CAR-T immunotherapies: biotechnological strategies to improve safety, efficacy and clinical outcome through CAR engineering

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Abstract

T cells engineered to express a chimeric antigen receptor (CAR) have re-shaped the way hematological malignancies are treated. Despite the overwhelming early clinical success, CAR-T therapies are associated with severe side-effects, disease relapse and often exhibit limited efficacy. In this Review article we summarize the most recent biotechnological advances that have been developed to enhance the efficacy and specificity of CAR-T therapies, as well as to address the key challenges associated with them. We place particular emphasis on the most recent clinical data that indicate which CAR-T populations are the most relevant to clinical success, and indicate how the molecular structure of the CAR receptor can affect clinical outcome. Finally, we outline what we believe is the next generation of immunotherapies.

Keywords: chimeric antigen receptor, immunotherapy, biotechnology

1. Introduction

Two autologous chimeric antigen receptor (CAR)-T cell therapies (Kymriah and Yescarta) were recently licensed by American and European agencies (FDA and EMA respectively). CAR-T cell therapies are a type of cancer immunotherapy in which a patient's T cells are genetically engineered in the laboratory so they can attack cancer cells. Both licensed products target CD19, an antigen expressed on B cells and leukemic cells. Kymriah (tisagenlecleucel) brought to market by Novartis and Yescarta (axicabtagene ciloleucel) brought to market by Kite Pharma/ Gilead, are indicated for the treatment of pediatric and relapse/refractory (R/R) B cell acute lymphoblastic leukemia (B-ALL) and certain lymphoma subtypes and adult refractory diffuse large B cell lymphoma (R/DLBCL) respectively. Remarkably, the overall response rate in the short term was 83% based on a single dose of Kymriah with patients entering into remission within 3 months of being treated (Grupp, 2018; Maude et al., 2018, 2014) (ELIANA, NCT02435849) (Table 2), extending to 76% and 70% at 12 months and 18 months respectively (Grupp, 2018). Similarly, the recipients of Yescarta had an overall response rate of 71% within 3 months (Locke et al., 2018, 2015; Neelapu et al., 2017) (ZUMA, NCT02348216) and 39% of patients remained in remission for 27 months (Locke et al., 2018; Neelapu, 2018).

Although CAR-T therapies have a remarkable life-saving potential they are nevertheless associated with severe toxicities (most notably cytokine release syndrome and neurotoxicities), high cost (> £200,000) and limited efficacy on cancers other than blood malignancies. Furthermore, despite the high short-term response rates a proportion of patients relapse after CAR-T treatment. As a result, further understanding of T and CAR-T cell biology is required in order to improve these immunotherapies.

The immune system is comprised of the innate and adaptive systems. While the innate system is the first line of defense and is poised to act rapidly, the adaptive system displays immunological memory and contains cells such as T and B cells that are highly specific for any pathogenic threat. T cells are defined by the expression of the T cell receptor (TCR) which interacts with the Major Histocompatibility Complex (MHC) (also termed human leukocyte-associated [HLA] antigens), found on the surface of antigen presenting cells (APCs) such as dendritic cells (DC). This interaction is necessary for the recognition of the pathogenic threat by the T cell and its subsequent activation (Figure 1). The immunogenic response elicited by the T cell is dependent on the type of MHC that is presented by the APC (class I MHC or class II MHC), which subsequently defines whether the T cell will differentiate into one of the two major T cell subsets, CD8+ (T cytotoxic) or CD4+ (T helper). While most T cells disappear after the threat has been eliminated, others will survive and form memory cells that can survive for years. Memory T cells are classified according to the expression of certain surface markers (Table 1) (Gattinoni et al., 2011, 2009; Gebhardt et al., 2009; Hofmann and Pircher, 2011; Lugli et al., 2013; Masopust et al., 2010; Sallusto et al., 1999).

This review outlines the challenges associated with CAR-T immunotherapies and focuses on the most recent biotechnological and genomic engineering advancements that have been developed in order to address such challenges. Furthermore the review summarizes the recent CAR-T clinical data that provide an indication as to which subsets of CAR-T cells have the most potential to expand within the patient and therefore provide sustainable remissions. Finally the review discusses alternative CAR immunotherapies by utilizing other cells of the immune system. The review is therefore intended for individuals with a biochemical engineering, biotechnology or similar background who wish to further understand CAR-T immunotherapies from a biological or biotechnological perspective.

2. CAR-T cells

2.1. CAR design

Genetically engineered T cells ectopically expressing CAR are referred to as CAR-T cells. Unlike TCRs, CARs enable highly-specific targeting of antigen in an MHC-independent manner. CARs comprise of extracellular, hinge/transmembrane and intracellular domains (Figure 2). The CAR design has evolved over the years in order to enhance safety and efficacy (Figure 2). First generation CARs were reported by Kuwana and Gross and later by Eshhar et al and only included the CD3ζ domain (Kuwana et al., 1987; Gross et al., 1989; Eshhar et al., 1993), which exhibited minimal response and persistence *in vivo* (Kershaw et al., 2006; Lamers et al., 2006; Till et al., 2008). Second generation CARs incorporated either CD28 or 4-1BB in addition to CD3ζ which led to increased efficacy and *in vivo* persistence (Finney et al., 2004, 1998; Imai et al., 2004; Milone et al., 2009). Third generation CAR constructs have been engineered to contain multiple co-stimulatory domains such as CD28 and 4-1BB in addition to CD3ζ (Carpenito et al., 2009; Pulè et al., 2005; Wang et al., 2007; Ying et al., 2015; Zhao et al., 2009) . While some studies suggest that third generation CARs have superior efficacy compared to second generation CARs (Carpenito et al., 2009; Zhong et al., 2010), other studies have shown opposite findings (Abate-Daga et al., 2014; Long et al., 2016). Notably, Kagoya et al increased CAR-T persistence and tumor control *in vivo* by constructing CAR-T cells containing Signal Transducer and Activator of Transcription 3 and 5 (STAT3/STAT5) activation motifs, thereby mimicking the cytokine-induced signal 3 (Kagoya et al., 2018).

2.2. How CAR co-stimulatory domains impact the clinical efficacy of CAR-T cells

As mentioned above, while first generation CARs exhibited limited capacity *in vivo*, second generation CARs showed greater persistence and tumor killing. Trials conducted with CARs incorporating CD28 or 4-1BB co-stimulatory domains have shown similar initial response rates in patients with ALL (Brentjens et al., 2013; Lee et al., 2015; Maude et al., 2014). However, differences between 4-1BB and CD28 CARs have been reported in patients with chronic lymphoblastic leukemia (CLL) and B-cell lymphoma. 4-1BB CARs appear superior (Maude et al., 2018; Porter et al., 2015; Turtle et al., 2016) by demonstrating persistence for longer than 4 years when compared to CD28 CARs (Kochenderfer et al., 2015; Neelapu et al., 2017) that in some cases exhibited persistence of 30 days (Brentjens et al., 2011; Davila et al., 2014; Lee et al., 2015; Maude et al., 2014).

As a result, efforts are made to understand the fundamental differences in the downstream signaling of 4-1BB and CD28. However, the variability in patient cohorts, clinical trial designs and CAR constructs make it difficult to make robust comparisons. Studies comparing 4-1BB and CD28 CAR-T cells in pre-clinical studies have partially attributed the clinical differences to the distinct metabolic properties that these two domains confer to CAR-T cells. Specifically, while CD28 CAR-T cells exhibited enhanced glycolysis, short-lived effector phenotype and augmented exhaustion phenotype (Chang et al., 2013; Frauwirth et al., 2002; Gubser et al., 2013; Kawalekar et al., 2016; Long et al., 2015), 4-1BB CAR-T cells demonstrated long-term survival, as well as increased mitochondrial biogenesis and oxidative respiration which was ultimately linked to increased frequency of central memory cells (Kawalekar et al., 2016).

The aforementioned studies indicate that 4-1BB domain is beneficial for CAR-T cell persistence. However, Silva et al showed that tonic (i.e. constitutive signaling with intermediate intensity) 4-1BB signaling can have adverse effects on CAR-T cells (Gomes-Silva et al., 2017). Specifically, tonic 4-1BB signaling resulted in increased CAR-T cell death that was augmented by the use of gamma-retroviral vectors (currently used in 41% of CAR-T clinical trials [reviewed in (Vormittag et al., 2018)]). These results indicate that 4-1BB may not be universally beneficial for CAR-T cells but rather, the outcome depends on the duration and intensity of the signaling. Finally, in support of the notion that signaling strength is the key determinant of CAR-T cell fate Salter et al carried a phosphoproteomic analysis (an analysis that identifies proteins that have been phosphorylated) in which they identified no major differences in signaling intermediates between 4-1BB and CD28 human CAR-T cells (Salter et al., 2018). Instead, they found that CD28 prompted a more rapid and intense activation of signaling events when compared to 4-1BB CAR-T cells.
Taken together, these studies highlight the importance of in-depth understanding of the signaling events that take place downstream of CAR as they could be key for achieving greater efficacy and persistence of CAR-T cells.

3. Optimal CAR-T subsets in clinical trials

An important factor governing CAR-T cell activity *in vivo* is the subset of T-cell input used. For both murine models and human clinical trials it has been demonstrated that less differentiated T cell subsets (T_{CM}, T_{SCM}) exhibit better persistence, expansion and anti-tumor activity (Gattinoni et al., 2005; Lugli et al., 2013; Crompton et al., 2014). Furthermore, retrospective studies have shown a correlation between increased persistence *in vivo* and a less differentiated T cell phenotype (Klebanoff et al., 2012; Sommermeyer et al., 2016). Xu et al analysed patients treated with CD19 CAR-T cells and identified a correlation between the level of T_{SCM} infused phenotype and *in vivo* expansion. There is also indication that presence of both $CD4^+$ and $CD8^+$ T cell subsets results in better anti-tumor responses (Church et al., 2014). Of note, Fraietta et al analysed CAR-T populations isolated from CLL patients that had either responded or not responded to the treatment and identified that CAR-T cells isolated from responders exhibited a gene profile associated with memory formation. In contrast, CAR-T cells isolated from non-responders exhibited a gene profile associated with exhaustion and effector phenotype. Notably, evaluation of T cell subsets at the point of leukapheresis indicated that responders had a higher frequency of stem-cell like T cells that could be used as predictor of clinical success (Fraietta et al., 2018a). The same group assessed one CLL patient whose disease regressed only after a second CD19 CAR-T infusion (Fraietta et al., 2018b). Analysis of the patient's CAR-T cells at their peak concentration showed that a) all CAR-T cells were derived almost exclusively from a single T cell clone, b) the CAR construct had been unintentionally inserted into one of the two copies of the gene *TET2* preventing the gene from producing a functional protein. Further analysis revealed that the patient's T cells had a pre-existing mutation in the other copy of *TET2*, leading to complete lack of functional TET2 protein in the CAR-T cells. TET2-disrupted CAR-T cells exhibited an altered epigenetic profile and at their peak concentration displayed a central memory phenotype, validating the correlation between successful clinical outcome and a less differentiated T cell phenotype. Finally, these results indicate that the progeny of a single CAR-T cell is sufficient to mediate potent anti-tumor effects.

4. Strategies to improve safety and efficacy of CAR-T cells

4.1. Challenges in CAR-T therapy

The success of the first CAR-T therapies is unprecedented. However, several challenges remain to be addressed. Patients successfully treated with CD19 CAR-T cells often exhibit profound B cell aplasia (diminished B cell numbers), owing to the fact that CAR-T cells directed against CD19 cannot discriminate between normal and tumor cells (Porter et al., 2011). B cell aplasia has been reported in 15% and 14-43% of patients treated with Yescarta and Kymriah respectively (Research, Approved products).

Similar on-target-off-tumor effects have been observed in CAR-T cells directed against other antigens such as Erb-B2 receptor tyrosine kinase 2 (ERBB2/HER2) and carbonic anhydrase IX (CAIX) where patients deceased due to respective pulmonary failure and hepatotoxicity (Lamers et al., 2013; Morgan et al., 2010). Apart from on-target-off-tumor effects, another commonly observed side effect is cytokine release syndrome (CRS) characterized by high fever, hypoxia, hypotension and/or multi-organ failure. The severity ranges from mild to life-threatening and is thought to correlate with tumor burden (Brentjens et al., 2013; Lee et al., 2015; Maude et al., 2014; Neelapu et al., 2017). In clinical trials using the CD19 CAR-T products Yescarta and Kymriah, 94% and 74% of patients respectively developed CRS (Research, Approved products). CAR-T-associated neurological toxicities including life-threatening reactions have also occurred in 58-72% of patients treated with Kymriah and in 87% of patients treated with Yescarta (Research, Approved products). Other challenges associated with CAR-T therapies include resistance to therapy or subsequent relapse due to antigen escape or CAR-T failure (Lee et al., 2015).
In clinical trials directed against B-ALL, disease relapses have been reported in 21-45% of patients subsequent CD19 CAR-T infusion (Grupp, 2018; Grupp et al., 2013; Lee et al., 2015; Maude et al., 2018, 2016, 2014; Park et al., 2015; Sotillo et al., 2015; Turtle et al., 2016). Multiple mechanisms of tumor escape from therapy have been reported and include downregulation or mutation of CD19, via selection of CD19 negative cells, or via lineage switching (Evans et al., 2015; Gardner et al., 2016; Grupp et al., 2013; Jacoby et al., 2016; Sotillo et al., 2015). Curiously, CD19 epitope loss has not been reported in CLL patients. Resistance to CAR-T therapies in CLL patients appears to be related to the failure of CAR-T cells to expand after infusion (Porter et al., 2015). Furthermore, a recent report of a patient enrolled in Novartis's phase I clinical trial (NCT01626495) testing the safety of their product has highlighted one of the many manufacturing challenges of CAR-T therapies (Ruella et al., 2018). In this case the CAR gene was unintentionally introduced into a single leukemic B cell during T cell manufacturing. As a result, its product bound to the epitope of CD19 on the surface of the leukemic cell, masking it from recognition and conferring resistance to the CD19 CAR-T treatment, ultimately leading to the death of the patient. Although the report stated that this occurrence was a rare event (1 out of 369 enrolled patients), it nevertheless illustrates the need for improved manufacturing technologies for identifying leukemic contaminants at the single cell level.
Finally other challenges, especially in the context of solid tumors, include immunosuppression and limited CAR-T persistence which will be discussed separately below. The severe side effects and relative inefficiencies of the current CAR-T treatments highlight the need for their improvement.

4.2. Improving safety & increasing specificity

4.2.1. Suicide genes

The side effects associated with CAR-T therapies highlight the need for the development of systems that will eliminate these cells if the side effects become life-threatening. Suicide switches such as inducible Caspas9 (iCasp9) and herpes simplex virus thymidine kinase (HSV-TK) (Figure 3A, B) allow inducible termination of CAR-T cells some of which are currently being evaluated in clinical trials (NCT03579927, NCT02414269, NCT02107963, NCT01822652). Alternative suicide genes include epitope tags that are recognized by FDA-approved monoclonal antibodies which induce T cell death via antibody-dependent or complement-dependent cytotoxicity. Examples include Cetuximab targeting a truncated form of the epithelial growth factor receptor (tEGFR) and Rituximab targeting the receptor CD20 (Griffioen et al., 2009; Vogler et al., 2010; Wang et al., 2011; Paszkiewicz et al., 2016; Tasian et al., 2017) (Figure 3C). The compact marker/suicide gene RQR8 that was developed by Philip et al was engineered to combine target epitopes from both CD20 and CD34 (Figure 3C) (Philip et al., 2014). RQR8 is currently tested in clinical trials (NCT03590574, NCT02746952). Additionally, EGFR- mediated CAR-T elimination is currently being tested in clinical trials targeting CD19 (NCT03085173, NCT03618381, NCT02051257, NCT03070327, NCT02028455, NCT02146924, NCT01865617), PD-1 (NCT02937844), EGFR806 (NCT03638167), CD171 (NCT02311621) and CD123 (NCT03114670, NCT02159495).

4.2.2. Inducible systems

The irreversible ablation of CAR-T cells may be counterproductive considering that persisting CAR-T cells are necessary to achieve long-lasting clinical success in patients, and that manufacturing of CAR-T cells is both expensive and laborious. Furthermore, the suicide gene in some of the CAR-T cells may not act quickly enough to eliminate the off-tumor toxicity as the death of target cells may take several minutes after induction of the suicide gene. Alternatively, non-cytotoxic, reversible systems may be useful for controlling adverse side effects without ablating the CAR-T population. For example, the CAR construct can be placed under the control of inducible expression systems that can turn the expression of the CAR construct on and off (Sakemura et al., 2016) (Figure 4A). Chemical inducers can also be used to control the assembly of the CAR receptor through drug dimerization domains**.** In these split receptors the antigen-binding and intracellular signaling domains assemble only in the presence of the dimerizing drug (Wu et al., 2015) (Figure 4B).

4.2.3. Logic states

In an effort to decrease the on-target-off-tumor effects as well as reduce tumor escape, strategies using multiple tumor-antigens for full activation of CAR-T cells are currently being developed (synNotch-AND gate; Figure 5A). CARs have also been engineered to discriminate between healthy and malignant tissue by incorporating inhibitory signals (iCARs) that dampen the T cell response when a healthy antigen is present (AND-NOT gate) (Figure 5B). As a result, CAR will be activated only when the cancer-specific antigen is present, and the healthy tissue-specific antigen is absent (Fedorov et al., 2013). Other combinatorial antigen approaches include CARs that can be activated in response to a cell expressing either antigen A or antigen B (OR gate) (Figure 5C). Others are based on the separation of the domains providing “signal 1” and “signal 2” necessary for T cell activation (dual CAR) (Figure 5D) that come together only when both antigens are present (Kloss et al., 2013). However reports have shown that activity has been detected in CD3ζ-only CARs (Pule et al., 2008). Another type of bi-specific CAR has been generated such that the extracellular portion of the CAR construct contains two linked scFvs with different antigen specificities. The CAR-T cells expressing the linked scFvs in tandem (tan CARs) are activated only in the presence of both antigens (Figure 5E) (Grada

et al., 2013). Tan CARs targeting CD19/CD22, CD19/CD20 and CD38/BCMA are currently being tested in clinical trials for adult and pediatric patients with lymphoma, B-ALL and myeloma (NCT03233854, NCT03241940, NCT03463928, NCT03271515, NCT03767751) (Fry et al., 2018).

Finally, CARs have also been split such that the antigen recognition domain is separated from the signaling motif of the CAR. This configuration uses a universal receptor allowing a large panel of antigens to be targeted without the need to re-engineer immune cells (Urbanska et al., 2012; Rodgers et al., 2016). In an effort to combine many of the aforementioned systems, Cho et al designed a split, universal, and programmable (SUPRA) CAR system that can integrate signals from multiple antigens and fine-tune T cell activation using AND logic gates in a cell type-specific manner (Cho et al., 2018).

4.3. Sensing tumor microenvironment

CAR-T cells have also been engineered to apply spatial control and exert their cytotoxic activity only once they reach the tumor tissue (Figure 6A) (Han et al., 2017). A feature of the tumor microenvironment is presence of oxygen in very low levels (hypoxia) (reviewed in (LaGory and Giaccia, 2016)). Consequently, CAR-T cells can be engineered to contain oxygen-sensing domains that degrade in normoxic (normal oxygen) conditions but remain stable in hypoxic conditions (Figure 6B) (Juillerat et al., 2017). Although these features have yet to be tested in human trials and there could be potential off-tumor effects due to certain hypoxic parts of the body, they nevertheless provide a framework for the creation of the next generation, decision-making CAR-T cells.

4.4. Improving expansion and persistence

After T cells home to the site of the tumor, they must undergo rapid expansion. Clinical outcome of CAR-T treatment is correlated with their expansion *in vivo* (Kalos et al., 2011; Maude et al., 2014; Porter et al., 2015). As discussed, CAR-T cells require signals 1, 2 as well as the cytokine-dependent signal 3 in order to exert potent anti-tumor functions. However, because the tumor microenvironment can be immunosuppressive such activating cytokines are often downregulated (Becker et al., 2013). In order to circumvent this effect, TRUCK CAR-T cells (T cells redirected for universal cytokine killing) have been engineered to be armored with activating cytokines (Figure 7A). Pre-clinical studies have shown that delivery of cytokines such as interleukin (IL)-12, IL-15, IL-18 and IL-21 can enhance CAR-T activity and re-shape the tumor micro-environment (Markley and Sadelain, 2010; Chinnasamy et al., 2012; Pegram et al., 2015; Krenciute et al., 2017; Hu et al., 2017; Avanzi et al., 2018). A phase I clinical trial is underway to test the effectiveness of IL-12-secreting CAR-T cells (NCT02498912) (Koneru et al., 2015). Caution must be exerted as exogenous expression of cytokines could counteract the therapeutic effect of CAR-T cells via an indirect induction of immunosuppressive mechanisms (Ahmadzadeh et al., 2009; Spolski et al., 2009). Finally, CAR-Ts can also be loaded with other molecules that can improve expansion or augment T cell function such as non-coding micro-RNAs that are physiologically involved in T cell regulation (Ohno et al., 2013).

4.5. Improving anti-immunosuppression

Immunosuppression is a major challenge for CAR-T therapies, especially in the case of solid tumors as it enables tumor cells to escape from immune responses (Beatty and Moon, 2014). Immune escape is mainly mediated via inhibitory receptors such as programmed cell death-1 (PD-1) and T-lymphocyte associated protein-4 (CTLA-4) and cancer cells often express ligands for these receptors. To overcome this challenge, combined therapies with PD-1 checkpoint inhibitors have been used with CAR-T cells in order to enhance the immune activity of the patient's T cells. Recent clinical reports have suggested that anti-PD-1 agents enhance the efficacy of CD19 CAR-T therapy in patients with DLBCL and ALL(Liu et al., 2016; Maude et al., 2017).

The efficacy of combinatorial PD-1 blockade and CD19 CAR-T is currently being tested in clinical trials (NCT02650999, NCT02926833, NCT02706405). Alternative approaches for disrupting the immunosuppressive pathways are currently being explored and include engineered dominant-negative receptors (NCT00889954) (Foster et al., 2008), "switch" receptors (Liu et al., 2016) and PD-1 monoclonal antibody- expressing CAR-T cells (NCT02873390, NCT02862028) (Suarez et al., 2016). Finally, many groups are in the process of generating CAR-T cells unresponsive to inhibitory signals by knocking-out the genes for PD-1 (NCT03545815), CTLA-4 or lymphocyte activation gene-3 (LAG-3) via the use of CRISPR–Cas9 (clustered regularly-inter- spaced short palindromic repeats–CRISPR-associated system 9) or TALENs (transcription activator-like effector nucleases (Figure 8A) (Menger et al., 2016; Ren et al., 2017; Zhang et al., 2017). Although inhibitory signal blockage may enhance anti-tumor activity of CAR-T cells, it could also increase toxicity owing to the reduction of T cell safety mechanisms which could ultimately result to uncontrolled activity of T cells. Notably, although CRISPR/Cas9 technology has been successfully applied to engineer T cells, a major issue with the system is that it is bacterially derived and therefore may be sufficiently immunogenic to interfere the delivery of CRISPR/Cas9– edited T cells (Charlesworth et al., 2018; Wagner et al., 2018). Furthermore, studies have indicated that cells amendable via CRISPR/Cas9 engineering may also be more susceptible to malignant transformation (Haapaniemi et al., 2018; Ihry et al., 2018).

4.6. Tumor stroma and homing

Advances have been made to tackle other issues regarding CAR-T efficacy in solid tumors. Such advances include directly modulating cells that surround the tumor in order to make it easier for CAR-T cells to infiltrate the periphery (Figure 8B) (Perera et al., 2017; Ruella et al., 2017), and to improve homing of CAR-T cells to the tumor by expressing chemokine receptors which will bind to tumor ligands (Figure 7B) (Di Stasi et al., 2009; Craddock et al., 2010; Moon et al., 2011; Siddiqui et al., 2016). Many of these emerging innovations remain to show feasibility and effectiveness in human clinical trials.

5. Alternative CAR immunotherapies

As the field of immunotherapies continues to develop, alternative avenues are currently being explored. Notable examples include a distinct and less common type of T cells, gamma delta T cells (γδ T). Unlike conventional T cells that are currently used for CAR-T therapies and express αβ TCR, γδ T cells express γδ TCR. Furthermore, while conventional T cells rely on specific antigens for target recognition, γδ T cells recognize "stress-antigens" which are indicative of malignant transformation or infection (Groh et al., 2002, 1998; Rincon-Orozco et al., 2005; Wrobel et al., 2007). This feature provides a distinct advantage in the context of

cancer therapy since they can recognize several types of cancer cells. Given that γδ T recognition does not depend on MHC presentation, these cells make an ideal candidate for allogeneic "off-the-shelf" therapies. TC Biopharm is currently in a phase II clinical trial utilizing autologous, genetically unmodified γδ T cells for the treatment of refractory malignant melanoma, renal cell cancer and non-small cell lung cancer (NCT02459067). Similarly, Incysus is expected to initiate a phase I clinical trial utilizing allogeneic (but haplo-identical), genetically unmodified γδ T cells (NCT03533816). Gadeta recently partnered up with Kite Pharma/Gilead to bring their innovative technology into clinical trials. Gadeta genetically engineers conventional αβ T cells to express γδ TCR thereby combining the advantages of both T cell sub-types. Clinical trials are expected to initiate soon. Finally, a spin-out of King's College London GammaDelta Therapeutics, is also entering the space by focusing on a specific sub-type of γδ T cell. While αβ T-based cell immunotherapies have shown limited capacity against solid tumors, γδ T cells have shown promise. Data from clinical trials remain to demonstrate whether γδ T cells are efficacious as an immunotherapy against solid and other tumors.

It is generally accepted that the tumor microenvironment can have immunosuppressive effects (Buckanovich et al., 2008; Spranger et al., 2015). Therefore immune cells that are more resilient against these effects should exert superior tumor-killing capacity within the immunosuppressive tumor microenvironment. Macrophages represent an attractive candidate as an immunotherapy against solid tumors. Myeloid cells such as macrophages are actively recruited to tumor sites to perform various anti- and pro-tumor functions (Jaiswal et al., 2009; Lee et al., 2013; Oosterling et al., 2005). As part of the innate immune system, macrophages directly kill target cells via phagocytosis. Furthermore, given that macrophages are professional APCs, they can elicit an adaptive immune response by directing T cells against tumor cells. Carisma Therapeutics, a spin-out of University of Pennsylvania, focuses on the development of macrophage-based immunotherapies and is the first technology to combine antigen recognition with the effector function of macrophages. Clinical development is anticipated to initiate in 2019. Similarly, MaxCyte is in the process of testing an autologous CAR-Macrophage (MCY-M11) product in a phase I clinical (NCT03608618).

NK cells are part of the innate immune system and as opposed to T cells, have the inherent ability to kill malignant cells without prior sensitization, therefore playing a key role in tumor imunosurveillance (Herberman et al., 1975; Kärre et al., 1986; Kiessling et al., 1975; Ljunggren and Kärre, 1985; Ruggeri et al., 2006, 2002). NK cells exert their cytotoxic effects based on a tight regulation of activating and inhibitory receptors found on their surface (Malmberg et al., 2017). Physiologically nearly all healthy cells express class I MHC molecules, which bind to the inhibitory receptors on the surface of NK cells and inhibit their killing capabilities. However, malignant cells often undergo surface marker changes such as loss/downregulation of their class I MHC and upregulation of damage-associated stress antigens. As a result, NK cells can kill tumor cells through these two pathways. On the one hand absence of class I MHC results in loss of inhibitory signals causing the activation of NK cells (Ljunggren and Kärre, 1990). On the other hand damage-associated signals on the surface of tumor cells bind to the activating receptors on NK cells thus triggering the cytotoxicity of NK cells which can target cell death both directly and indirectly. Finally, similarly to γδ T cells, NK cells do not require MHC recognition thus making them ideal for an "off-the-shelf" therapy. Clinical trials using NK-based immunotherapies have been initiated for blood malignancies (NCT03056339, NCT01974479, NCT02944162, NCT02742727, NCT02892695, NCT00995137) as well as for metastatic solid tumors (NCT03415100, NCT02839954). These trials have used NKs derived

from various sources such as peripheral blood for autologous therapies or peripheral blood, umbilical cord blood and NK cell-line NK-92 for allogeneic therapies.

On the allogeneic off-the-shelf front, clinical trials in phase I led by Cellectis are testing the clinical efficacy and safety of their genetically modified allogeneic CAR-T products UCART19 (NCT02735083) and UCART123 (NCT03190278) for hematological malignancies. More of their allogeneic CAR-T products such as UCART22, UCARTCS1 and UCART38 are also underway to clinical trials. Celyad who has autologous CAR-T products currently tested in phase I clinical trials (NCT03018405, NCT03612739), has recently received approval to initiate clinical trials using allogeneic CAR-T cells in patients with colorectal cancer (NCT03692429).

Finally, Fate therapeutics has generated induced pluripotent stem cell (iPSCs) master cell banks to subsequently generate iPSC-derived CAR-T or CAR-NK cells providing provide off-the-shelf immunotherapies. Notable examples include the allogeneic CAR-T FT819 defined by the integration of CD19 CAR into the T cell receptor alpha locus (*TRAC*) providing both antigen specificity while eliminating the potential of graft-versus-host disease (GvHD) mediated via the lack of TCR, and the expression of a non-cleavable form of CD16 to address tumor antigen escape. CD16 mediates activation of NK cells by binding to the Fc portion of monoclonal antibodies thereby mediating antibody-dependent cellular cytotoxicity (ADCC). As a result, NK recognition of an antibody-coated target cell results in rapid NK activation and lysis of the target cell. In the presence of monoclonal antibodies targeting tumor cells, ADCC can be activated as an additional mechanism to target them. FT519 is another allogeneic CAR-NK product that in addition to the aforementioned features contains the additional modality of IL-15 expression to provide self-stimulating signals for enhanced NK function and persistence. The results of these pre-clinical studies demonstrated that iPSC-derived, off-the-shelf immunotherapies could be used effectively for the treatment of malignancies in an allogeneic setting (Clarke, 2018; Kaufman, 2018). These products remain to be tested in human clinical trials.

6. Concluding remarks and future outlook

In the past year, the CD19 CAR-T products Kymriah and Yescarta have gained approval by the European Medicines Agency (EMA) for their use in the European Union following their approval by the FDA. While Kymriah was granted approval by the UK's National Institute for Healthcare and Excellence (NICE) for its use to treat children and adults up to 25 years suffering from B-ALL, it was turned down for its use in adults suffering from DLBCL, a disease affecting more than 4,800 people in the UK (https://lymphoma-action.org.uk). This decision was largely based on the drug's listed price of £282,000. Similarly, NICE initially turned down Gilead's Yescarta for the treatment of adult DLBCL patients but eventually reached a deal after agreeing on a confidential discount on its listed price of £300,000. The significant costs associated with personalized cell and gene therapies, at present, represents a critical barrier to adoption. However, as the emerging scientific breakthroughs in recent years (e.g. genome editing) are integrated into process development, manufacturing platforms and technologies can be optimized to reduce costs whilst maintaining clinical efficacy. Moreover, with the emergence of allogeneic CAR-T therapies currently entering Phase 1 clinical trials and significant investment in such approaches, this is likely to significantly reduce the cost of manufacture through economies of scale, presenting a different manufacturing paradigm and business model to autologous CAR-T production. However, the safety and efficacy of an off-the-shelf CAR-T therapy is yet to be proven and technical challenges associated with sourcing

appropriate donors and preservation of incoming starting material and outgoing final product necessary to uncouple the logistics from the manufacturing process must be overcome.

Currently CAR-T treatments are available for a very small proportion of cancer patients as ALL incidence in the UK represents less than 1% of all cancers, corresponding to 800 patients (https://www.cancerresearchuk.org). The issue of high cost will become exacerbated if and when CAR-T treatments either become available for larger patient cohorts such as those suffering from breast and prostate cancer, which are the first and second most common cancers in the UK (15% and 13% respectively corresponding to 55,000 and 47,000 patients) (https://www.cancerresearchuk.org), or if CAR-T therapies are utilized as a first-line treatment. Aside from the manufacturing challenges, addressing the challenges surrounding resistance to treatment, disease relapse, severe side effects and limited persistence, will require a holistic understanding of the biological complexity of these immunotherapies. Importantly, the clinical trials used to bring these products to market comprise of small patient cohorts and are inherently difficult to compare due to differences in trial design. Clinical trials implementing a direct comparison between the approved products would offer tremendous insight.

Finally, although CAR-T therapies are transforming the management of hematological malignancies there are several hurdles that remain to be overcome in order to successfully utilize immunotherapies for solid tumors.

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Figure 1 TCR structure and T cell activation. The TCR/CD3 complex comprises of variable α and β chains associated with one transmembrane CD3γε heterodimer, one transmembrane CD3δε heterodimer and one transmembrane CD3ζζ homodimer (Nurieva et al., 2009). T cell activation is initiated when the TCR/CD3 complex on the T cell interacts with the MHC molecule on the APC surface. For full T cell activation signals 1 and 2 are required from the TCR/CD3 (signal 1) and the co-stimulatory receptors (signal 2) CD28 (binding to CD80 and CD86), 4-1BB (binding to 4-1B ligand), ICOS (binding to ICOS ligand), CD27 (binding to CD70) and OX40 (binding to OX40 ligand). Signal 3 which is transmitted by cytokine receptors is crucial for survival and differentiation. The binding of peptide-MHC complexes to the TCR initiate a signaling cascade propagated through the cytosol and the nucleus of the cell reviewed in (Malissen et al., 2014). TM, transmembrane; ICOS, inducible co-stimulator; CD, cluster of differentiation.

Figure 2 CAR generations. CAR comprises of an extracellular, a hinge/transmembrane and an intracellular domain. The extracellular portion of the CAR consists of only the variable regions of the light and heavy chain of an antibody that are fused via a flexible linker (scFv). The flexible hinge domain, used alone or in conjunction with a spacer, is a short peptide fragment that provides conformational freedom to facilitate binding to the target antigen on the tumor cell and together with the transmembrane domain bridge the scFv to the intracellular domain. The intracellular domain comprises of a TCR-derived CD3ζ domain and one or more co-stimulatory domains for intracellular signaling, depending on the generation of the CAR construct. First generation CARs contain a single CD3ζ domain. Second and third generation CARs incorporate one or more co-stimulatory domains respectively. While the vast amount of studies have looked into CD28 and 4-1BB, other co-stimulatory domains are currently being evaluated (Foster et al., 2017; Song et al., 2012).

Figure 3 Inducible switches. **A**, Addition of a dimerizing drug activates iCaspase 9 (iCasp9) signaling pathway which results in apoptosis of CAR-T cells (Straathof et al., 2005). **B**, CAR-T cells expressing the herpes simplex virus thymidine kinase (HSV-TK) suicide gene can be eliminated by the administration of ganciclovir (GCV) which is metabolized into a toxic purine analogue. **C**, CAR-T cells expressing truncated epithelial growth factor receptor (tEGFR) or CD20 can be tagged with the monoclonal antibodies Cetuximab or Rituximab that bind tEGFR and CD20 respectively. RQR8 is a minimal marker/suicide gene combining epitopes from CD20 (to bind Rituximab for elimination of CAR-T cells) and CD34 (to serve as a selection marker) (Labanieh et al., 2018).

Figure 4 Inducible systems. **A**, The Tet ON system is controlled by the addition or removal of tetracycline that allows the reversible expression of CD19 CAR. In the presence of tetracycline (Tet), the reverse tetracycline-controlled trans activator (rtTA) induces the expression of the CD19 CAR. **B**, CAR domains are split in individual, non-functional receptors attached on an FK506 binding protein (FKBP) domain and an FKBP12-rapamycin binding domain (FRB). CAR assembly can be subsequently induced via the addition of a dimerizing drug such as rapamycin.

Figure 5 Logic states. **A**, Upon binding antigen A, synNotch undergoes conformational changes that result in the release of the transcription factor (TF). TF in turn translocates to the nucleus of the cell and induces the expression of CAR for antigen B (Roybal et al., 2016). **B**,

Inhibitory CAR (iCAR) reduces T cell response in the presence of a healthy antigen due to the presence of an inhibitory domain such as PD-1 or CTLA-4 that is fused to the antigen-binding domain recognizing the healthy antigen. CAR-T response occurs only in the simultaneous presence of a tumor associated antigen (TAA) and the absence of the healthy antigen (AND-NOT Gate). **C**, OR Gate CARs can be activated in the presence of either antigen A or antigen B. **D**, Dual CARs express two separate CARs with different ligand binding targets. One CAR contains only the domain for signal 1 while the second CAR contains only the co-stimulatory domains. CAR-T activation occurs only in the presence of both antigens. **E**, Tandem CARs (Tan CARs) express a single CAR consisting of two linked scFvs in tandem fused to co-stimulatory and signal 1 domains. Similarly to dual CARs, CAR-T activation occurs only in the presence of both antigens adapted from (Labanieh et al., 2018).

Figure 6 Tumor microenvironment-sensing (TME) CARs. **A**, Masked CARs are kept in an OFF state through a targetable peptide that masks the scFv region of the CAR. Once CAR-Ts reach the TME, tumor-specific proteases cleave the peptide and expose the scFv. **B**, Oxygen-sensing CARs contain a hypoxia-inducible factor (HIF) domain that is targeted for degradation in the presence of oxygen (normoxia). CAR expression is stabilized in the hypoxic (low oxygen) conditions of the TME adapted from (Labanieh et al., 2018).

Figure 7 Armored CARs. **A**, CAR-T cells armored with anti-tumor cytokines. Cytokine expression may be constitutive or induced subsequent of T cell activation. Localized production of pro-inflammatory cytokines can recruit other immune cells to tumor sites where they may enhance anti-tumor activity. IL, interleukin. **B**, CAR-T cells may be engineered to express chemokine receptors to enhance trafficking into tumor tissue and homing to tumor cells.

Figure 8 Strategies improving efficacy and anti-immunosuppression. **A**, CAR-T cells engineered to be resistant to immunosuppression by lacking expression of immune checkpoint molecules (e.g. PD-1, CTLA-4, LAG-3, TIM-3), by expressing immune checkpoint switch receptors or dominant-negative receptors, or by being administered with antibodies or inhibitors that result in reduction of immune checkpoint signaling. **B**, CAR-T cells can be engineered to target cells of the tumor microenvironment (TME) that may therefore enhance the infiltration of CAR-T cells directed against tumor cells. Examples include cancer-associated fibroblasts, tumor endothelial cells, tumor-associated macrophages and T regulatory cells. TIM-3; T-cell immunoglobulin protein-3.

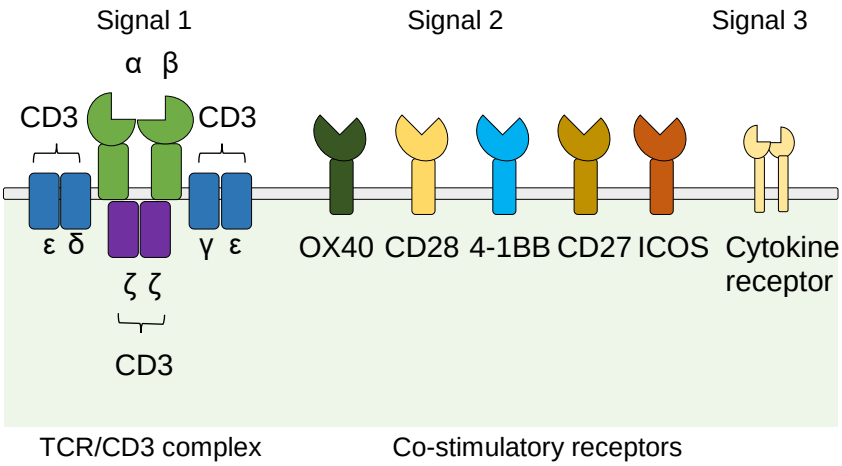

Signal 1
Signal 2
Signal 3
α β
CD3
CD3
ε δ
γ ε
ζ ζ
CD3
OX40 CD28 4-1BB CD27 ICOS
Cytokine receptor
TCR/CD3 complex
Co-stimulatory receptors

Figure 1

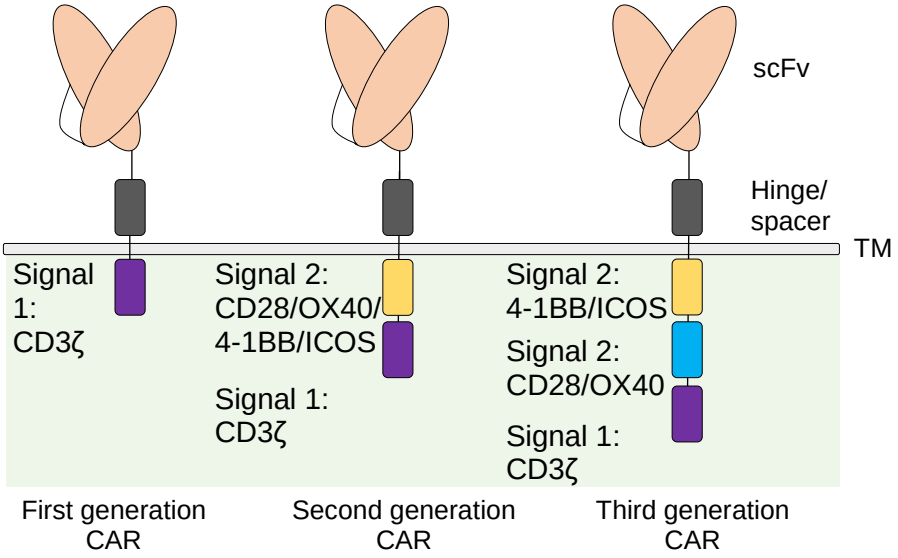
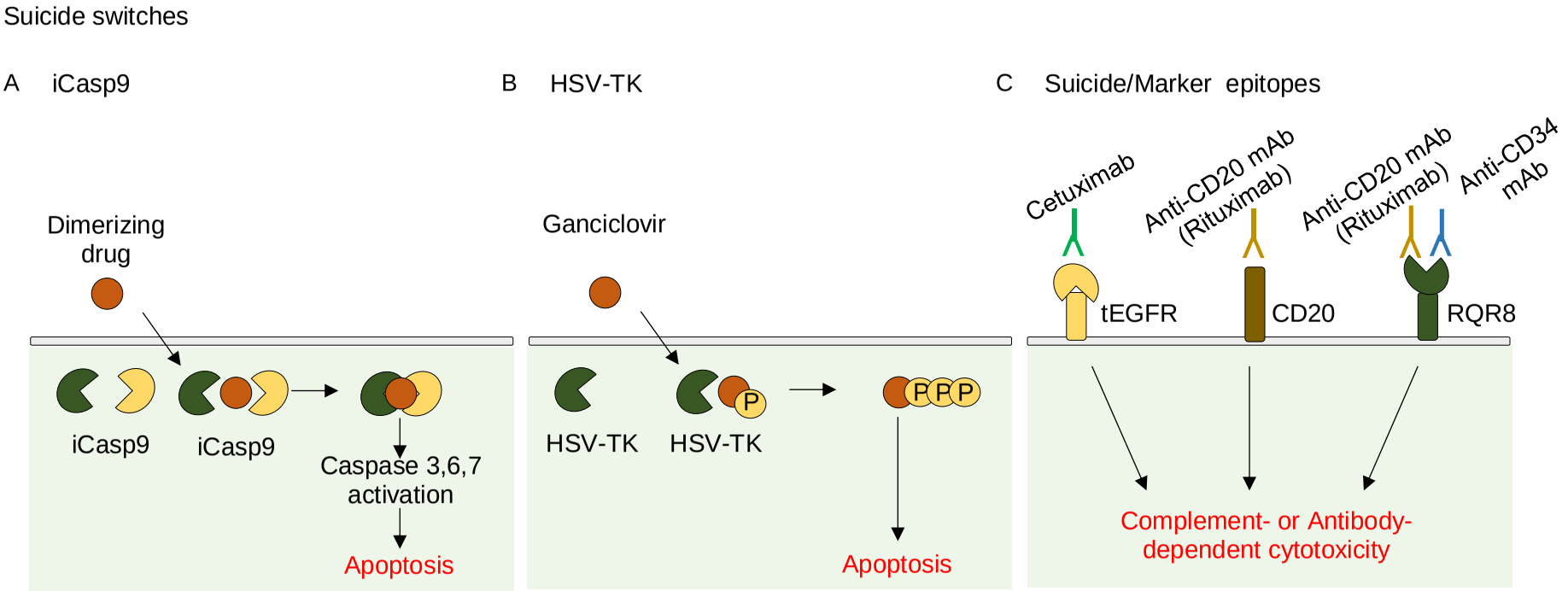

scFv
Hinge/
spacer
TM
Signal
1:
CD3ζ
Signal 2:
CD28/OX40/
4-1BB/ICOS
Signal 1:
CD3ζ
Signal 2:
4-1BB/ICOS
Signal 2:
CD28/OX40
Signal 1:
CD3ζ
First generation
CAR
Second generation
CAR
Third generation
CAR

Figure 2

Figure 3


Suicide switches
A iCasp9
B HSV-TK
C Suicide/Marker epitopes
Dimerizing drug
iCasp9
iCasp9
Caspase 3,6,7 activation
Apoptosis
Ganciclovir
HSV-TK
HSV-TK
P
P P P
Apoptosis
Cetuximab
Anti-CD20 mAb (Rituximab)
Anti-CD20 mAb (Rituximab)
Anti-CD34 mAb
tEGFR
CD20
RQR8
Complement- or Antibody-dependent cytotoxicity

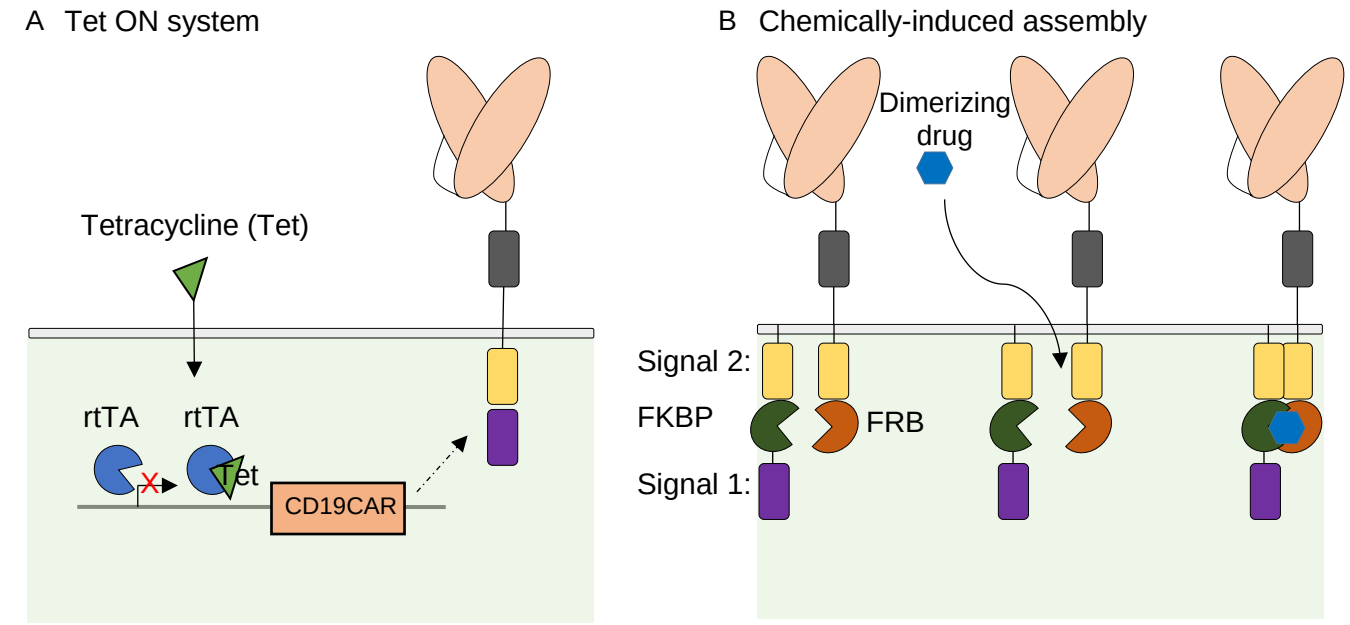

A Tet ON system
Tetracycline (Tet)
rtTA
rtTA
Tet
CD19CAR
B Chemically-induced assembly
Dimerizing drug
Signal 2:
FKBP
FRB
Signal 1:

Figure 4

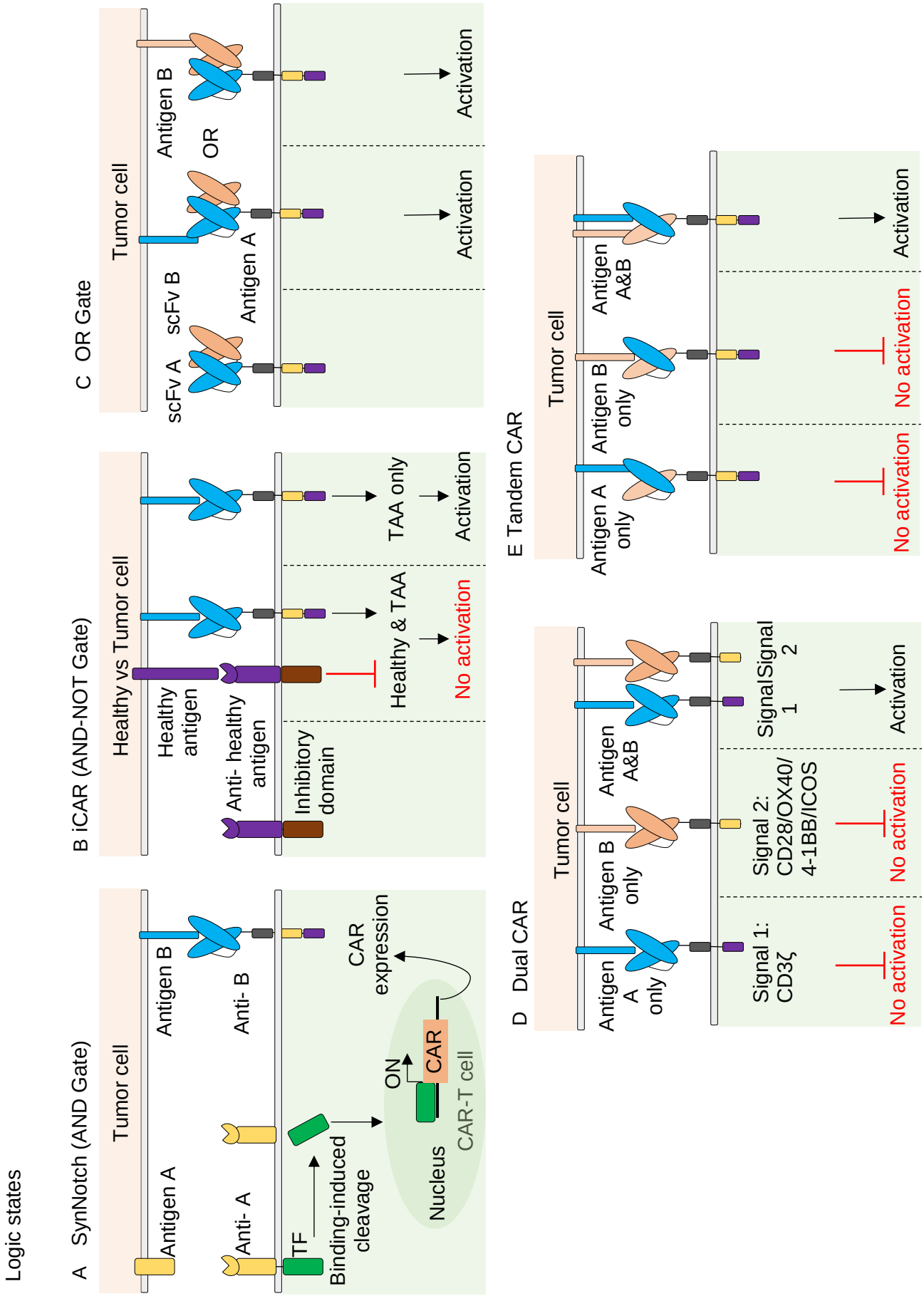

Logic states
A SynNotch (AND Gate)
Tumor cell
Antigen A
Anti- A
TF
Binding-induced cleavage
Nucleus
ON
CAR
CAR-T cell
Antigen B
Anti- B
CAR expression
B iCAR (AND-NOT Gate)
Healthy vs Tumor cell
Healthy antigen
Anti- healthy antigen
Inhibitory domain
Healthy & TAA
No activation
TAA only
Activation
C OR Gate
Tumor cell
scFv A
scFv B
OR
Antigen A
Antigen B
Activation
Activation
D Dual CAR
Tumor cell
Antigen A only
Antigen B only
Antigen A&B
Signal 1: CD3ζ
Signal 2: CD28/OX40/ 4-1BB/ICOS
Signal 1
Signal 2
Activation
No activation
No activation
E Tandem CAR
Tumor cell
Antigen A only
Antigen B only
Antigen A&B
Activation
No activation
No activation

Figure 5

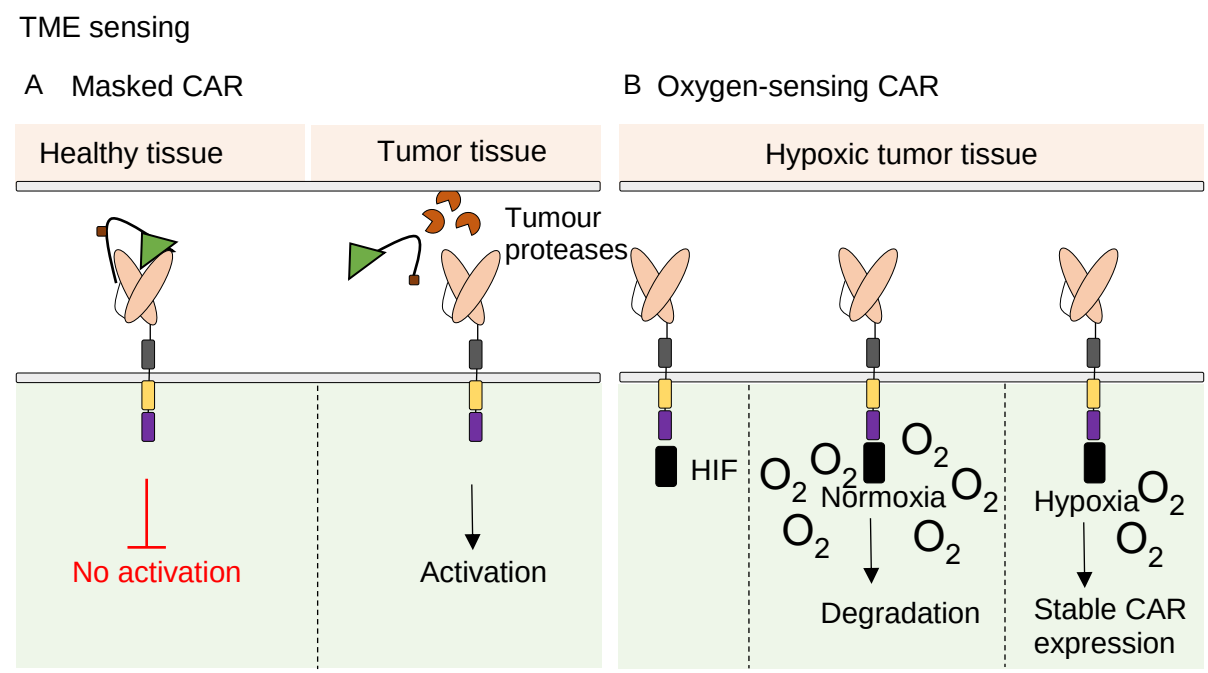

TME sensing
A Masked CAR
B Oxygen-sensing CAR
Healthy tissue
Tumor tissue
Hypoxic tumor tissue
Tumour proteases
No activation
Activation
HIF
Normoxia
Degradation
Hypoxia
Stable CAR expression

Figure 6

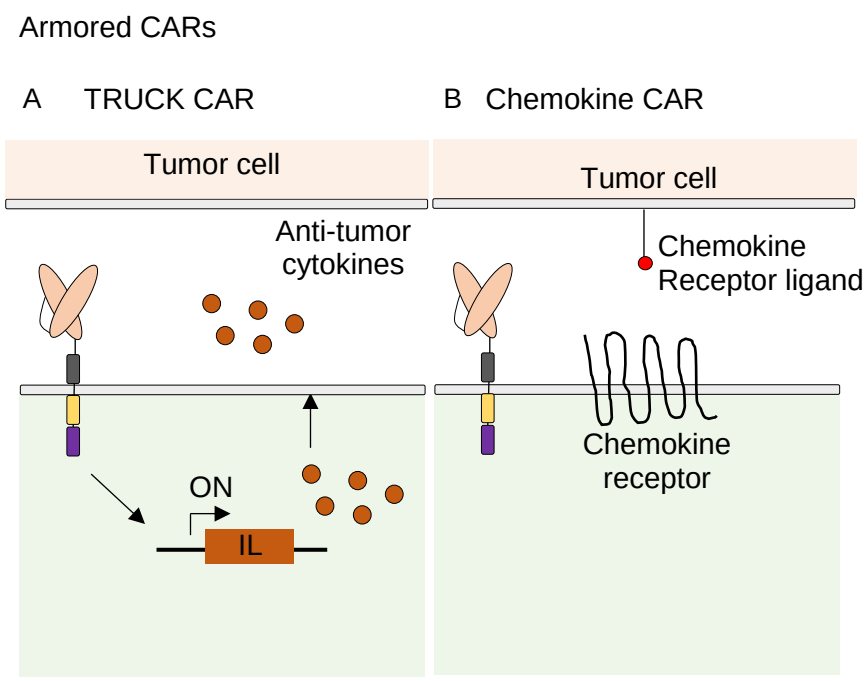
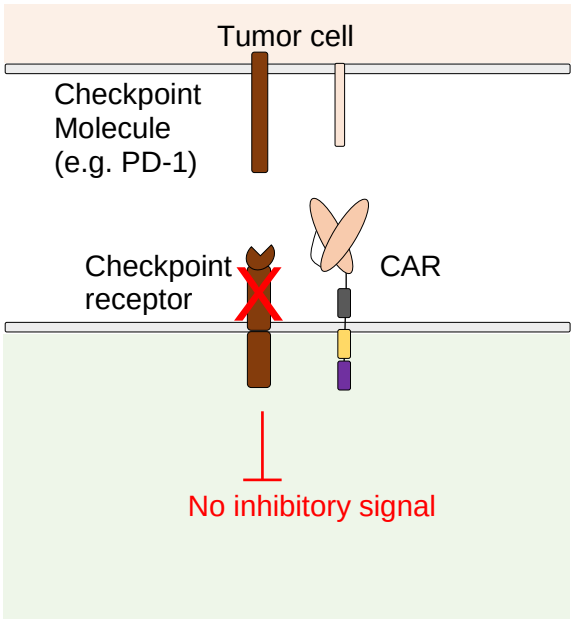

Armored CARs
A TRUCK CAR
B Chemokine CAR
Tumor cell
Anti-tumor
cytokines
ON
IL
Tumor cell
Chemokine
Receptor ligand
Chemokine
receptor

Figure 7

A Anti- immunosuppression CARs

B Anti-TME-cell directed CARs



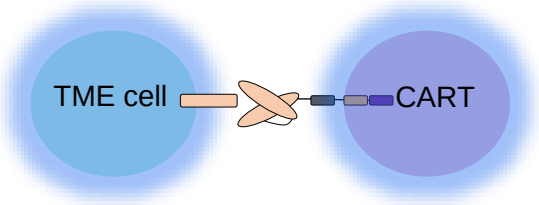


Figure 8

Table 1 Heterogeneity of the human T cell compartment. The combinatorial expression of multiple markers on the cell surface, as determined by multicolor flow cytometry, allows to identify 6 major T cell subsets. T cells that have not yet encountered an antigen and are therefore not activated, are called naïve T (T_N) cells. Activated T cells rapidly proliferate and exert effector functions such as cell-mediated cytotoxicity and cytokine production. While differentiating (through activation) from T_N, T_{SCM} to T_{CM}, $T_{RM/TM}$, T_{EM} and culminating in T_{EF} cells, memory T cells progressively lose or acquire specific functions such as self-renewal and survival. T_N; naïve, T_{SCM}; stem cell memory, T_{CM}; central memory, $T_{RM/TM}$; resident memory/ tissue memory, T_{EM}; effector memory, T_{EF}; effector; CD, cluster of differentiation.

	T_N	T_{SCM}	T_{CM}	$T_{RM/TM}$	T_{EM}	T_{EF}
CD45RO	-	-	+	+	+	-
CD45RA	+	+	-	-	-	+
CCR7	+	+	+	-	-	-
CD62L	+	+	+	-	-	-
CD28	+	+	+	+	-	-
CD95	-	+	+	+	+	+

Table 2 Summary of clinical trials cited. iCasp9; i Caspase9, GD2; disialoganglioside, TRBC1; T cell receptor beta constant 1, , EGFRt; truncated epithelial growth factor receptor, BCMA; B cell maturation antigen, PD-1; programmed cell death-1; PD-L1; programmed cell death-ligand 1, HER2; human epidermal growth factor 2, NK; natural killer, FOLFOX; folinic acid, fluorouracil, oxaliplatin.

Clinical trial number (NCT)	Enrolled patients	Phase of clinical trial	Sponsor	Modality
NCT02435849, ELIANA, CTL019	81	II	Novartis Pharmaceuticals	CD19 CAR
NCT02348216, ZUMA-1, KTE-C19	290	I/II	Kite, A Gilead Company	CD19 CAR
NCT01626495	76	I/II	University of Pennsylvania	CD19 CAR
NCT03579927	36	I/II	M.D. Anderson Cancer Center	CD19 CAR, iCasp9
NCT02414269	66	I	Memorial Sloan Kettering Cancer Center	Mesothelin CAR, iCasp9
NCT02107963	15	I	National Cancer Institute (NCI)	GD2 CAR,iCasp9
NCT01822652, GRAIN	11	I	Baylor College of Medicine	GD2 CAR,iCasp9
NCT03590574	55	I/II	Autolus	TRBC1 CAR, RQR8
NCT02746952, CALM, UCART19	40	I	Institut de Recherches Internationales Servier	CD19 CAR, RQR8, allogeneic
NCT03085173	37	I	Memorial Sloan Kettering Cancer Center	CD19 CAR, EGFRt
NCT03618381	36	I	Seattle Children's Hospital	CD19 CAR, EGFRt
NCT02051257	51	I	City of Hope Medical Center	CD19 CAR, EGFRt
NCT03070327	36	I	Memorial Sloan Kettering Cancer Center	BCMA CAR, EGFRt
NCT02028455	80	I/II	Seattle Children's Hospital	CD19 CAR, EGFRt
NCT02146924	22	I	City of Hope Medical Center	CD19 CAR, EGFRt

NCT01865617	203	I/II	Fred Hutchinson Cancer Research Center	CD19 CAR, EGFRt
NCT02937844	20	I	Beijing Sanbo Brain Hospital	PD-1 CAR, EGFRt
NCT03638167	36	I	Seattle Children's Hospital	EGFR CAR, EGFRt
NCT02311621	40	I	Seattle Children's Hospital	CD171 CAR, EGFRt
NCT03114670	20	I	Affiliated Hospital to Academy of Military Medical Sciences	CD123 CAR, EGFRt
NCT02159495	42	I	City of Hope Medical Center	CD123CAR, EGFRt
NCT03233854	57	I	Crystal Mackall, MD	CD19/CD22 CAR
NCT03241940	50	I	Crystal Mackall, MD	CD19/CD22 CAR
NCT03463928	10	I	Chinese PLA General Hospital	CD19/CD22 CAR
NCT03271515	20	I	Beijing Doing Biomedical Co., Ltd.	CD19/CD20 CAR
NCT03767751	80	I/II	Chinese PLA General Hospital	CD38/BCMA CAR
NCT02498912	30	I	Memorial Sloan Kettering Cancer Center	MUC16^{ecto}CAR/EGFRt/ IL-12-secreting
NCT02650999	12	I/II	Abramson Cancer Center of the University of Pennsylvania	PD-1 block (pembrolizumab)/CD19 CAR
NCT02926833, ZUMA-6	37	I/II	Kite, A Gilead Company	PD-L1 block (atezolizumab)/ CD19 CAR
NCT02706405	42	I	Fred Hutchinson Cancer Research Center	PD-L1 block (durvalumab)/ CD19 CAR

NCT00889954	20	I	Baylor College of Medicine	HER2 CAR/dominant-negative TGFbeta
NCT02873390	20	I/II	Ningbo Cancer Hospital	PD-1 antibody/EGFR CAR
NCT02862028	20	I/II	Shanghai International Medical Center	PD-1 antibody/EGFR CAR
NCT03545815	10	I	Chinese PLA General Hospital	CRISPR/Cas9-mediated PD-1 knockout/Mesothelin CAR
NCT02459067	8	II	TC Biopharm	γδ T
NCT03533816	38	I	University of Alabama at Birmingham	γδ T
NCT03608618, CARMA	15	I	MaxCyte, Inc.	Mesothelin macrophage CAR
NCT03056339	36	I/II	M.D. Anderson Cancer Center	CD19 NK CAR, cord-blood-derived
NCT01974479	20	I	National University Health System, Singapore	CD19 CAR NK
NCT02944162	10	I/II	PersonGen BioTherapeutics (Suzhou) Co., Ltd.	CD33 CAR NK
NCT02742727	10	I/II	PersonGen BioTherapeutics (Suzhou) Co., Ltd.	CD7 CAR NK
NCT02892695	10	I/II	PersonGen BioTherapeutics (Suzhou) Co., Ltd.	CD19 CAR NK, NK-92-derived
NCT00995137	14	I	St. Jude Children's Research Hospital	CD19 CAR NK
NCT03415100	30	I	The Third Affiliated Hospital of Guangzhou Medical University	G2D CAR NK
NCT02839954	10	I/II	PersonGen BioTherapeutics	MUC1 CAR NK

			(Suzhou) Co., Ltd.	
NCT02735083, UCART19	200	I	Institut de Recherches Internationales Servier	CD19 CAR, RQR8, allogeneic
NCT03190278, UCART123	162	I	Cellectis S.A.	CD123 CAR, allogeneic
NCT03018405, THINK, CYAD-01	146	I/II	Celyad	CAR-T, expressing NK activating receptor (NKR-2 CART)
NCT03612739, EPITHINK	15	I	Celyad	NKR-2 CART, allogeneic, 5-azacytidine
NCT03692429, alloSHRINK	36	I	Celyad	NKR-2 CART, allogeneic, FOLFOX chemotherapy