, Yong-Chen Lu, Li Jia, Jared J. Gartner, et al. 2019. «Immunologic Recognition of a Shared P53 Mutated Neoantigen in a Patient with Metastatic Colorectal Cancer». *Cancer Immunology Research* 7 (4): 534–43. https://doi.org/10.1158/2326-6066.CIR-18-0686.

Loenen, Marleen M. van, Renate de Boer, Avital L. Amir, Renate S. Hagedoorn, Gerdien L. Volbeda, Roelof Willemze, Johannes J. van Rood, J. H. Frederik Falkenburg, e Mirjam H. M. Heemskerk. 2010. «Mixed T Cell Receptor Dimers Harbor Potentially Harmful Neoreactivity». *Proceedings of the National Academy of Sciences of the United States of America* 107 (24): 10972–77. https://doi.org/10.1073/pnas.1005802107.

Louis, Chrystal U., Barbara Savoldo, Gianpietro Dotti, Martin Pule, Eric Yvon, G. Doug Myers, Claudia Rossig, et al. 2011. «Antitumor Activity and Long-Term Fate of Chimeric Antigen Receptor-Positive T Cells in Patients with Neuroblastoma». *Blood* 118 (23): 6050–56. https://doi.org/10.1182/blood-2011-05-354449.

Lowe, Anson W., Mari Olsen, Ying Hao, Sum P. Lee, Kyu Taek Lee, Xin Chen, Matt van de Rijn, e Patrick O. Brown. 2007. «Gene Expression Patterns in Pancreatic Tumors, Cells and Tissues». *PLOS ONE* 2 (3): e323. https://doi.org/10.1371/journal.pone.0000323.

Lu, Peihua, Ying Liu, Junfang Yang, Xian Zhang, Xiao Yang, Hui Wang, Lin Wang, et al. 2022. «Naturally Selected CD7 CAR-T Therapy without Genetic

Manipulations for T-ALL/LBL: First-in-Human Phase 1 Clinical Trial». *Blood* 140 (4): 321–34. https://doi.org/10.1182/blood.2021014498.
Mahe, Etienne, Tevor Pugh, e Suzanne Kamel-Reid. 2018. «T Cell Clonality Assessment: Past, Present and Future». *Journal of Clinical Pathology* 71 (3): 195–200. https://doi.org/10.1136/jclinpath-2017-204761.
Maher, John, Renier J. Brentjens, Gertrude Gunset, Isabelle Rivière, e Michel Sadelain. 2002. «Human T-Lymphocyte Cytotoxicity and Proliferation Directed by a Single Chimeric TCRzeta /CD28 Receptor». *Nature Biotechnology* 20 (1): 70–75. https://doi.org/10.1038/nbt0102-70.
Majzner, Robbie G., e Crystal L. Mackall. 2018. «Tumor Antigen Escape from CAR T-Cell Therapy». *Cancer Discovery* 8 (10): 1219–26. https://doi.org/10.1158/2159-8290.CD-18-0442.
Majzner, Robbie G., Skyler P. Rietberg, Elena Sotillo, Rui Dong, Vipul T. Vachharajani, Louai Labanieh, June H. Myklebust, et al. 2020. «Tuning the Antigen Density Requirement for CAR T-Cell Activity». *Cancer Discovery* 10 (5): 702–23. https://doi.org/10.1158/2159-8290.CD-19-0945.
Mamonkin, Maksim, Rayne H. Rouce, Haruko Tashiro, e Malcolm K. Brenner. 2015. «A T-Cell-Directed Chimeric Antigen Receptor for the Selective Treatment of T-Cell Malignancies». *Blood* 126 (8): 983–92. https://doi.org/10.1182/blood-2015-02-629527.
Manfredi, Francesco, Beatrice Claudia Cianciotti, Alessia Potenza, Elena Tassi, Maddalena Noviello, Andrea Biondi, Fabio Ciceri, Chiara Bonini, e Eliana Ruggiero. 2020. «TCR Redirected T Cells for Cancer Treatment: Achievements, Hurdles, and Goals». *Frontiers in Immunology* 11: 1689. https://doi.org/10.3389/fimmu.2020.01689.
Manieri, Nicholas A., Eugene Y. Chiang, e Jane L. Grogan. 2017. «TIGIT: A Key Inhibitor of the Cancer Immunity Cycle». *Trends in Immunology* 38 (1): 20–28. https://doi.org/10.1016/j.it.2016.10.002.
Manne, Ashish, Ashwini Esnakula, Laith Abushahin, e Allan Tsung. 2021. «Understanding the Clinical Impact of MUC5AC Expression on Pancreatic Ductal Adenocarcinoma». *Cancers* 13 (12): 3059. https://doi.org/10.3390/cancers13123059.
Mansilla-Soto, Jorge, Justin Eyquem, Sascha Haubner, Mohamad Hamieh, Judith Feucht, Noémie Paillon, Andrés Ernesto Zucchetti, et al. 2022. «HLA-Independent T Cell Receptors for Targeting Tumors with Low Antigen Density». *Nature Medicine* 28 (2): 345–52. https://doi.org/10.1038/s41591-021-01621-1.
Mantovani, Alberto, Federica Marchesi, Alberto Malesci, Luigi Laghi, e Paola Allavena. 2017. «Tumour-Associated Macrophages as Treatment Targets in Oncology». *Nature Reviews. Clinical Oncology* 14 (7): 399–416. https://doi.org/10.1038/nrclinonc.2016.217.
Mao, Yixiang, Jianjun Shen, Yue Lu, Kevin Lin, Huamin Wang, Yanan Li, Ping Chang, Mary G. Walker, e Donghui Li. 2017. «RNA sequencing analyses reveal novel differentially expressed genes and pathways in pancreatic cancer». *Oncotarget* 8 (26): 42537–47. https://doi.org/10.18632/oncotarget.16451.
Mastaglio, Sara, Pietro Genovese, Zulma Magnani, Eliana Ruggiero, Elisa Landoni, Barbara Camisa, Giulia Schiroli, et al. 2017. «NY-ESO-1 TCR Single Edited Stem and Central Memory T Cells to Treat Multiple Myeloma without Graft-versus-Host Disease». *Blood* 130 (5): 606–18. https://doi.org/10.1182/blood-2016-08-732636.
Maurer, Carlo, Sam R. Holmstrom, Jing He, Pasquale Laise, Tao Su, Aqeel Ahmed, Hanina Hibshoosh, et al. 2019. «Experimental Microdissection Enables Functional Harmonisation of Pancreatic Cancer Subtypes». *Gut* 68 (6): 1034–43. https://doi.org/10.1136/gutjnl-2018-317706.

Maus, Marcela V., Andrew R. Haas, Gregory L. Beatty, Steven M. Albelda, Bruce L. Levine, Xiaojun Liu, Yangbing Zhao, Michael Kalos, e Carl H. June. 2013. «T Cells Expressing Chimeric Antigen Receptors Can Cause Anaphylaxis in Humans». *Cancer Immunology Research* 1 (1): 26–31. https://doi.org/10.1158/2326-6066.CIR-13-0006.

McGuirk, Joseph, Carlos R. Bachier, Michael Russell Bishop, P. Joy Ho, Hemant S. Murthy, Michael J. Dickinson, Joseph E. Maakaron, et al. 2021. «A phase 1 dose escalation and cohort expansion study of the safety and efficacy of allogeneic CRISPR-Cas9–engineered T cells (CTX110) in patients (Pts) with relapsed or refractory (R/R) B-cell malignancies (CARBON).» *Journal of Clinical Oncology* 39 (15_suppl): TPS7570–TPS7570. https://doi.org/10.1200/JCO.2021.39.15_suppl.TPS7570.

Milella, Michele, Claudio Bassi, Ugo Boggi, Oronzo Brunetti, Alessandro Cavaliere, Stefano Crippa, Ferdinando De Vita, et al. 2022. «Evolving pancreatic cancer treatment: From diagnosis to healthcare management». *Critical Reviews in Oncology/Hematology* 169 (gennaio): 103571. https://doi.org/10.1016/j.critrevonc.2021.103571.

Moffitt, Richard A., Raoud Marayati, Elizabeth L. Flate, Keith E. Volmar, S. Gabriela Herrera Loeza, Katherine A. Hoadley, Naim U. Rashid, et al. 2015. «Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma». *Nature genetics* 47 (10): 1168–78. https://doi.org/10.1038/ng.3398.

Morgan, Doris Anne, Francis W. Ruscetti, e Robert Gallo. 1976. «Selective in Vitro Growth of T Lymphocytes from Normal Human Bone Marrows». *Science* 193 (4257): 1007–8. https://doi.org/10.1126/science.181845.

Morgan, Richard A., Nachimuthu Chinnasamy, Daniel Abate-Daga, Alena Gros, Paul F. Robbins, Zhili Zheng, Mark E. Dudley, et al. 2013. «Cancer Regression and Neurological Toxicity Following Anti-MAGE-A3 TCR Gene Therapy». *Journal of Immunotherapy (Hagerstown, Md.: 1997)* 36 (2): 133–51. https://doi.org/10.1097/CJI.0b013e3182829903.

Morgan, Richard A., Mark E. Dudley, John R. Wunderlich, Marybeth S. Hughes, James C. Yang, Richard M. Sherry, Richard E. Royal, et al. 2006. «Cancer Regression in Patients After Transfer of Genetically Engineered Lymphocytes». *Science (New York, N.Y.)* 314 (5796): 126–29. https://doi.org/10.1126/science.1129003.

Morgan, Richard A., Mark E. Dudley, Yik Y. L. Yu, Zhili Zheng, Paul F. Robbins, Marc R. Theoret, John R. Wunderlich, Marybeth S. Hughes, Nicholas P. Restifo, e Steven A. Rosenberg. 2003. «High Efficiency TCR Gene Transfer into Primary Human Lymphocytes Affords Avid Recognition of Melanoma Tumor Antigen Glycoprotein 100 and Does Not Alter the Recognition of Autologous Melanoma Antigens». *Journal of Immunology (Baltimore, Md.: 1950)* 171 (6): 3287–95. https://doi.org/10.4049/jimmunol.171.6.3287.

Morgan, Richard A, James C Yang, Mio Kitano, Mark E Dudley, Carolyn M Laurencot, e Steven A Rosenberg. 2010. «Case Report of a Serious Adverse Event Following the Administration of T Cells Transduced With a Chimeric Antigen Receptor Recognizing ERBB2». *Molecular Therapy* 18 (4): 843–51. https://doi.org/10.1038/mt.2010.24.

Morris, Emma C., Sattva S. Neelapu, Theodoros Giavridis, e Michel Sadelain. 2022. «Cytokine Release Syndrome and Associated Neurotoxicity in Cancer Immunotherapy». *Nature Reviews Immunology* 22 (2): 85–96. https://doi.org/10.1038/s41577-021-00547-6.

Mosmann, T. R., L. Li, e S. Sad. 1997. «Functions of CD8 T-Cell Subsets Secreting Different Cytokine Patterns». *Seminars in Immunology* 9 (2): 87–92. https://doi.org/10.1006/smim.1997.0065.

Müller, Sören, Susanne Raulefs, Philipp Bruns, Fabian Afonso-Grunz, Anne Plötner, Rolf Thermann, Carsten Jäger, et al. 2015. «Next-Generation Sequencing Reveals Novel Differentially Regulated mRNAs, lncRNAs, miRNAs, sdRNAs and a piRNA in Pancreatic Cancer». *Molecular Cancer* 14 (aprile): 94. https://doi.org/10.1186/s12943-015-0358-5.

Muul, L. M., P. J. Spiess, E. P. Director, e S. A. Rosenberg. 1987. «Identification of Specific Cytolytic Immune Responses against Autologous Tumor in Humans Bearing Malignant Melanoma». *Journal of Immunology (Baltimore, Md.: 1950)* 138 (3): 989–95.

Nagl, Laurenz, Lena Horvath, Andreas Pircher, e Dominik Wolf. 2020. «Tumor Endothelial Cells (TECs) as Potential Immune Directors of the Tumor Microenvironment – New Findings and Future Perspectives». *Frontiers in Cell and Developmental Biology* 8. https://www.frontiersin.org/articles/10.3389/fcell.2020.00766.

Noviello, Maddalena, Francesco Manfredi, Eliana Ruggiero, Tommaso Perini, Giacomo Oliveira, Filippo Cortesi, Pantaleo De Simone, et al. 2019. «Bone Marrow Central Memory and Memory Stem T-Cell Exhaustion in AML Patients Relapsing after HSCT». *Nature Communications* 10 (1): 1065. https://doi.org/10.1038/s41467-019-08871-1.

Obenauf, Anna C., e Joan Massagué. 2015. «Surviving at a distance: organ specific metastasis». *Trends in cancer* 1 (1): 76–91. https://doi.org/10.1016/j.trecan.2015.07.009.

Oliveira, Giacomo, Eliana Ruggiero, Maria Teresa Lupo Stanghellini, Nicoletta Cieri, Mattia D'Agostino, Raffaele Fronza, Christina Lulay, et al. 2015. «Tracking Genetically Engineered Lymphocytes Long-Term Reveals the Dynamics of T Cell Immunological Memory». *Science Translational Medicine* 7 (317): 317ra198. https://doi.org/10.1126/scitranslmed.aac8265.

Oliveira, Giacomo, Kari Stromhaug, Susan Klaeger, Tomasz Kula, Dennie T. Frederick, Phuong M. Le, Juliet Forman, et al. 2021. «Phenotype, Specificity and Avidity of Antitumour CD8+ T Cells in Melanoma». *Nature* 596 (7870): 119–25. https://doi.org/10.1038/s41586-021-03704-y.

O'Rourke, Donald M., MacLean P. Nasrallah, Arati Desai, Jan J. Melenhorst, Keith Mansfield, Jennifer J. D. Morrissette, Maria Martinez-Lage, et al. 2017. «A Single Dose of Peripherally Infused EGFRvIII-Directed CAR T Cells Mediates Antigen Loss and Induces Adaptive Resistance in Patients with Recurrent Glioblastoma». *Science Translational Medicine* 9 (399): eaaa0984. https://doi.org/10.1126/scitranslmed.aaa0984.

Ostuni, Renato, Franz Kratochvill, Peter J. Murray, e Gioacchino Natoli. 2015. «Macrophages and Cancer: From Mechanisms to Therapeutic Implications». *Trends in Immunology* 36 (4): 229–39. https://doi.org/10.1016/j.it.2015.02.004.

Ottaviano, Giorgio, Christos Georgiadis, Soragia Athina Gkazi, Farhatullah Syed, Hong Zhan, Annie Etuk, Roland Preece, et al. 2022. «Phase 1 Clinical Trial of CRISPR-Engineered CAR19 Universal T Cells for Treatment of Children with Refractory B Cell Leukemia». *Science Translational Medicine* 14 (668): eabq3010. https://doi.org/10.1126/scitranslmed.abq3010.

Overman, Michael J., Sara Lonardi, Ka Yeung Mark Wong, Heinz-Josef Lenz, Fabio Gelsomino, Massimo Aglietta, Michael A. Morse, et al. 2018. «Durable Clinical Benefit With Nivolumab Plus Ipilimumab in DNA Mismatch Repair-Deficient/Microsatellite Instability-High Metastatic Colorectal Cancer». *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 36 (8): 773–79. https://doi.org/10.1200/JCO.2017.76.9901.

Overman, Michael J., Ray McDermott, Joseph L. Leach, Sara Lonardi, Heinz-Josef Lenz, Michael A. Morse, Jayesh Desai, et al. 2017. «Nivolumab in Patients with Metastatic DNA Mismatch Repair-Deficient or Microsatellite Instability-High

Colorectal Cancer (CheckMate 142): An Open-Label, Multicentre, Phase 2 Study». *The Lancet. Oncology* 18 (9): 1182–91. https://doi.org/10.1016/S1470-2045(17)30422-9.

Park, Wungki, Akhil Chawla, e Eileen M. O'Reilly. 2021. «Pancreatic Cancer: A Review». *JAMA* 326 (9): 851–62. https://doi.org/10.1001/jama.2021.13027.

Parkhurst, Maria R., James C. Yang, Russell C. Langan, Mark E. Dudley, Debbie-Ann N. Nathan, Steven A. Feldman, Jeremy L. Davis, et al. 2011. «T Cells Targeting Carcinoembryonic Antigen Can Mediate Regression of Metastatic Colorectal Cancer but Induce Severe Transient Colitis». *Molecular Therapy: The Journal of the American Society of Gene Therapy* 19 (3): 620–26. https://doi.org/10.1038/mt.2010.272.

Paulson, K. G., V. Voillet, M. S. McAfee, D. S. Hunter, F. D. Wagener, M. Perdicchio, W. J. Valente, et al. 2018. «Acquired Cancer Resistance to Combination Immunotherapy from Transcriptional Loss of Class I HLA». *Nature Communications* 9 (1): 3868. https://doi.org/10.1038/s41467-018-06300-3.

Peng, Junya, Bao-Fa Sun, Chuan-Yuan Chen, Jia-Yi Zhou, Yu-Sheng Chen, Hao Chen, Lulu Liu, et al. 2019. «Single-Cell RNA-Seq Highlights Intra-Tumoral Heterogeneity and Malignant Progression in Pancreatic Ductal Adenocarcinoma». *Cell Research* 29 (9): 725–38. https://doi.org/10.1038/s41422-019-0195-y.

Poirot, Laurent, Brian Philip, Cécile Schiffer-Mannioui, Diane Le Clerre, Isabelle Chion-Sotinel, Sophie Derniame, Pierrick Potrel, et al. 2015. «Multiplex Genome-Edited T-cell Manufacturing Platform for "Off-the-Shelf" Adoptive T-cell Immunotherapies». *Cancer Research* 75 (18): 3853–64. https://doi.org/10.1158/0008-5472.CAN-14-3321.

Potenza, Alessia, Chiara Balestrieri, Martina Spiga, Luca Albarello, Federica Pedica, Francesco Manfredi, Beatrice Claudia Cianciotti, et al. 2023. «Revealing and Harnessing CD39 for the Treatment of Colorectal Cancer and Liver Metastases by Engineered T Cells». *Gut*, giugno, gutjnl-2022-328042. https://doi.org/10.1136/gutjnl-2022-328042.

Preece, Roland, Andrea Pavesi, Soragia Athina Gkazi, Kerstin A. Stegmann, Christos Georgiadis, Zhi Ming Tan, Jia Ying Joey Aw, Mala K. Maini, Antonio Bertoletti, e Waseem Qasim. 2020. «CRISPR-Mediated Base Conversion Allows Discriminatory Depletion of Endogenous T Cell Receptors for Enhanced Synthetic Immunity». *Molecular Therapy. Methods & Clinical Development* 19 (dicembre): 149–61. https://doi.org/10.1016/j.omtm.2020.09.002.

Prinzing, Brooke, Caitlin C. Zebley, Christopher T. Petersen, Yiping Fan, Alejandro Allo Anido, Zhongzhen Yi, Phuong Nguyen, et al. 2021. «Deleting DNMT3A in CAR T cells prevents exhaustion and enhances antitumor activity». *Science Translational Medicine* 13 (620): eabh0272. https://doi.org/10.1126/scitranslmed.abh0272.

Provasi, Elena, Pietro Genovese, Angelo Lombardo, Zulma Magnani, Pei-Qi Liu, Andreas Reik, Victoria Chu, et al. 2012. «Editing T Cell Specificity towards Leukemia by Zinc Finger Nucleases and Lentiviral Gene Transfer». *Nature Medicine* 18 (5): 807–15. https://doi.org/10.1038/nm.2700.

Qasim, Waseem, Hong Zhan, Sujith Samarasinghe, Stuart Adams, Persis Amrolia, Sian Stafford, Katie Butler, et al. 2017. «Molecular Remission of Infant B-ALL after Infusion of Universal TALEN Gene-Edited CAR T Cells». *Science Translational Medicine* 9 (374): eaaj2013. https://doi.org/10.1126/scitranslmed.aaj2013.

Qi, Lina, Jiani Chen, Biting Zhou, Kailun Xu, Kailai Wang, Zhihao Fang, Yingkuan Shao, Ying Yuan, Shu Zheng, e Wangxiong Hu. 2021. «HomeoboxC6 Promotes Metastasis by Orchestrating the DKK1/Wnt/β-Catenin Axis in Right-Sided Colon Cancer». *Cell Death & Disease* 12 (4): 1–13. https://doi.org/10.1038/s41419-021-03630-x.

Qian, Jianfei, Yuhuan Zheng, Chengyun Zheng, Lijuan Wang, Hong Qin, Sungyoul Hong, Haiyan Li, et al. 2012. «Active vaccination with Dickkopf-1 induces protective and therapeutic antitumor immunity in murine multiple myeloma». *Blood* 119 (1): 161–69. https://doi.org/10.1182/blood-2011-07-368472.

Rafiq, Sarwish, Oladapo O. Yeku, Hollie J. Jackson, Terence J. Purdon, Dayenne G. van Leeuwen, Dylan J. Drakes, Mei Song, et al. 2018. «Targeted Delivery of a PD-1-Blocking scFv by CAR-T Cells Enhances Anti-Tumor Efficacy in Vivo». *Nature Biotechnology* 36 (9): 847–56. https://doi.org/10.1038/nbt.4195.

Rasaiyaah, Jane, Christos Georgiadis, Roland Preece, Ulrike Mock, e Waseem Qasim. 2018. «TCRαβ/CD3 Disruption Enables CD3-Specific Antileukemic T Cell Immunotherapy». *JCI Insight* 3 (13): e99442, 99442. https://doi.org/10.1172/jci.insight.99442.

Rees, Holly A., Alexis C. Komor, Wei-Hsi Yeh, Joana Caetano-Lopes, Matthew Warman, Albert S. B. Edge, e David R. Liu. 2017. «Improving the DNA Specificity and Applicability of Base Editing through Protein Engineering and Protein Delivery». *Nature Communications* 8 (1): 15790. https://doi.org/10.1038/ncomms15790.

Rees, Holly A., e David R. Liu. 2018. «Base editing: precision chemistry on the genome and transcriptome of living cells». *Nature reviews. Genetics* 19 (12): 770–88. https://doi.org/10.1038/s41576-018-0059-1.

Reeves, Emma, e Edward James. 2017. «Antigen Processing and Immune Regulation in the Response to Tumours». *Immunology* 150 (1): 16–24. https://doi.org/10.1111/imm.12675.

Ren, Jiangtao, Xiaojun Liu, Chongyun Fang, Shuguang Jiang, Carl H. June, e Yangbing Zhao. 2017. «Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition». *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 23 (9): 2255–66. https://doi.org/10.1158/1078-0432.CCR-16-1300.

Ribas, Antoni, e Jedd D. Wolchok. 2018. «Cancer Immunotherapy Using Checkpoint Blockade». *Science (New York, N.Y.)* 359 (6382): 1350–55. https://doi.org/10.1126/science.aar4060.

Robbins, Paul F., Richard A. Morgan, Steven A. Feldman, James C. Yang, Richard M. Sherry, Mark E. Dudley, John R. Wunderlich, et al. 2011. «Tumor Regression in Patients with Metastatic Synovial Cell Sarcoma and Melanoma Using Genetically Engineered Lymphocytes Reactive with NY-ESO-1». *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 29 (7): 917–24. https://doi.org/10.1200/JCO.2010.32.2537.

Rohatgi, Anjali, e John M. Kirkwood. 2021. «Beyond PD-1: The Next Frontier for Immunotherapy in Melanoma». *Frontiers in Oncology* 11. https://www.frontiersin.org/articles/10.3389/fonc.2021.640314.

Rosenberg, S. A., B. S. Packard, P. M. Aebersold, D. Solomon, S. L. Topalian, S. T. Toy, P. Simon, M. T. Lotze, J. C. Yang, e C. A. Seipp. 1988. «Use of Tumor-Infiltrating Lymphocytes and Interleukin-2 in the Immunotherapy of Patients with Metastatic Melanoma. A Preliminary Report». *The New England Journal of Medicine* 319 (25): 1676–80. https://doi.org/10.1056/NEJM198812223192527.

Rosenberg, S. A., P. Spiess, e R. Lafreniere. 1986. «A New Approach to the Adoptive Immunotherapy of Cancer with Tumor-Infiltrating Lymphocytes». *Science (New York, N.Y.)* 233 (4770): 1318–21. https://doi.org/10.1126/science.3489291.

Rosenberg, Steven A., Michael T. Lotze, Linda M. Muul, Susan Leitman, Alfred E. Chang, Stephen E. Ettinghausen, Yvedt L. Matory, et al. 1985. «Observations on the Systemic Administration of Autologous Lymphokine-Activated Killer Cells and Recombinant Interleukin-2 to Patients with Metastatic Cancer». *New*

England Journal of Medicine 313 (23): 1485–92. https://doi.org/10.1056/NEJM198512053132327.
Rousseau, A., C. Parisi, e F. Barlesi. 2023. «Anti-TIGIT Therapies for Solid Tumors: A Systematic Review». *ESMO Open* 8 (2): 101184. https://doi.org/10.1016/j.esmoop.2023.101184.
Royal, Richard E., Catherine Levy, Keli Turner, Aarti Mathur, Marybeth Hughes, Udai S. Kammula, Richard M. Sherry, et al. 2010. «Phase 2 Trial of Single Agent Ipilimumab (Anti-CTLA-4) for Locally Advanced or Metastatic Pancreatic Adenocarcinoma». *Journal of Immunotherapy (Hagerstown, Md.: 1997)* 33 (8): 828–33. https://doi.org/10.1097/CJI.0b013e3181eec14c.
Rucki, Agnieszka A., Qian Xiao, Stephen Muth, Jianlin Chen, Xu Che, Jennifer Kleponis, Rajni Sharma, Robert A. Anders, Elizabeth M. Jaffee, e Lei Zheng. 2017. «Dual Inhibition of Hedgehog and c-Met Pathways for Pancreatic Cancer Treatment». *Molecular Cancer Therapeutics* 16 (11): 2399–2409. https://doi.org/10.1158/1535-7163.MCT-16-0452.
Ruess, Dietrich A., Kivanc Görgülü, Sonja M. Wörmann, e Hana Algül. 2017. «Pharmacotherapeutic Management of Pancreatic Ductal Adenocarcinoma: Current and Emerging Concepts». *Drugs & Aging* 34 (5): 331–57. https://doi.org/10.1007/s40266-017-0453-y.
Ruggiero, Eliana, Erica Carnevale, Aaron Prodeus, Zulma Irene Magnani, Barbara Camisa, Ivan Merelli, Claudia Politano, et al. 2022. «CRISPR-Based Gene Disruption and Integration of High-Avidity, WT1-Specific T Cell Receptors Improve Antitumor T Cell Function». *Science Translational Medicine* 14 (631): eabg8027. https://doi.org/10.1126/scitranslmed.abg8027.
Ruggiero, Eliana, Jan P. Nicolay, Raffaele Fronza, Anne Arens, Anna Paruzynski, Ali Nowrouzi, Gökçe Ürenden, et al. 2015. «High-Resolution Analysis of the Human T-Cell Receptor Repertoire». *Nature Communications* 6 (settembre): 8081. https://doi.org/10.1038/ncomms9081.
Sahai, Erik, Igor Astsaturov, Edna Cukierman, David G. DeNardo, Mikala Egeblad, Ronald M. Evans, Douglas Fearon, et al. 2020. «A Framework for Advancing Our Understanding of Cancer-Associated Fibroblasts». *Nature Reviews Cancer* 20 (3): 174–86. https://doi.org/10.1038/s41568-019-0238-1.
Sahin, Ibrahim H., Harold Elias, Joanne F. Chou, Marinela Capanu, e Eileen M. O'Reilly. 2018. «Pancreatic adenocarcinoma: insights into patterns of recurrence and disease behavior». *BMC Cancer* 18 (1): 769. https://doi.org/10.1186/s12885-018-4679-9.
Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, e M. Toda. 1995. «Immunologic Self-Tolerance Maintained by Activated T Cells Expressing IL-2 Receptor Alpha-Chains (CD25). Breakdown of a Single Mechanism of Self-Tolerance Causes Various Autoimmune Diseases». *Journal of Immunology (Baltimore, Md.: 1950)* 155 (3): 1151–64.
Salter, Alexander I., Anusha Rajan, Jacob J. Kennedy, Richard G. Ivey, Sarah A. Shelby, Isabel Leung, Megan L. Templeton, et al. 2021. «Comparative Analysis of TCR and CAR Signaling Informs CAR Designs with Superior Antigen Sensitivity and in Vivo Function». *Science Signaling* 14 (697): eabe2606. https://doi.org/10.1126/scisignal.abe2606.
Scheikl-Gatard, Tanja, Caroline Tosch, François Lemonnier, e Ronald Rooke. 2017. «Identification of new MUC1 epitopes using HLA-transgenic animals: implication for immunomonitoring». *Journal of Translational Medicine* 15 (1): 154. https://doi.org/10.1186/s12967-017-1254-0.
Scheper, Wouter, Sander Kelderman, Lorenzo F. Fanchi, Carsten Linnemann, Gavin Bendle, Marije A. J. de Rooij, Christian Hirt, et al. 2019. «Low and Variable Tumor Reactivity of the Intratumoral TCR Repertoire in Human Cancers». *Nature Medicine* 25 (1): 89–94. https://doi.org/10.1038/s41591-018-0266-5.

Schiroli, Giulia, Anastasia Conti, Samuele Ferrari, Lucrezia Della Volpe, Aurelien Jacob, Luisa Albano, Stefano Beretta, et al. 2019. «Precise Gene Editing Preserves Hematopoietic Stem Cell Function Following Transient P53-Mediated DNA Damage Response». *Cell Stem Cell* 24 (4): 551-565.e8. https://doi.org/10.1016/j.stem.2019.02.019.

Schmitt, Thomas M., David H. Aggen, Ingunn M. Stromnes, Michelle L. Dossett, Sarah A. Richman, David M. Kranz, e Philip D. Greenberg. 2013. «Enhanced-Affinity Murine T-Cell Receptors for Tumor/Self-Antigens Can Be Safe in Gene Therapy despite Surpassing the Threshold for Thymic Selection». *Blood* 122 (3): 348–56. https://doi.org/10.1182/blood-2013-01-478164.

Schuster, Stephen J., Jakub Svoboda, Elise A. Chong, Sunita D. Nasta, Anthony R. Mato, Özlem Anak, Jennifer L. Brogdon, et al. 2017. «Chimeric Antigen Receptor T Cells in Refractory B-Cell Lymphomas». *The New England Journal of Medicine* 377 (26): 2545–54. https://doi.org/10.1056/NEJMoa1708566.

Shafer, Paul, Lauren M. Kelly, e Valentina Hoyos. 2022. «Cancer Therapy With TCR-Engineered T Cells: Current Strategies, Challenges, and Prospects». *Frontiers in Immunology* 13. https://www.frontiersin.org/articles/10.3389/fimmu.2022.835762.

Shah, Payal D., Alexander C. Huang, Xiaowei Xu, Robert Orlowski, Ravi K. Amaravadi, Lynn M. Schuchter, Paul Zhang, et al. 2023. «Phase I Trial of Autologous RNA-electroporated cMET-directed CAR T Cells Administered Intravenously in Patients with Melanoma and Breast Carcinoma». *Cancer Research Communications* 3 (5): 821–29. https://doi.org/10.1158/2767-9764.CRC-22-0486.

Shakiba, Mojdeh, Paul Zumbo, Gabriel Espinosa-Carrasco, Laura Menocal, Friederike Dündar, Sandra E. Carson, Emmanuel M. Bruno, et al. 2021. «TCR signal strength defines distinct mechanisms of T cell dysfunction and cancer evasion». *Journal of Experimental Medicine* 219 (2): e20201966. https://doi.org/10.1084/jem.20201966.

Sheridan, Cormac. 2017. «First Approval in Sight for Novartis' CAR-T Therapy after Panel Vote». *Nature Biotechnology* 35 (8): 691–93. https://doi.org/10.1038/nbt0817-691.

Shi, Donghua, Yaoping Shi, Ahmed O. Kaseb, Xingxing Qi, Yuan Zhang, Jiachang Chi, Qing Lu, et al. 2020. «Chimeric Antigen Receptor-Glypican-3 T-Cell Therapy for Advanced Hepatocellular Carcinoma: Results of Phase I Trials». *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 26 (15): 3979–89. https://doi.org/10.1158/1078-0432.CCR-19-3259.

Shibuya, A., D. Campbell, C. Hannum, H. Yssel, K. Franz-Bacon, T. McClanahan, T. Kitamura, et al. 1996. «DNAM-1, a Novel Adhesion Molecule Involved in the Cytolytic Function of T Lymphocytes». *Immunity* 4 (6): 573–81. https://doi.org/10.1016/s1074-7613(00)70060-4.

Siegel, Rebecca L., Kimberly D. Miller, Hannah E. Fuchs, e Ahmedin Jemal. 2022. «Cancer Statistics, 2022». *CA: A Cancer Journal for Clinicians* 72 (1): 7–33. https://doi.org/10.3322/caac.21708.

Simoni, Yannick, Etienne Becht, Michael Fehlings, Chiew Yee Loh, Si-Lin Koo, Karen Wei Weng Teng, Joe Poh Sheng Yeong, et al. 2018. «Bystander CD8+ T Cells Are Abundant and Phenotypically Distinct in Human Tumour Infiltrates». *Nature* 557 (7706): 575–79. https://doi.org/10.1038/s41586-018-0130-2.

Sohal, Davendra P. S., Field F. Willingham, Massimo Falconi, Kara L. Raphael, e Stefano Crippa. 2017. «Pancreatic Adenocarcinoma: Improving Prevention and Survivorship». *American Society of Clinical Oncology Educational Book*, fasc. 37 (maggio): 301–10. https://doi.org/10.1200/EDBK_175222.

Stadtmauer, Edward A., Joseph A. Fraietta, Megan M. Davis, Adam D. Cohen, Kristy L. Weber, Eric Lancaster, Patricia A. Mangan, et al. 2020. «CRISPR-

Engineered T Cells in Patients with Refractory Cancer». *Science (New York, N.Y.)* 367 (6481): eaba7365. https://doi.org/10.1126/science.aba7365.
Steele, Nina G., Eileen S. Carpenter, Samantha B. Kemp, Veerin R. Sirihorachai, Stephanie The, Lawrence Delrosario, Jenny Lazarus, et al. 2020. «Multimodal Mapping of the Tumor and Peripheral Blood Immune Landscape in Human Pancreatic Cancer». *Nature Cancer* 1 (11): 1097–1112. https://doi.org/10.1038/s43018-020-00121-4.
Sterner, Robert C., e Rosalie M. Sterner. 2021a. «CAR-T Cell Therapy: Current Limitations and Potential Strategies». *Blood Cancer Journal* 11 (4): 1–11. https://doi.org/10.1038/s41408-021-00459-7.
———. 2021b. «CAR-T Cell Therapy: Current Limitations and Potential Strategies». *Blood Cancer Journal* 11 (4): 1–11. https://doi.org/10.1038/s41408-021-00459-7.
Straub, Adrian, Simon Grassmann, Sebastian Jarosch, Lena Richter, Philipp Hilgendorf, Monika Hammel, Karolin I. Wagner, Veit R. Buchholz, Kilian Schober, e Dirk H. Busch. 2023. «Recruitment of Epitope-Specific T Cell Clones with a Low-Avidity Threshold Supports Efficacy against Mutational Escape upon Re-Infection». *Immunity* 56 (6): 1269-1284.e6. https://doi.org/10.1016/j.immuni.2023.04.010.
Stromnes, Ingunn M., Thomas M. Schmitt, Ayaka Hulbert, J. Scott Brockenbrough, Hieu Nguyen, Carlos Cuevas, Ashley M. Dotson, et al. 2015. «T cells engineered against a native antigen can surmount immunologic and physical barriers to treat pancreatic ductal adenocarcinoma». *Cancer cell* 28 (5): 638–52. https://doi.org/10.1016/j.ccell.2015.09.022.
Suarez, Eloah Rabello, De Kuan Chang, Jiusong Sun, Jianhua Sui, Gordon J. Freeman, Sabina Signoretti, Quan Zhu, e Wayne A. Marasco. 2016. «Chimeric Antigen Receptor T Cells Secreting Anti-PD-L1 Antibodies More Effectively Regress Renal Cell Carcinoma in a Humanized Mouse Model». *Oncotarget* 7 (23): 34341–55. https://doi.org/10.18632/oncotarget.9114.
Sung, Hyuna, Jacques Ferlay, Rebecca L. Siegel, Mathieu Laversanne, Isabelle Soerjomataram, Ahmedin Jemal, e Freddie Bray. 2021. «Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries». *CA: A Cancer Journal for Clinicians* 71 (3): 209–49. https://doi.org/10.3322/caac.21660.
Sykulev, Y., M. Joo, I. Vturina, T. J. Tsomides, e H. N. Eisen. 1996. «Evidence That a Single Peptide-MHC Complex on a Target Cell Can Elicit a Cytolytic T Cell Response». *Immunity* 4 (6): 565–71. https://doi.org/10.1016/s1074-7613(00)80483-5.
Tang, Yuchen, Zixiang Zhang, Yaocheng Tang, Xinyu Chen, e Jian Zhou. 2018. «Identification of Potential Target Genes in Pancreatic Ductal Adenocarcinoma by Bioinformatics Analysis». *Oncology Letters* 16 (2): 2453–61. https://doi.org/10.3892/ol.2018.8912.
Tian, Shao-bo, Kai-xiong Tao, Jia Hu, Zhi-bo Liu, Xue-liang Ding, Ya-nan Chu, Jin-yuan Cui, et al. 2017. «The Prognostic Value of AGR2 Expression in Solid Tumours: A Systematic Review and Meta-Analysis». *Scientific Reports* 7 (1): 15500. https://doi.org/10.1038/s41598-017-15757-z.
Togashi, Yosuke, Kohei Shitara, e Hiroyoshi Nishikawa. 2019. «Regulatory T Cells in Cancer Immunosuppression — Implications for Anticancer Therapy». *Nature Reviews Clinical Oncology* 16 (6): 356–71. https://doi.org/10.1038/s41571-019-0175-7.
Tran, Eric, Paul F. Robbins, Yong-Chen Lu, Todd D. Prickett, Jared J. Gartner, Li Jia, Anna Pasetto, et al. 2016. «T-Cell Transfer Therapy Targeting Mutant KRAS in Cancer». *The New England Journal of Medicine* 375 (23): 2255–62. https://doi.org/10.1056/NEJMoa1609279.

Tsuchida, Connor A., Nadav Brandes, Raymund Bueno, Marena Trinidad, Thomas Mazumder, Bingfei Yu, Byungjin Hwang, et al. 2023. «Mitigation of Chromosome Loss in Clinical CRISPR-Cas9-Engineered T Cells». *Cell* 186 (21): 4567-4582.e20. https://doi.org/10.1016/j.cell.2023.08.041.

Tsukagoshi, Mariko, Satoshi Wada, Seiko Hirono, Shintaro Yoshida, Erica Yada, Tetsuro Sasada, Ken Shirabe, Hiroyuki Kuwano, e Hiroki Yamaue. 2018. «Identification of a Novel HLA-A24-Restricted Cytotoxic T Lymphocyte Epitope Peptide Derived from Mesothelin in Pancreatic Cancer». *Oncotarget* 9 (59): 31448–58. https://doi.org/10.18632/oncotarget.25837.

Tunnacliffe, A, R Kefford, C Milstein, A Forster, e T H Rabbitts. 1985. «Sequence and Evolution of the Human T-Cell Antigen Receptor Beta-Chain Genes». *Proceedings of the National Academy of Sciences of the United States of America* 82 (15): 5068–72. https://doi.org/10.1073/pnas.82.15.5068.

Turchiano, Giandomenico, Geoffroy Andrieux, Julia Klermund, Georges Blattner, Valentina Pennucci, Melina El Gaz, Gianni Monaco, et al. 2021. «Quantitative Evaluation of Chromosomal Rearrangements in Gene-Edited Human Stem Cells by CAST-Seq». *Cell Stem Cell* 28 (6): 1136-1147.e5. https://doi.org/10.1016/j.stem.2021.02.002.

Valkenburg, Kenneth C., Amber E. de Groot, e Kenneth J. Pienta. 2018. «Targeting the Tumour Stroma to Improve Cancer Therapy». *Nature Reviews. Clinical Oncology* 15 (6): 366–81. https://doi.org/10.1038/s41571-018-0007-1.

Vlachogiannis, Georgios, Somaieh Hedayat, Alexandra Vatsiou, Yann Jamin, Javier Fernández-Mateos, Khurum Khan, Andrea Lampis, et al. 2018. «Patient-Derived Organoids Model Treatment Response of Metastatic Gastrointestinal Cancers». *Science (New York, N.Y.)* 359 (6378): 920. https://doi.org/10.1126/science.aao2774.

Wang, Xinjie, Zhiwei Liu, GuangLei Li, Lu Dang, Shisheng Huang, Lei He, Yu'e Ma, et al. 2020. «Efficient Gene Silencing by Adenine Base Editor-Mediated Start Codon Mutation». *Molecular Therapy* 28 (2): 431–40. https://doi.org/10.1016/j.ymthe.2019.11.022.

Watanabe, Keisuke, Seitaro Terakura, Anton C. Martens, Tom van Meerten, Susumu Uchiyama, Misa Imai, Reona Sakemura, et al. 2015. «Target Antigen Density Governs the Efficacy of Anti-CD20-CD28-CD3 ζ Chimeric Antigen Receptor-Modified Effector CD8+ T Cells». *Journal of Immunology (Baltimore, Md.: 1950)* 194 (3): 911–20. https://doi.org/10.4049/jimmunol.1402346.

Watanabe, Norihiro, Feiyan Mo, Rong Zheng, Royce Ma, Vanesa C. Bray, Dayenne G. van Leeuwen, Juntima Sritabal-Ramirez, et al. 2023. «Feasibility and Preclinical Efficacy of CD7-Unedited CD7 CAR T Cells for T Cell Malignancies». *Molecular Therapy: The Journal of the American Society of Gene Therapy* 31 (1): 24–34. https://doi.org/10.1016/j.ymthe.2022.09.003.

Webber, Beau R., Cara-lin Lonetree, Mitchell G. Kluesner, Matthew J. Johnson, Emily J. Pomeroy, Miechaleen D. Diers, Walker S. Lahr, et al. 2019a. «Highly Efficient Multiplex Human T Cell Engineering without Double-Strand Breaks Using Cas9 Base Editors». *Nature Communications* 10 (1): 5222. https://doi.org/10.1038/s41467-019-13007-6.

Webber, Beau R., Cara-Lin Lonetree, Mitchell G. Kluesner, Matthew J. Johnson, Emily J. Pomeroy, Miechaleen D. Diers, Walker S. Lahr, et al. 2019b. «Highly Efficient Multiplex Human T Cell Engineering without Double-Strand Breaks Using Cas9 Base Editors». *Nature Communications* 10 (1): 5222. https://doi.org/10.1038/s41467-019-13007-6.

Wellhausen, Nils, Ryan P. O'Connell, Stefanie Lesch, Nils W. Engel, Austin K. Rennels, Donna Gonzales, Friederike Herbst, et al. 2023. «Epitope Base Editing CD45 in Hematopoietic Cells Enables Universal Blood Cancer Immune Therapy». *Science Translational Medicine* 15 (714): eadi1145. https://doi.org/10.1126/scitranslmed.adi1145.

Wilde, Susanne, Daniel Sommermeyer, Bernhard Frankenberger, Matthias Schiemann, Slavoljub Milosevic, Stefani Spranger, Heike Pohla, Wolfgang Uckert, Dirk H. Busch, e Dolores J. Schendel. 2009. «Dendritic cells pulsed with RNA encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity». *Blood* 114 (10): 2131–39. https://doi.org/10.1182/blood-2009-03-209387.

Xie, Yuan-Hong, Ying-Xuan Chen, e Jing-Yuan Fang. 2020. «Comprehensive Review of Targeted Therapy for Colorectal Cancer». *Signal Transduction and Targeted Therapy* 5 (1): 1–30. https://doi.org/10.1038/s41392-020-0116-z.

Xu, Zhihong, Tony C. Y. Pang, Adele C. Liu, Srinivasa P. Pothula, Alpha Raj Mekapogu, Chamini J. Perera, Takashi Murakami, et al. 2020. «Targeting the HGF/c-MET Pathway in Advanced Pancreatic Cancer: A Key Element of Treatment That Limits Primary Tumour Growth and Eliminates Metastasis». *British Journal of Cancer* 122 (10): 1486–95. https://doi.org/10.1038/s41416-020-0782-1.

Ying, Zhitao, Xue F. Huang, Xiaoyu Xiang, Yanling Liu, Xi Kang, Yuqin Song, Xiaokai Guo, et al. 2019. «A Safe and Potent Anti-CD19 CAR T Cell Therapy». *Nature Medicine* 25 (6): 947–53. https://doi.org/10.1038/s41591-019-0421-7.

Yu, Jiali, Michael D. Green, Shasha Li, Yilun Sun, Sara N. Journey, Jae Eun Choi, Syed Monem Rizvi, et al. 2021. «Liver metastasis restrains immunotherapy efficacy via macrophage-mediated T cell elimination». *Nature medicine* 27 (1): 152–64. https://doi.org/10.1038/s41591-020-1131-x.

Yu, Xin, Kristin Harden, Lino C. Gonzalez, Michelle Francesco, Eugene Chiang, Bryan Irving, Irene Tom, et al. 2009. «The Surface Protein TIGIT Suppresses T Cell Activation by Promoting the Generation of Mature Immunoregulatory Dendritic Cells». *Nature Immunology* 10 (1): 48–57. https://doi.org/10.1038/ni.1674.

Yu, Yi, Thomas C. Leete, David A. Born, Lauren Young, Luis A. Barrera, Seung-Joo Lee, Holly A. Rees, Giuseppe Ciaramella, e Nicole M. Gaudelli. 2020. «Cytosine Base Editors with Minimized Unguided DNA and RNA Off-Target Events and High on-Target Activity». *Nature Communications* 11 (1): 2052. https://doi.org/10.1038/s41467-020-15887-5.

Zhang, Hongyan, Jiangyang Chi, Jia Hu, Tiantian Ji, Zhen Luo, Caihong Zhou, Lifeng Huang, et al. 2021. «Intracellular AGR2 Transduces PGE2 Stimuli to Promote Epithelial-Mesenchymal Transition and Metastasis of Colorectal Cancer». *Cancer Letters* 518 (ottobre): 180–95. https://doi.org/10.1016/j.canlet.2021.06.025.

Zhang, Qing, Jiacheng Bi, Xiaodong Zheng, Yongyan Chen, Hua Wang, Wenyong Wu, Zhengguang Wang, et al. 2018. «Blockade of the Checkpoint Receptor TIGIT Prevents NK Cell Exhaustion and Elicits Potent Anti-Tumor Immunity». *Nature Immunology* 19 (7): 723–32. https://doi.org/10.1038/s41590-018-0132-0.

Zhang, Qiong-wen, Lei Liu, Chang-yang Gong, Hua-shan Shi, Yun-hui Zeng, Xiao-ze Wang, Yu-wei Zhao, e Yu-quan Wei. 2012. «Prognostic Significance of Tumor-Associated Macrophages in Solid Tumor: A Meta-Analysis of the Literature». *PloS One* 7 (12): e50946. https://doi.org/10.1371/journal.pone.0050946.

Zhang, Yongping, Xingying Zhang, Chen Cheng, Wei Mu, Xiaojuan Liu, Na Li, Xiaofei Wei, Xiang Liu, Changqing Xia, e Haoyi Wang. 2017. «CRISPR-Cas9 Mediated LAG-3 Disruption in CAR-T Cells». *Frontiers of Medicine* 11 (4): 554–62. https://doi.org/10.1007/s11684-017-0543-6.

Zhang, Zhen, Shasha Liu, Bin Zhang, Liang Qiao, Yi Zhang, e Yi Zhang. 2020. «T Cell Dysfunction and Exhaustion in Cancer». *Frontiers in Cell and Developmental Biology* 8 (febbraio): 17. https://doi.org/10.3389/fcell.2020.00017.

Zhang, Zhiheng, Hongzhen Li, Yibin Deng, Kathleen Schuck, Susanne Raulefs, Nadja Maeritz, Yuanyuan Yu, et al. 2021. «AGR2-Dependent Nuclear Import of RNA Polymerase II Constitutes a Specific Target of Pancreatic Ductal

Adenocarcinoma in the Context of Wild-Type P53». *Gastroenterology* 161 (5): 1601-1614.e23. https://doi.org/10.1053/j.gastro.2021.07.030.
Zhao, Lan, Hongya Zhao, e Hong Yan. 2018. «Gene expression profiling of 1200 pancreatic ductal adenocarcinoma reveals novel subtypes». *BMC Cancer* 18 (1): 603. https://doi.org/10.1186/s12885-018-4546-8.
Zhao, Yangbing, Zhili Zheng, Paul F. Robbins, Hung T. Khong, Steven A. Rosenberg, e Richard A. Morgan. 2005. «Primary Human Lymphocytes Transduced with NY-ESO-1 Antigen-Specific TCR Genes Recognize and Kill Diverse Human Tumor Cell Lines». *Journal of Immunology (Baltimore, Md.: 1950)* 174 (7): 4415–23. https://doi.org/10.4049/jimmunol.174.7.4415.
Zhou, Changyang, Yidi Sun, Rui Yan, Yajing Liu, Erwei Zuo, Chan Gu, Linxiao Han, et al. 2019. «Off-Target RNA Mutation Induced by DNA Base Editing and Its Elimination by Mutagenesis». *Nature* 571 (7764): 275–78. https://doi.org/10.1038/s41586-019-1314-0.
Zhou, Hui, Zhongtao Liu, Yongxiang Wang, Xiaoyong Wen, Eric H. Amador, Liqin Yuan, Xin Ran, et al. 2022. «Colorectal Liver Metastasis: Molecular Mechanism and Interventional Therapy». *Signal Transduction and Targeted Therapy* 7 (1): 1–25. https://doi.org/10.1038/s41392-022-00922-2.

