Harnessing costimulatory domains for the structural design of transgenic T cell receptors to improve T cell-based immunotherapy

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Abstract

The use of T cell based immunotherapy has been growing over the years and a number of chimeric antigen receptor (CAR)-modified T cell therapies have been approved for clinical use. These therapies have shown impressive results for hematological cancers. However, going from hematological cancers into solid tumors has been hard. Therefore, better designs should be tested to achieve effective anti-tumor responses against solid tumors. The major reason for the failure of T cell based immunotherapy to clear solid tumors is their immunosuppressive tumor microenvironment (TME). In this review many promising ongoing designs to better T cell based immunotherapy to overcome the obstacles posed by the TME and previous designed therapies will be brought forward. Both CAR and TCR based designs will be discussed. Each with the aim to achieve superior efficacy against hematological and solid tumors.

Layman summary

T cells, also known as T lymphocytes, are a type of white blood cell that play a key role in the immune system's ability to fight off threats such as bacteria and viruses. However, danger can also come from our own body in form of cancer cells. When cancer cells develop in the body, they can evade detection by the immune system. They do this in multiple ways, such as creating physical barriers which makes it hard for immune cells to reach the cancer. Or they start giving of 'don't kill me' signals which confuse the immune cells and causes them to not identify the cancer cells as a threat. However, cancer cells will still express proteins related to them being cancers cells on their surface called antigens. These can be recognize and bound by T cells allowing them to overcome the barriers created by the cancer cells. Once the T cells bind to the cancer cells, they release chemical signals, which can kill the cancer cells directly or recruit other immune cells to help in the fight. T cell based immunotherapy uses the ability of T cells to attack the cancers cells by modifying them genetically to express proteins that can better target and kill the cancer cells. One of these special proteins is the chimeric antigen receptor (CAR), added to the T cells. These T cells then turn into CAR-T cells. This CAR is a special receptor able to recognize a cancer-protein (antigen) of a cancer cell. In addition, researchers can also add a cancer-specific TCRs (T cell receptors) to target and kill cancer cells. This is a protein able to do the same thing as a CAR but works slightly different. Both the CAR and the TCR are designed by researchers and therefore they can be changed. New designs for both CARs and TCRs are being developed to make them better at targeting and killing cancer cells, overcoming the barriers. These new designs include specific intracellular protein parts to better activate them, making the CAR-T cells and TCR-T cells more durable and long-lasting. This activation is called co-stimulation and can be achieved by adding additional signaling molecules to the CAR-T and TCR-T cells. These signaling molecules can be introduced in the CAR or TCRs or can be added as a separate

protein. Each of these designs has their own special feature such as stronger activation, more cell expansion , persisting longer in the body, or generating the ability to work in a hostile environment. Overall, these new designs are aimed at making T cell immunotherapy more effective and widely available to patients with cancer.

Introduction

The use of immune-based therapies to combat cancer has been increasing over the years[1,2]. A multitude of different therapies have been developed *e.g.* the administration of cytokines, checkpoint inhibitors or the infusion of cancer specific-antibodies[1]. One of the more recent developments is the use of genetically engineered T cell-based therapies. T lymphocytes are a key component of the adaptive immune system which express a diverse set of antigen-specific receptors. This enables them to target and kill a broad set of pathogenic entities including cancerous cells. With genetic tools, researchers can modify the T cells to express synthetic molecules to redirect T cell killing capability to specifically target tumor cells.

The two main molecules used to target cancer cells are transgenic αβ TCRs and CARs. The major difference between both types of receptors is major histocompatibility complex (MHC) dependence and the ability of TCRs to recognize both surface and intracellular proteins. Under normal circumstances T cells are activated by cells presenting antigens to the TCR via their MHC protein. These presented antigens can originate either from the cell-surface or from intracellular proteins. However, CARs are only able to recognize surface/extracellular proteins as no MHC protein is involved in the recognition process and an antibody-like binding domain is used. The TCRs used for immunotherapy are highly specific for known tumor-associated antigens which allow for reproducible targeting of specific tumors. An example is the αβTCR specific for HLA-A2-restricted cancer-testis antigen (Ag) NY-ESO-1[3]. In addition, promising results have been obtained with melanoma, multiple myeloma and viral-associated malignancies[4]. However, T cell activation is limited when engaging cancers cells due to evasion of immune surveillance[5]. This in turn causes TCR engagement at the tumor site to result in incomplete T cell activation and transient antitumor effects.

Several CARs designs, called generations, have been developed during the last years, (Fig1). A first-generation CAR is composed of the single-chain variable fragment (scFv) of an antibody fused to a transmembrane domain and a (signal 1) activating intracellular CD3ζ domain containing 3 immunoreceptor tyrosine-based activation motifs (ITAM)[6]. These ITAMs are involved in the initiation of a variety of signaling pathways involved in T cell activating and normally found in the TCR (Fig2A-C). The first generation of CAR-T cells were only able to target the tumor however because of the lack of an additional co-stimulatory signal 2 generating domain separate from the CD3ζ domain these T cells got exhausted fast[7]. This resulted in a lack of sustained pro-inflammatory signaling which reduced the anti-tumor function. Therefore, subsequent CAR designs had an additional costimulatory domain (signal 2) added from proteins such as CD28 or 4-1BB to generate a sustained anti-tumor response[8,9] . These CAR-T cells are known as the second generation. This method was found to work well for hematological based cancers such as CD19 expressing B-cell lymphomas[10] and the recently Ide-cell, an autologous B cell maturation antigen (BCMA) targeting CAR-T cells was approved to treat multiple myeloma[11]. However, going from liquid hematological based cancers to solid tumors has

been a major hurdle. Non-hematological solid tumors pose a unique challenge to treatment with engineered T cells because of the increased barriers provided by their associated tumor microenvironment (TME)[12]. Crucially, the TME suppress the ability of cellular therapies to function normally because solid tumors are often surrounded by physical barriers which limit tumor infiltration by T cells[5].In addition, the TME is highly immunosuppressive which causes T cell dysfunction and T cell exhaustion, an aspect which this review will mostly focus on. Examples of immunosuppressive molecules are Programmed cell death protein 1 (PD-1) ligands (PD-L1) and Transforming growth factor β (TGF-β)[13,14]. To overcome these intrinsic barriers of solid tumors T cell-based immunotherapies should be engineered in such a way that they either overcome the suppressive nature of the TME or that they can utilize it to improve T cell fitness. To surmount the TME and better T cell-based therapies, the activating costimulatory signal and pathways utilized by signal 2 are of interest and have proven to work. Signal 2 is mandatory to properly activate T cells otherwise the T cells will become anergic[15]. The orthodox signal 2 receptor complex is CD28-CD80/86 [16]. CD28-mediated stimulation causes the activation of NFAT, NF-kβ and AP-1 pathways. This then increases the expression of CD25 (IL2R-α) and multiple proinflammatory cytokines, including IL2, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF), through stabilization of mRNA. These cytokines provide further co-stimulation which leads to enhanced proliferation, survival, and effector function of T cells. This will potentiate the anti-tumor function of the T cells. In addition to CD28 other T cell-related co-stimulatory receptors are known such as 4-1BB[17] or OX40[18]. These receptors with their activating intracellular domains (ICD) and other costimulatory ICDs have therefore entered the spotlight to be evaluated in different designs for CAR or TCR-based T cell therapies.

Since the beginning of transgenic T cell-based immunotherapy different receptors and ICD designs have been investigated. Which have proven to enhance the first generation of CAR-T cell-based immunotherapy against hematological cancers. In addition, because of the recent focus on overcoming the solid tumor TME innovative designs for both TCR and CAR-T-based therapies using the considerable diversity in costimulatory domains have been investigated. This review will discuss different methods and approaches of improving co-stimulation for gene-engineered T cell-based therapies. Focusing on their effect on the four key features of T cell-based therapies, cytotoxic capability, cancer sensitivity, persistence of T cells and T cell fitness.

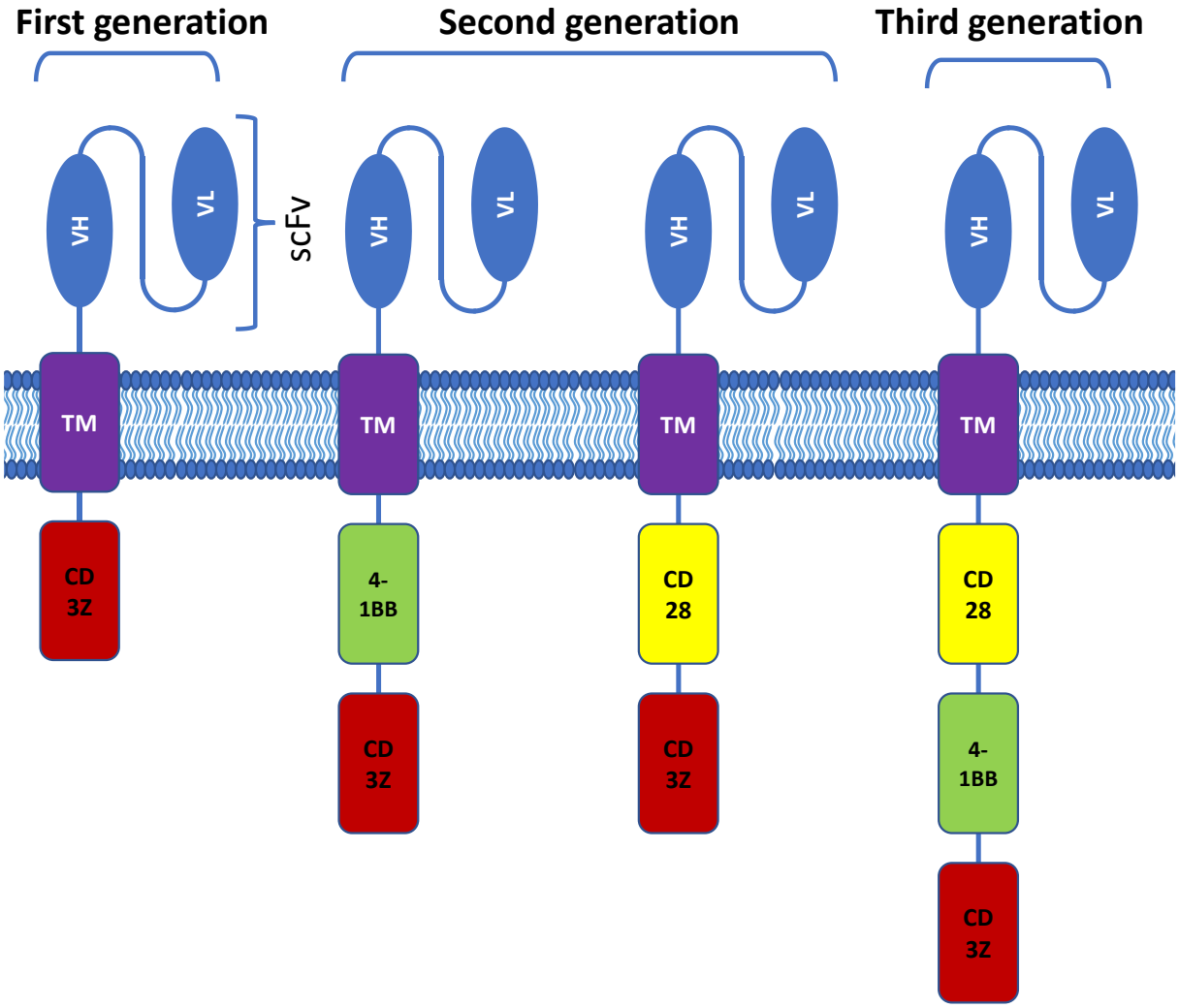

Figure1. *Schematic overview showing the difference in costimulatory domains of first, second and third generation CAR designs. Blue= single-chain variable fragment (scFv) with shown variable light (VL) and variable heavy (VH) chains, Purple= Transmembrane domain (TM), Red= CD3Z, Green = 4-1BB intracellular domain, Yellow= CD28 intracellular domain.*

Utilization of co-stimulation domains in *CARs*

As mentioned before the most common co-stimulation ICDs for CAR-T cells are CD28 and 4-1BB[19,20]. First-generation CARs could target cancer cells however lacked sustained anti-tumor function. After the addition of CD28 or 4-1BB ICDs the newly designed CARs were able to specifically lyse the tumor cells, with improved persistence and antitumor effect. Both the CD28 and 4-1BB ICD carrying CARs showed superior tumoricidal activity than first generation CARs. However, 4-1BB showed a decrease in susceptibility to exhaustion and better persistence than CD28-based CARs.[17] Building on this research, new designs were made utilizing both CD28 and 4-1BB in one CAR design[21]. Instead of using a full-length CD28 ICD, the design contains a full-length 4-1BB ICD with a truncated CD28 domain only containing the PYAP signaling motif. The results demonstrated that combining 4-1BB with an optimized form of CD28 increased the CAR-T cells effectiveness compared to a single co-stimulatory domain. The cells were less exhausted and improved antitumor activity with increased persistence. However, this paper only looked at Raji-CD19 tumors which is a model system used for hematological cancers thereby generating no data on solid-tumor performance. Another approach is to use a different domain combination than CD28 and 4-1BB. Instead of 4-1BB, OX40 was used together with CD28[22(p40)]. This combination showed a better response *in vivo* with an increase in persistence, *in vivo* expansion and proliferation compared to a CD28-4-1BB ICD combination. In addition, high production of IFN-γ, TNF-α and IL2 was measured. The CD28-OX40 showed higher survival in a rechallenge mice tumor xenograft model, with secondary T cell re-expansion. Furthermore, after 240+ days mouse tissues showed the presence of CAR-T cells in multiple organs. In addition, no apparent off-target cytotoxicity was observed. These results indicate that the double CD28-OX40 CAR has high potency for clinical translation. Expanding further on the use of different ICDs is a CAR design utilizing a truncated cytoplasmic tail of IL2 receptor B (IL2RB) and a STAT-3 binding YXXQ motif[23]. Under normal cytokine activation, the IL2RB generates STAT-3 activation and is involved in T cell proliferation, effector differentiation and memory formation. However, constitutive cytokine expression has been shown to pose a risk for serious adverse events such as cytokine release syndrome[24]. Therefore, this design tries to deliver cytokine related signals via antigen engagement. When comparing this design to a single CD28 or 4-1BB domain it showed superior in vivo persistence and antitumor effects for both hematological and solid tumor models. Most designs have looked only at T cell derived ICDs related to signal 2. However, when using a novel approach called CAR Pooling, investigators were able to identify B cell-activating factor (BAFF) receptor as having a superior intracellular signaling domain compared to 4-1BB or CD28[25]. By changing the 4-1BB ICD for the BAFF ICD the CAR-T cells gained enhanced cytotoxic activity against multiple myeloma.

As the BAFF paper shows other non T cell related ICDs are highly usable to potentiate T cell-based therapies[25]. One of these novel ICD is the toll/interleukin-1 receptor domain of Toll-like receptor 2 (TRL2)[26]. TLR2 is highly expressed on activated and memory T cells. Normally, TLR2 signaling is able increases T cell expansion and cytokine production, 2 key features for good anti-tumor function. Therefore, the researchers hypothesized that TLR2 signaling could potentiate the antitumor function of CAR-T cells. CARs specific for CD19 and mesothelin were generated. Different from other designs the TLR2 domain was introduced to the 3'end of the CD3ζ chain. Both CARs also carried a CD28 ICD after the scFv. When comparing CAR-T cells with (T2-CD19-CAR) and without the additional TLR2 domain it was found to enhance antitumor function for both leukemia and solid tumors. Further

experiments with xenografted B-ALL tumors in mice indicated that TLR2 carrying CARs induced a higher survival rate. When the TLR2 domain was added to mesothelin-specific CARs (T2-M-CAR) the same cytokine pattern and ratio effect were observed. Furthermore, T2-M-CAR-T cells showed a significantly stronger effect on reducing tumor weight compared non TLR2 carrying CARs. In addition, a clinical trial was performed on a patient with relapsed and refractory B-ALL. After a single dose of 5*10^4/kg of T2-CD19-CAR-T cells was administrated, the patient had a complete remission. The T2-CD19-CAR-T cells were also detected in the cerebrospinal fluid, indicating a potential role of T2-CD19-CAR-T cells in eradicating leukemia in the central nervous system. Overall, this design shows that other non-T cell/signal 2 specific together with a T cell specific ICD can be used to potentiate CARs. Both for leukemia and solid tumors. As can be observed from these papers, a lot of different designs show great potential to potentiate CAR-T cells.

CAR-TCR hybrid designs

CARs are synthetic receptors that allow for HLA-independent activation of T cells. However, they are only able to recognize external Ag. In addition to this, CARs expressing T cells are unable to eliminate tumors with low Ag levels[27]. CARs are however not equal in their antigen sensitivity and the above-mentioned methods are trying to enhance CARs. However, TCRs are highly sensitive and therefore could be used to create a T cell-based therapy that is sensitive at low Ag levels. In this line, new approaches that combine CAR targeting with TCR signaling have been described. One example are the HLA-independent T cell (HIT) receptors[28], in which the scFv from a CAR is incorporated into the TCR-CD3 complex using the *TRAC* locus editing with CRISPR/Cas9 [28] (Fig2A), combining a CAR-like recognition with the natural intracellular signaling capabilities of a TCR. Because of this, the receptor is more sensitive than a normal CAR with only a CD28 ICD . In addition, it is able reducing exhaustion caused by tonic signaling of CARs[28,29]. HIT receptor carrying cells were able to outperform CAR carrying cells in cytotoxic ability when targeting low CD19 expressing tumor cells *in vitro*, while they showed similar cytokine levels when the antigen expression was high. When assessed *in vivo* against the same tumor cells HIT-T cells performed better or matched CAR-T cells at higher CD19 levels. Therefore, the hypothesis was evaluated that providing costimulatory support to HIT-T cells would enable the latter to always outperform the CAR cells by extending their persistence, as the CAR had an advantage afforded by the incorporation of a costimulatory domain (CD28). Consistent with earlier findings, incorporation of both CD28 and 4-1BB ICD in the HIT receptor improved survival of mice treated with HIT-T cells when compared to CARs. Thus, T cells expressing the HIT receptor alone provided sensitive tumor recognition however needed CD28/4-1BB based co-stimulation to significantly prolong mouse survival. This opens new prospects for targeting tumors with low-density antigens while keeping strong cytotoxic capabilities.

A similar design was pioneered by Lin *et al*[30]. Their so-called synthetic T cell receptor and antigen receptor (STAR) incorporated again the antigen-recognition domain of an antibody and constant regions of TCR that engages endogenous CD3 signaling machinery. However, instead of performing *TRAC* locus editing with CRISPR/Cas9 they transduce via the lentiviral method. This leaves the endogenous TCR intact. Therefore the STAR uses murine TCR α/β constant regions to limit mispairing. One of the main benefits of the STAR is the usage of endogenous CD3 subunits which potentially has 10 ITAMs instead of the 3 ITAMs contributed by CD3ζ in a conventional CAR design. In solid xenografted mouse tumor models, STAR-T cells outperform or equal CAR-T cells in anti-tumor

efficacy. In addition, during Ag-free expansion, STAR did not trigger tonic signaling and activation-induced cell death or dysfunction which was observed with CARs. Furthermore, costimulatory signaling domains were fused to the intracellular tails of STAR. The OX40 ICD enhanced IL-2 production and proliferation while continuously stimulated with antigen resulting in a stronger and more sensitive response than CAR signaling. Like the before mentioned HIT receptor, STAR-T cells were responsive to cells with a broad range of antigen density which the CAR-T cells were not. This again boosted tumor-killing efficiency and lowers the risk of antigen loss induced resistance. In conclusion, Lin *et al* showed a multifunctional TCR based T cell therapy that demonstrates potent and lasting antitumor effects in multiple solid tumor models. However, further humanization of STAR is needed to minimize possible host-versus-graft disease risk.

CoCar stimulation for TCR based therapy

The previous papers have looked at αβTCRs which are the most common TCR. However, γδTCRs have been getting attention because of their MHC-unrestricted manner of differentiating between healthy and tumor cells, as γδTCRs recognize metabolic changes in cells via molecular patterns[31]. The most abundant γδT cell subset in human peripheral blood cells express the Vγ9Vδ2 TCR (hereafter Vδ2+ γδT cells) which recognizes increased phosphoantigen production on target cells. Multiple clinical trials have attempted to stimulate autologous Vδ2+ γδT cells directly however no substantial antitumoral activity was reported[31]. In addition, transfer of *ex vivo* expanded Vδ2+ γδT cells showed no effect. Therefore, to optimize Vδ2+ γδT cells for cancer, neuroblastoma, recognition and cytotoxicity a co-stimulatory CAR (coCar) specific for a neuroblastoma related Ag G2 was introduced into the Vδ2+ γδT cells (Fig2B)[32]. This generates a T cell which has specific recognition of target cells via its TCR and only gets a costimulatory signal upon encountering a cancer cell. The design goal is to limit off-target activation, limiting patient toxicity. Two different endodomains were assessed, CD28 and DAP10. DAP10 is normally involved in NKG2D signal transduction which is involved in the immunosurveillance of malignant cells[33]. When CD28 and DAP10 were compared the CD28 containing CAR was found to induce T cell activation without TCR engagement. Thereby generating both a signal 1 and 2 and tonic signaling. DAP10 was found to only induce activation when the TCR was engaged. This was tested using a murine G2 expressing cell which is unable to activate the γδTCR. The G2/DAP10 + Vγ9Vδ TCR combination provides more precise control than conventional CD3ζ containing CARs which can induce cytotoxicity in the presence of the target antigen alone. In addition, the design shows the tendency to lower T cell exhaustion due to reduced tonic CAR signaling. However, the study is limited by the fact that no xenografted assays were performed.

A similar design using a coCAR and an tumor-specific αβTCR was also investigated[34]. To overcome the lack of signal 2 which is known to be reduced in the TME[7]. The transgenic TCR was survivin-specific, a common tumor Ag[35]. In addition, a coCAR was transduced specific for CD19 with a CD28, 4-1BB or OX40+CD28 ICDs The CAR did not have the cytotoxic CD3ζ chain which is normally part of CARs (Fig2C). This design enables the T cells to be specific for the TCR epitope and in addition, receive the signal 2 when it needs to be activated, without the need for an antigen presenting cell (APC). From all coCAR designs, the OX40-CD28 ICDs combination (coCAR3) had the strongest INF-γ, *in vitro* tumor control and T cell expansion compared to non coCAR expressing and single domain coCAR expressing T cells. CoCAR3 T cells showed no off-target activation against a survivin knock-out cancer cell line. In addition, when coCAR3 co-cultured with heterogeneous CD19 expressing tumor cells, it was sensitive to all conditions. However, T cells showed lessened cytotoxicity with lowering CD19 levels. The coCAR3 + TCR carrying T cells were evaluated *in vivo* leukemia model yielding an improved median

survival rate of 35 days. Overall indicating that coCAR3 can increase the cytotoxicity, activity, and antitumor response of T cells. Adding coCARs seem to help TCR based T cell therapy overcome the TME suppressive nature. This is due to creating the ability to obtain signal 2 without the need for an APC which are known to be dysfunctional within the TME. This type of design offers promising features that can enhance T cell-based immunotherapy.

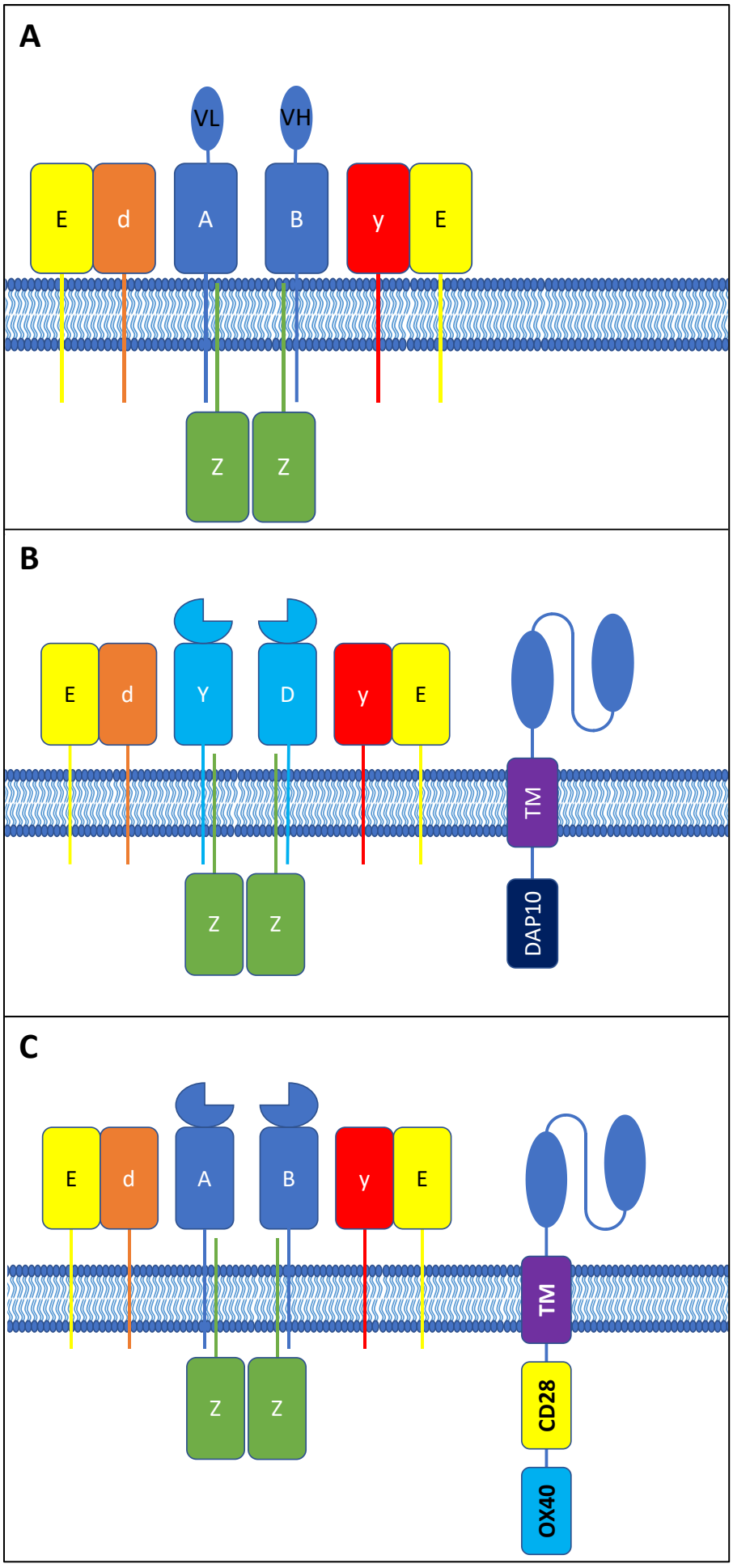

Figure2: *Schematic overview of different TCR based designs.* ***A*** *Shown is a HIT receptor. Instead of the variable alpha and beta chains the receptor contains Variable-Light (VL), and Heavy (VH) chains derived from scFv. TM= transmembrane domain.* ***B*** *Shown is a γδTCR together with a co-expressed G2 specific CAR carrying the DAP10 co-stimulatory motif.* ***C*** *Shown is a survivin specific αβTCR together with coCAR3. coCAR3 is CD19 specific and has CD28 and OX40 as co-stimulatory domains.*

Co-receptor enhancement of T cell function

In addition to optimizing the Ag recognizing receptor investigators have looked at producing a second non-CAR related signal . The goal of the second signal is to promote T cell fitness. T cell fitness is important to achieve strong antitumor responses[6]. When a T cell is recursively exposed to tumor cells it will eventually be exhausted and unable to fulfill its function. In addition, solid tumors generate the immunosuppressive TME which further negatively impacts T cell fitness[6]. To improve T cell fitness, investigators have focused on providing costimulatory signals which induce activation and increased anti-tumor function. One of the designs is using a receptor composed of ICOSL extracellular and transmembrane region fused with 4-1BB ICD (ICOSL-4-1BB) (Fig3A)[36]. Normally ICOS, a protein of the B7 superfamily, is expressed on the surface of T cells and involved in the activation of T cells. When co-expressing an ICOSL-4-1BB the T cells can activate the (CAR-)T cells in two manners. Firstly, the ICOSL is bound by the natural ICOS expressed on the surface of the (CAR-)T cell. This generates an agonistic effect on the other cell. In addition, when the ICOSL is bound by ICOS the internal 4-1BB domain is activated, generating a 4-1BB signal. Therefore, this receptor can enhance the activation of itself and neighboring (CAR-)T cells. Furthermore, tumor infiltrating lymphocytes (TILs) are also activated by ICOSL, enhancing their anti-tumor functionality. When administered against GPC3-positive solid tumors xenografted into mice, the ICOSL-4-1BB CAR-T cells prolonged mice survival in comparison to non-co-expressing CAR-T cells, suggesting that this kind of design has the potential to potentiate CAR-T cells and possibly naturally occurring TILs.

A different design was investigated for CAR-T cells targeting CD20. Instead of using a receptor that is activated, the design used a constitutively co-expressed full-length $OX40^{18(p40)}$. OX40 was chosen because of its enhanced proliferation the most compared to the other co-stimulatory receptors. The 20BBZ-OX40 CAR-T cells showed improved anti-tumor capability and less T cell exhaustion compared with the second generation CD20 based CAR-T cells that only include the 4-1BB costimulatory domain. In addition, the cells had better persistence and activity in mouse tumor models. When given to four patients with diffuse large B cell lymphoma, two experienced complete remission. Moreover, for three patients the CAR-T cell number in circulation at the peak increased more than 100-fold, indicating robust T cell proliferation of the original population. In addition, no severe side-effects were observed,. This pilot study shows that the constitutively co-expressed OX40 receptor is a viable design to potentiate CAR-T cells against OX40L expressing cancer cells. However, this approach remains untested against non-hematological solid tumors.

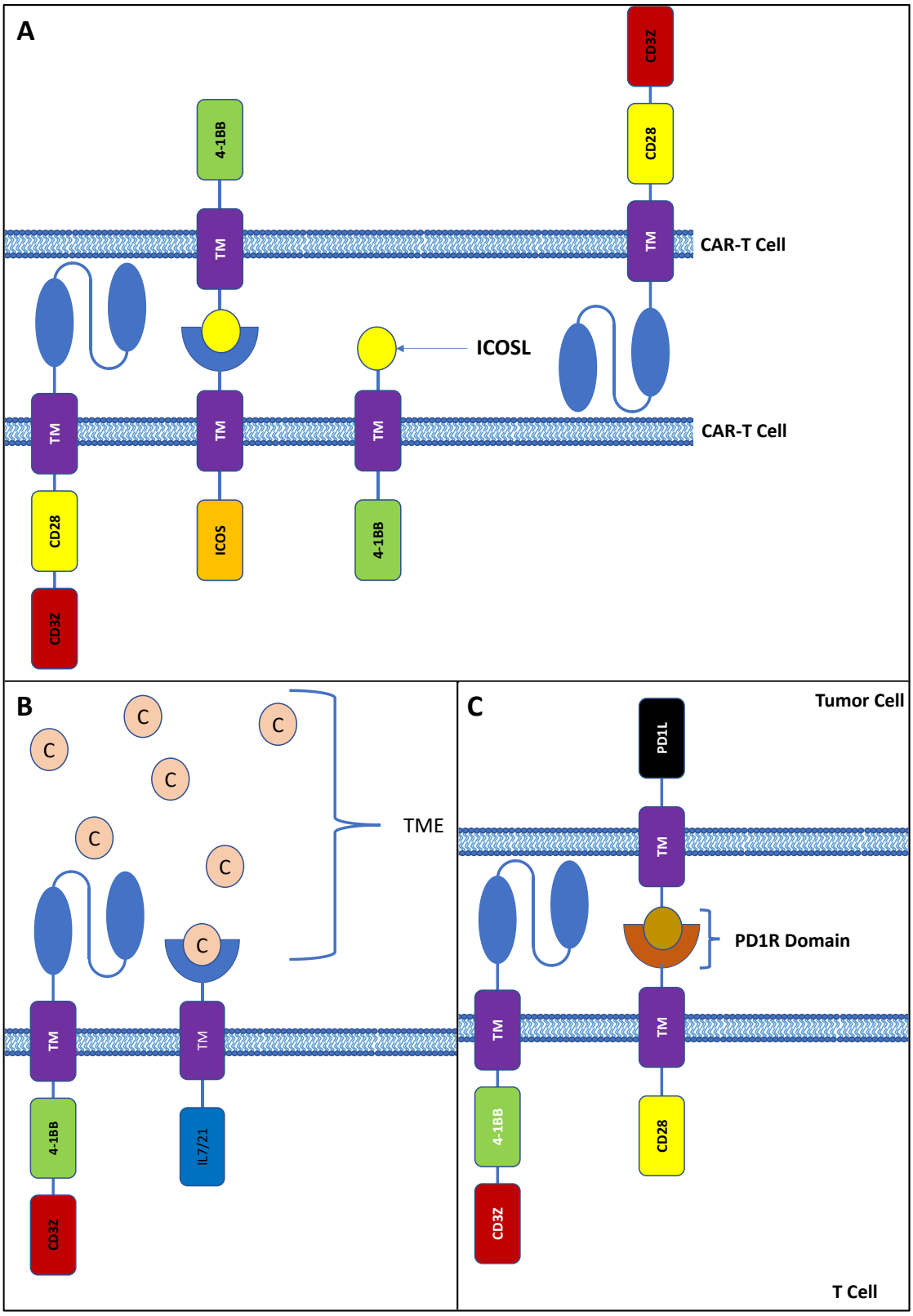

Figure3*: Schematic overview of three different co-stimulation designs.* ***A****. Shown are two ICOSL overexpressing CAR-T cells which can be activated by the ICOSL and can activate neighboring T cells.* ***B.*** *Shown is an inverted cytokine receptor which turns immunosuppressive signal (TGF-B) into an activating signal (IL7/IL21).* ***C.*** *Shown is a chimeric switch receptor that turns a PD-1L signal from a tumor cell into a CD28 costimulatory signal. TM= Transmembrane.*

Inverted/chimeric receptors

Separate from co-expression receptors are so-called inverted or chimeric receptors. These receptors are specifically designed to work in a T cell suppressive environment such as the TME of solid tumors. The principle behind this design is to change the inhibitory signal received into an activating signal. This is done by coupling the extracellular recognition domain of an inhibitory receptor to a pro-inflammatory intercellular domain. An example of this design is an inverted cytokine receptor (ICR)(Fig3B). CAR T cells were generated to target pancreatic cancer using a PSCA-specific CAR[37]. In addition to this CAR, an ICR specific for IL4 was co-expressed. Under normal circumstances, IL4 is an immunosuppressive cytokine and one of the strategies pancreatic tumors employ to limit the cytotoxic activity of tumor-targeting cells. By fusing an IL7 receptor (IL7R) endodomain to the IL4 receptor (IL4R) exodomain the normal immunosuppressive signal is inverted into a pro-inflammatory signal which promotes T cell proliferation [38]. This inverted receptor caused the CAR/ICR T cells to thrive in an IL4 rich TME resulting in enhanced anti-tumor activity. Furthermore, the CAR/ICR T cells remained both Ag and cytokine dependent for activation thereby limiting off-target activation. When the CAR/ICR T cells were compared to normal CAR-T cells they showed superior proliferation and tumor killing capacity in mice xenografted tumors. In addition to IL7, IL21 has been reported to promote T cell mediated tumor rejection[39]. Hence, researchers designed an ICR based on IL4R with an IL21 receptor endodomain[40]. This ICR has the same principle as the IL4/IL7 design however it promotes Th-17-like polarization via STAT3 phosphorylation in the presence of IL4. This resulted in elevated RORyt expression which is the critical linage transcriptional factor of the Th17 cell lineage. Consistently, CXCR3, the chemokine receptor expressed on Th1/Th17 cells was highly expressed on ICR carrying CAR-T cells. The previous study on IL4/IL7 ICR showed an attenuated tumor killing effect and superior proliferation in mice. This same effect was observed for the IL4/IL21 ICR CAR-T cells in mice with IL4/IL21 ICR showing a superior effect compared to IL4/IL7 ICR and non ICR carrying CAR-T cells. Altogether, the IL4/IL21 ICR showed a potent anti-tumor effect and long-term persistence *in vivo*. Thus, IL4/IL21 ICR can be seen as a promising design against IL-4 rich solid tumor cancers.

TGF-B is a soluble immunosuppressive cytokine commonly present in the solid tumor TME[13]. TGF-B is involved in the promotion of tumor invasion, metastasis and inhibits T cell activation and proliferation. Therefore, it is important to limit the effects of TGF-B on T cell function. Many strategies have been designed to overcome TGF-B. One example is the chimeric switch receptor TGF-B/IL7 ICR (Fig3B) [41]. As mentioned before IL7 is a potent pro-inflammatory cytokine which enhances T cell anti-tumor effects. By combining the extracellular domain of TGF-B with the intracellular signaling domain of IL7 the suppressive nature of TGF-B is changed into an IL7R-mediated immune activating signal. Allowing T cells to function within a TGF-B rich TME typically present in solid tumors[13]. While the ICR was co-expressed with a CAR targeting CD19 improved IFN-y and TNF-a production was measured when exposed to a TGF-B rich environment compared to non-ICR controls. In addition, superior cytotoxicity was observed. When ICR co-expressing CAR-T cells were intravenously injected into mice with xenografted Daudi-Fluc tumors, the anti-tumor effect was far superior showing a 100% survival rate compared to controls. Additionally, Daudi cells are known to express TFG-B showing the effects of the ICR compared to non ICR-CAR-T cells[42]. Moreover, all mice were tumor-free at the experimental endpoint of day 84. Indicating a reduced tumor recurrence rate

caused by either superior cytotoxicity or persistence of T cells. The TGF-B/IL7 ICR seems promising to overcome TGF-B suppression in the solid TME.

One of the best-known immunosuppressive receptors is PD-1. PD-1 ligand, so called PD-1L, is often overexpressed by tumors which influences the suppressive nature of the TME. Therefore, by utilizing the high expression of PD-1L in the TME investigators wanted to change the suppression nature of the PD-1L into a T cell costimulatory activation signal. Different designs have been made *e.g* a PD-1 receptor with a CD28 costimulatory endodomain (Fig3C). This type of receptor is known as a chimeric switch receptor (CSR). CAR-T cells specific for c-Met, a gastric cancer Ag, were found to have enhanced killing ability in addition to increasing the secretion of INF-γ and TNF-α compared to non CSR carrying CAR-T cells[14]. The CSR was specifically correlated with giving CAR-T cells a long-term anti-tumor effect in mice. Furthermore, no increase in off-target toxicity was observed when given to mice with xenografted tumors. In addition, a different group looked at the effects of a PD-1/CD28 CSR co-expressed with a third-generation CD-19/CD28/4-1BB CAR[43] in patients with PD-L1-positive B cell lymphoma. This phase 1b study showed that this design had superior T cell proliferation, cytokine production and better killing capability of PD-L1+ B-cell lymphoma cells *in vivo* and *in vitro*. Additionally, no severe neurologic toxicity or cytokine release syndrome was observed. Therefore, this study demonstrates real-life application of CSRs to enhance known transgenic T cell therapies.

Another CSR was designed based on T cell immunoreceptor with Ig and ITIM domains (TIGIT)[44]. Normally this is a co-inhibitory receptor expressed by T and NK cells which when activated, decreases the cytokine production and effector function of these cells. Ligands for TIGIT are known to be present within the TME of some solid tumors such gastric cancers decreasing the effectiveness of T cells [45]. Researchers designed a CSR which was composed of the TIGIT exodomain fused to the signaling domain of CD28 which enabled positive signaling upon binding of TIGIT ligands. The TIGIT-CRS was co-transduced together with a MART1-specific TCR or CD19/4-1BB-CAR into human T cells. TIGIT-CSR was found to enhance the secretion of cytokines important for anti-tumor function, TNF-α, IFN-γ and IL2 when compared to control transduced cells and the control TIGIT-CSR, which does not have a CD28 signaling domain. Furthermore, the TIGIT-CSR was found to mitigate the effects of prolonged antigen exposure, thereby limiting T cell exhaustion. In addition, when TIGIT-CSR expressing MART1-TCR T cells were given to mice with xenografted tumors, superior anti-tumor cytotoxicity and higher survival were observed. Illustrating TIGIT-CSR could delay tumor growth and prolong survival. This shows that the co-expression of TIGIT-CSR can improve both tumor-specific TCR and CAR-T cells function and activation phenotype.

Previously research has shown that Mesothelin (MSLN)-specific TCR-engineered T cells preferentially accumulate within established tumors, delay tumor growth and prolong the survival of mice models[46]. However, this design showed low T cell persistence and antitumor activity was not found to be sustained. Therefore a new design tried to overcome an important aspect of the TME, Fas/FasL signaling which can induce activation-induced cell death, an apoptotic mechanism that normally regulates T cell expansion however within the TME it has been associated with decreasing T cell function[47]. To overcome FasL-mediated immune evasion and enhance T cell response a CSR based on the Fas extracellular binding domain fused to a 4-1BB co-stimulatory domain was co-expressed with the MSLN-specific TCR. When exposing $ID8_{VEGF}$ tumor-bearing mice to MSLN-specific T cells or CSR + and MSLN-TCR+ T cells, the T cells engineered to express both showed better persistence in the ovarian TME and improved survival of the mice. Furthermore, the ICR expressing T cells exhibited

enhanced proliferation, cytokine production and specific lysis of ovarian tumor cells *in vitro*. This research shows a viable strategy to enhance TCR based therapy with CSRs that target tumors overexpressing certain immunosuppressive ligands or cytokines.

The CSR/ICR as a design can provide co-stimulatory signals which are often limited within the TME without the need for constitutive expression of cytokines. Therefore, the CSR/ICR design should be seen as one of the main ways the effectiveness of T cells-based therapies should be enhanced in the future.

Armored/dominant negative receptor cells

A different approach to overcoming the suppressive nature of the TME is by generating armored T cells. Armored T cells are T cells which co-express a dominant negative receptor separate from a tumor specific CAR or TCR[48]. dominant negative (DN) indicates that the receptor can bind its ligand however it is unable to transduce a signal. T cells normally express TGF-BRII to bind TGF-B. By binding TGF-B, T cell functionality is inhibited on a multitude of fronts such as proliferation and repressing the cytotoxic functions of CD8+ T cells. By overexpressing the DN TGF-βRII it endows the CAR-T cells with resistance to the inhibitory effects of TGF-β. Under normal circumstances TGF-β is recognized by the TGF-βR which is a heterodimer made up from TGF-βRI and TGF-βRII[49]. By overexpression of DN TGF-βRII the natural expressed TGF-βRII needs to compete for dimerization. This results in a dysfunctional TGF-βR because of the missing internal signaling domains of TGF-βRII. Research found that this design allowed BCMA specific CAR-T cells to maintain high functionality after prolonged exposure to TGF-β. However, it did not confer any additional stimuli as is possible with a CSR or ICR. A different approach is to create an inducible DN TGF-βRII instead of overexpressing it[50]. By creating an inducible expression of the CAR together with the DN receptor the goal is to limit T cell exhaustion and overcome the negative effects of TGF-β on CAR-T activation. Comparable results were observed for T cell expansion while in the presence of TGF-β as in the previously discussed research. In addition, the inducible nature of both the CAR and the DN receptor showed equivalent tumor lysis, superior CAR-T expansion and reduced expansion compared to the constitutive expression.

Discussion

The initial success of adoptive T cell therapies in treating leukemia and lymphomas was an exciting advancement in cancer treatment. However, the step from hematological based cancers to solid tumors has been paved with difficulty. The intricate interactions between immune cells, tumor cells, cytokines, and other immune related molecules in the TME have forced T cell-based immunotherapy to adapt and provide innovative designs to overcome the suppressive nature of the TME. A wide variety of unique designs have been brought forth each with different enhancing effects. Using the variety of possible co-stimulating intracellular signaling motifs/ICDs unique designs have been pioneered. The use of both CD28+ 4-1BB ICDs has shown increased anti-tumor functionality compared to single ICD use against hematological cancers. It, however, seems surpassed by other domain combinations such as the CD28-OX40. In addition, both the single domain CD28 and 4-1BB seem to be bested by BAFF in direct comparison. This shows that our current understanding of which single or ICD combination should be questioned as more effective and cancer specific co-stimulatory signaling is possible. The incorporation of costimulatory ICDs in CARs itself should also not have been seen as the endpoint of T cell augmentation. This has been proven by the impressive results provided by the different co-, switch-, T cell - and hybrid T cell receptors utilizing different ICDs and TME

interactions to improve anti-cancer functionality, T cell persistence, fitness, and sensitivity. While CARs as recognition receptors have been in the spotlight compared to TCR based platforms, both γδTCR and hybrid TCR platforms should be taken seriously on their potential. The hybrid TCR platforms incorporate the Ag HLA-independent tumor recognition of CARs with the stronger effect of TCR signaling, less tonic signaling, incorporation of costimulatory domains and increased Ag sensitivity. This gives an edge in anti-tumor activity vs second/third generation CARs. An interesting addition to this design could be an ISR/ICR further potentiating its ability in the TME. γδTCR remains an interesting option due to its inherent HLA-independent TCR activation. Adding a coCAR allows their anti-tumor function to increase and create more precise targeting of tumor cells. Looking at Co-receptor enhancement the self and neighbor-activating capacity of the ICOS-4-1BB receptor shows great promise in enhancing both transgenic T cells and naturally occurring TILs. The constitutively co-expressed OX40 receptor holds less potential as its constitutively expression should generate enhanced T cell exhaustion. In addition, it is OX40L overexpressing cancers specific, lowering its functionality compared to other more universal activating designs. Creating a more inducible manner of co-stimulation should be tried to be designed to lessen T cell exhaustion, *e.g.* the inducible DN TGF-βRII. This holds for all co-expressing receptors. Switch receptors are focused on overcoming specific TME rich in certain specific immune suppressing molecules such as IL4, TGF-β, PD-1L, FAS or TIGIT. All switch receptors showed exciting potential in enhancing T cells carrying a cancer specific TCR or CAR. In addition, the ISR/ICR as a design can provide co-stimulatory signals which are often limited within the TME without the need for constitutive expression of cytokines which is known to generate cytokine release syndrome and neurotoxicity. Here is where the armored design seems less promising. Because even though it is not susceptible to certain immunosuppressive molecules, it is unable to turn it into a potentiating signal. Therefore, it is fully dependent on (co-) stimulation from a CAR or TCR. One troublesome aspect of all these designs is that no side-to-side comparison has been made. Each new design has mostly been compared to the first of second-generation CARs. New research should focus on combining and comparing different designs. These novel designs each hold promise to allow gene-engineered T cell-based therapies to overcome the barriers created by the TME. Understanding and refining the use of different co-stimulation ICDs and methods is critical to enhancing the future application of T cell-based therapies. Each design should be utilized to personalize T cell therapy for the patient's respective tumor. In the future, more research should be focused on combining certain design types like the coCAR or ISR/ICR with the enhanced (hybrid-) TCR/CARs to overcome the barriers put up by the solid TME.

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