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REVIEW ARTICLE

CAR-T Cells: Next Generation Cancer Therapeutics

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Abstract | Chimeric antigen receptors (CAR) are synthetic receptors consisting of recognition domains derived from antibodies coupled to the signaling domains of T cells. CAR-modified T-cell therapies have shown dramatic remissions in the treatment of B-cell-derived malignancies. This review examines the different factors involved in CAR-T cell design such as design of the synthetic receptor, choice of T-cell subset and tumor target. Further, we discuss the promise of the initial clinical trials in hematological malignancies and the obstacles for translation of CAR-T cell therapies in solid tumors. The review also describes the use of safety circuits designed in CAR-T cells to minimize off-tumor toxicity. The combination of these approaches will help facilitate effective translation of CAR-T cell therapies.

1 Introduction

Adoptive T-cell therapies represent the next generation of personalized cancer therapies, where autologous T cells isolated from the blood of the patient are enriched or re-engineered in vitro to produce tumor-specific T cells and then are transferred back into the patient. The two main types of adoptive T-cell therapies are T-cell receptor (TCR) and chimeric antigen receptor (CAR)-based adoptive T-cell therapies where each of these modalities have a distinct set of advantages[1]. Adoptively transferred T cells with naturally occurring or engineered/affinity-enhanced TCRs are able to target both intracellular and extracellular proteins although in a major histocompatibility complex (MHC)-restricted fashion. Chimeric antigen receptors (CARs) are hybrid receptors consisting of the single-chain fragment variable (scFv) of antibodies coupled to the signaling domain of a T-cell receptor. Since the recognition domain of a CAR-modified T cell is antibody-based, they can recognize only extracellular targets but they do so in a MHC-independent context and are not limited by the HLA makeup of the patient[2].

2 Receptor Optimization

The earliest CARs or T bodies consisted of the fusion of the scFv domain, spacer region, transmembrane domain coupled to the signaling components consisting of immunoreceptor tyrosine-based activation motif (ITAM) chains of the CD3ζ or Fc receptor (FcRγ) capable of promoting T-cell activation (Fig. 1)[3–5]. These first generation CARs were tested clinically in hematological malignancies targeting the CD20 antigen in relapsed or refractory B-cell lymphomas and in solid tumors targeting the L1-cell adhesion molecule (L1-CAM) in glioblastomas and the α-folate receptor (FR) in patients with metastatic ovarian cancer[6–8]. The anti-tumor activity in these early clinical trials with first generation CARs was disappointing possibly due to lack of cytokine response in the context of repeated antigen exposure but provided insights that expansion and persistence of adoptively transferred T cells is a key determinant of anti-tumor efficacy[9].

The next generation of CAR receptors were engineered with costimulatory endodomains derived from either the CD27, CD28, 4-1BB, ICOS or OX-40 molecules in tandem with the activation domain of the first generation CARs giving rise to either second-generation CARs with a single costimulation or third generations CARs with multiple tandem costimulation domains in the same CAR construct (Fig. 1)[10–15]. The addition of in situ costimulation greatly boosted the efficacy of first generation CARs leading to higher cytokine production, T-cell proliferation and anti-tumor efficacy in preclinical mouse models;

Adoptive T cell therapy: T cells that are transferred back into patients after ex vivo manipulation.

Major histocompatibility complex: Cell surface protein that presents foreign peptides for recognition by T-cell receptors.

Costimulation: Second signal from antigen presenting cell to rescue T cell from anergy after antigen-specific activation through T-cell receptor (TCR).

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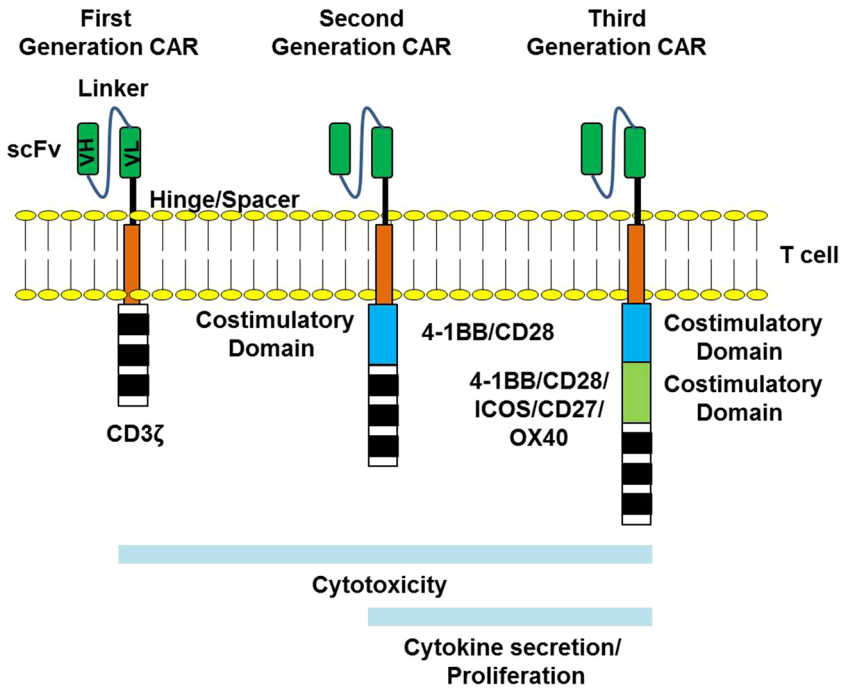

Figure 1: Structure of chimeric antigen receptors comprises of the single-chain fragment variable region (scFV) of the antibody as the recognition domain connected to a spacer and transmembrane region, which is connected to the CD3ζ activation domain in first generation CARs or in tandem with the CD3ζ activation domain and one (second generation) or two costimulatory domains (third generation).

however, the optimal costimulation combination has not been determined[10, 12, 16, 17]. The second-generations CAR best characterized in literature are the CARs with either CD28 or 4-1BB costimulation where the CD28s generation CAR has been associated with robust initial effector function but lower persistence while the 4-1BB CAR depicts higher resistance to activation-induced cell death and longer persistence in vivo. Further, the choice of costimulation has been proposed to alter cell fate in the CAR-modified T cells where CAR activation with CD28 costimulation leads to a more glycolytic phenotype with an increase in effector memory T cells while 4-1BB costimulation leads to increase in the generation of central memory T cells with enhanced respiratory capacity, mitochondrial biogenesis and fatty acid oxidation[18]. The inclusion of the CD28 costimulatory domain has also been shown to increase the propensity of antigen independent tonic signaling in CAR-T cells, which has also been correlated with decreased in vivo functionality[19].

Activation-induced cell death: Programmed cell death of T cells due to repeated triggering of TCR.

Further, CD27 costimulation in the second-generation CARs has also shown to prevent antigen-induced cell death through upregulation of the anti-apoptotic Bcl-X_L protein similar to 4-1BB CARs warranting an evaluation of these costimulatory molecules while ICOS signaling in CD4 Th17 cells has been shown to increase anti-tumor efficacy[10, 20]. Preclinical data from third generation CARs has been conflicting with studies indicating that two costimulatory modules CD28 and 41-BB in tandem improved CAR-T cell signaling and in vivo functionality, whereas other studies indicated that the tandem modules CD28 and OX-40 increase activation-induced cell death and decreased anti-tumor efficacy[21–24]. The optimal costimulation is likely to be different in CD8 and CD4 CAR-T cells and may also be affected by the level of antigen expression, scFv affinity and the inhibitory tumor environment.

3 CAR-T Cells—Affinity and Spacer Modifications

The efficacy of the CAR-T cell has been shown to be dependent on the affinity of the antibody from which the scFv of the CAR receptor is derived. Previous studies have shown that CAR-T cells derived from antibodies of higher affinities have increased in vitro cytokine production and proliferation resulting in higher vivo anti-tumor activity[25, 26]. However, reports have shown high-affinity CARs do not discriminate between high and low levels of target expression[25, 27–29]. CAR-T cells targeting EGFR and Erbb2 built from lower affinity-tuned scFvs were able to eradicate EGFR

and Erbb2 high tumor cells as effectively as high-affinity CARs while sparing normal cells expressing lower amounts of these targets, which were also targeted by high-affinity CARs[27, 28, 30]. The choice of a high-affinity- versus affinity-tuned scFv moiety will likely depend on the level of target expression on the tumor cells and the exclusivity of antigen expression on tumor cells.

The spacer region between the scFv and the transmembrane domain affects CAR-T cell function and has been shown to be dependent on the location of the target epitope on the tumor cell, which binds to the CAR-T cell. The spacer sequences have largely been derived from the fragment crystallizable region (Fc region) of either IgG1 or IgG4 antibody, where the short spacer consist of only the hinge region (12 amino acids), intermediate consists of hinge-CH_3 (119 amino acids), and the long spacer consists of hinge-CH_2-CH_3 region (229 amino acids)[26, 31]. CAR-T cells recognizing a membrane distal epitope on the tumor cell have been shown to possess superior in vitro anti-tumor activity and in vivo tumor eradication with a short spacer region compared to an intermediate or long-spacer region[26, 32, 33]. However, CAR-T cells that recognize an epitope proximal to the tumor membrane require a long spacer for optimal cytolytic activity and cytokine secretion[34]. However, multiple studies demonstrated that long-spacer CARs that possess the hinge–CH_2–CH_3 region of IgG1 or IgG4 Fc receptor do not persist in vivo even in tumor-free mice due to premature activation-induced cell death of the CAR-T cells caused by interaction with Fc domain with Fc receptor-bearing myeloid cells[31, 35]. Modifying the region in the CH_2 domain of the long spacer abrogated the interaction with the Fc receptor restoring the in vivo persistence and anti-tumor activity of the long-spacer CAR-T cell[35, 36].

Further with the advent of protein engineering, multiple groups are testing non-scFv-based protein scaffolds such as Designed Ankyrin Repeat Proteins and adnectins and high-affinity tumor-specific peptides as recognition motifs in CARs[37–41]. These scFv- and non-scFv-based scaffolds will improve the breadth of screening available for new candidate targets. However, factors such as host immunogenicity and cross-reactivity will have to be evaluated for these scaffolds.

4 T-cell Subset

The pool of T cells used for manufacturing CAR-T cells comprises of naïve T cells (T_N) and antigen-experienced memory T cells, which consists of central memory (T_{CM}) and effector memory T cells (T_{EM}). Preclinical studies have shown that adoptively transferred antigen-specific T cells derived from the central memory pool persist longer than effector memory T cells in the rhesus macaque[42]. Heavily pretreated patients who are candidates for CAR-T cell clinical trials possess a lot of heterogeneity in the number of CD8 and CD4 T cells and a higher frequency of effector memory T cells[43]. Preclinical work conducted in human xenograft mouse models demonstrated that CAR-T cells derived from naïve CD4 T cells and central memory CD8 T cells demonstrated the highest anti-tumor activity at a suboptimal T-cell dose[43, 44]. CAR-T cells transduced into naïve and central memory T cells retained a higher percentage of central memory CAR-T cells at the end of culture compared to CAR-T cells derived from effector memory T cells. Further, CD4 CAR-T cells augmented proliferation of central memory CD8 T cells with naïve CD4 CAR-T cells providing the greatest enhancement of CD8 CAR-T cell proliferation[43]. The combination of naïve CD4 T cells and central memory CD8 T cells in 1:1 ratio also correspondingly exhibited synergistic activity in mouse tumor models promoting superior anti-tumor clearance compared to CAR-T cells derived solely from CD8, CD4 or unselected PBMCs[43]. These studies provide strong rationale to use defined cell compositions for CAR-T cell products in clinical trials to achieve uniform efficacy of the CAR-T cell product for a defined dose[45].

5 Choice of Targets and Normal Tissue Expression

The choice of target has been critical to the translation of CAR-T cell therapies. For CAR-T cell therapy to be successful, one assumes that the target antigen has to be expressed at high levels and homogenously by the tumor while normal tissue expression has to be low or restricted to non-vital tissues. This dogma has worked favorably in regard to targeting B-cell-derived hematological malignancies due to the targeting of the lineage antigen CD19, which is involved in B-cell receptor signaling as B-cell malignancies derived from all stages of B-cell maturation express high and homogenous levels of CD19[46]. Further, the off-tumor targeting of normal B cells leads to B-cell aplasia, which is a manageable side-effect with intravenous immunoglobulin (IVIG) therapy[47]. Other targets pursued in B-cell malignancies such as CD20 and CD22 have a similar restricted expression in the normal

Immunogenicity: Ability of an epitope to evoke humoral or cell-mediated immune responses.

B cell aplasia: Low numbers or absence of mature B cells.

B-cell lineage[48, 49]. CAR-T cell therapy in other hematological malignancies such as acute myeloid leukemia (AML) do not have the advantage of tumor-restricted targets and targeting of AML targets such as CD123 or CD33 is often associated with significant hematopoietic toxicities due to expression on normal hematopoietic cells[50–52].

The choice of CAR-T cell target is much more restricted in solid tumors as few tumor targets such as EGFR variant III or abnormally glycosylated Mucin1 are expressed solely in tumors and not in normal tissues[53, 54]. A few cases illustrate the importance of considering normal tissue toxicity when choosing tumor targets. ERBB2 directed CAR-T cells generated to target ERBB2 high tumors caused respiratory failure and multi-organ dysfunction in a patient attributable to low expression of ERBB2 on lung epithelial cells while carboxy-anhydrase-IX (CAIX)-specific CAR-T cells targeting metastatic renal cell carcinoma resulted in liver toxicity[55, 56]. Early trials in two patients with mesothelin-targeted CARs on the other hand showed anti-tumor activity without clinical toxicity to normal pleural cells expressing the target[57]. Further, the safety of ROR1-directed CARs has been tested in normal human primates with similar ROR1 normal tissue expression as humans and no toxicities were observed in this model[58, 59]. Strategies such as the choice of affinity-tuned CARs to discriminate high versus low expression in target cells, intrapleural or local administration for mesothelin CAR-T cells and the evaluation of safety in preclinical animal models with similar normal tissue expression will help refine the choice of antigen for CAR-T cell therapies[27, 28, 30, 60]. A list of hematological and solid tumor targets currently being pursued preclinically and in clinical trials is described elsewhere with the predominant clinical targets summarized in Table 1[61, 62].

6 Clinical Trials in Hematological Malignancies

B-cell derived cancers were the ideal frontier for clinical testing of CAR-T cells due to the uniform expression of the B-cell lineage marker CD19 on disparate B-cell derived cancers from different stages of B-cell development and different clinical behaviors. Furthermore, since CD19 expression is restricted to the B-cell compartment, off-tumor normal tissue toxicity caused by CD19-specific CAR-T cells is restricted to normal B cells causing B-cell aplasia[47].

Multiple PhaseI/II clinical trials have demonstrated an impressive complete response rate (70–93%) in relapsed or refractory acute lymphoblastic leukemia (B-ALL) derived from immature B cells in both adult and pediatric patients[45, 63–67]. CD19-specific CAR-T cells have also demonstrated high overall response for other lymph node malignancies such as chronic lymphocytic leukemia (CLL), non-Hodgkin lymphoma (NHL) and diffuse large B-cell lymphoma (DBCL) that arise from mature B cells though the

Table 1: Clinical CAR targets in hematological and solid tumors.

CAR target	Malignancies	Clinical trial (phase)
CD19	Non-Hodgkin lymphoma (NHL), acute lymphoblastic leukemia (B-ALL), chronic lymphocytic leukemia (CLL), diffuse large B cell lymphoma (DBCL), mantle cell lymphoma (MCL)	FDA approved for childhood ALL and NHL
CD20	DBCL, MCL, follicular lymphoma (FL)	Phase I/II (NCT02965157)
CD22	FL, NHL, B-ALL	Phase I (NCT02315612)
NKG2D	Leukemia	Phase I (NCT02203825)
BCMA	Multiple myeloma	Phase I (NCT02546167)
CD138	Multiple myeloma	Phase I/II (NCT01886976)
Lewis Y	Multiple myeloma	Phase I (NCT01716364)
ROR1	CLL, MCL, B-ALL, triple negative breast cancer (TNBC), lung adenocarcinomas	Phase I (NCT02706392)
EGFR EGFR variant III	Advanced solid tumors Glioblastoma	Phase I/II (NCT01869166) Phase I/II (NCT01454596)
c-met	TNBC	Phase I (NCT01837602)
HER2	Sarcoma, metastatic cancer, solid tumors	Phase I/II (NCT00924287)
Mesothelin	Cervical, pancreatic, ovarian, mesothelioma, lung cancer	Phase I/II (NCT01583686)

complete rate has been lower than B-ALL[68–73]. The potent anti-tumor activity of CD19-CARs in the clinical trials was accompanied by life-threatening toxicities, most commonly cytokine release syndrome (CRS) due to the elevated levels of inflammatory cytokines especially in patients with high tumor burden[74, 75]. Neurological toxicities have also been reported in patients following infusion of CD19 CAR-T cells due to the increased permeability of the blood brain barrier due to high cytokine levels[75]. Corticosteroids or IL-6 receptor blockade with tocilizumab has been shown to cause an immediate reversal of CRS with tocilizumab being used as the front line therapy for CRS as prolonged use of corticosteroids causes ablation of the CAR-T cells limiting their long-term function[64]. The use of a defined cell composition (CD4/CD8 ratio) of CAR-T cells enabled risk stratified dosing based on the patient tumor burden, which decreased the off-tumor toxicities[45].

CD8 T-cell-mediated anti-CAR responses have developed in some patients due to the immunogenic murine scFv sequences used in the initial CD19 CAR trials, which has severely limited T-cell persistence in patients[45]. The addition of fludarabine to the cyclophosphamide lymphodepleting regimen prior to CAR-T cell transfer significantly improved CAR-T cell persistence and delayed anti-CAR immune responses[45, 67, 71]. The use of fully human scFvs as recognition domains for CARs should also reduce host immune responses against the CAR construct[76]. A minority of patients treated with CD19 CAR-T cells relapse with tumors that have lost the CAR-T cell epitope due to alternative splicing or acquire CD19-negative myeloid switch phenotype, which facilitates immune escape of the tumor[77, 78]. This has led to the targeting of other B-cell markers such as CD20 or CD22 or the development of bispecific CAR T cells[79–81].

Another promising target that is being currently targeted by CAR-T cells is the B-cell maturation antigen (BCMA) in multiple myeloma, an incurable malignancy derived from plasma cells. Preclinical mouse models depicted promising anti-tumor clearance of $BCMA^{+}$ tumor cells and significant anti-myeloma activity was observed in early results from clinical trials with toxicities similar to the CD19 CAR-T cells[82, 83]. The promising results in clinical trials has generated a lot of promise for CAR-T cell therapy in the treatment of hematological malignancies and has led to the approval of CD19-targeted CAR-T cell therapies by the Food and Drug Administration (FDA) in United States for patients with certain types of B-ALL and NHL.

7 Challenges in Solid Tumors

The remarkable success of CAR-T cells in patient with advanced hematological malignancies has generated considerable anticipation about their efficacy in solid tumors. Solid tumors, however, are inherently more difficult to target for multiple reasons. Tumor-unique CAR-T cell antigens which are not expressed in normal tissues but show homogenous expression in the tumor are rare. Neoepitopes such as EGFR variant III in glioblastomas or abnormally glycosylated Mucin 1 present good targeting opportunities in a minority of cancers[53, 54]. Choosing CAR moieties of intermediate affinity to differentiate high tumor expression while sparing normal cells with lower target expression has also reduced the potential for off-tumor toxicity[27, 28, 30]. Another challenge that CAR-T cells face in solid tumors is their ability to traffic and infiltrate solid tumors. This challenge has been overcome in by intra-tumoral or regional administration of CAR-T cells in disease indications such as glioblastoma or pleural malignancies[84, 85]. Further, other groups are incorporating chemokine receptors such as CXCR2 or CCR2b into CAR-T cells to promote selective migration of T cells to tumors secreting the corresponding cytokines CXCL5 or CCL2[86, 87].

One of the big barriers to the success of CAR-T cells in solid tumors is the inhospitable immunesuppressive environment. Late-stage solid tumors have evolved numerous suppressive pathways and cell types to evade the endogenous immune system. Solid tumors are infiltrated by highly immunesuppressive cells such as regulatory T cells, myeloid-derived suppressor cells, plasmacytoid dendritic cells and tumor-associated macrophages[88]. These cell populations secrete numerous inhibitory cytokines such as transforming growth factor (TGF-β), prostaglandin E2 (PGE-2), interleukin4 (IL-4) and interleukin10 (IL-10), which dampen the cytotoxic activity of the adoptively transferred T cell and skews the immune response towards a Th2 phenotype. Further, adoptively transferred T cells can be inhibited by engagement of their coinhibitory receptors (e.g. CTLA-4, PD-1, LAG-3, TIM-3) by the corresponding immunesuppressive ligands expressed by tumor cells or other immunesuppressive cells. T cells are also significantly inhibited by the hypoxic environment in the tumor and the lack of nutrients such as arginine and

Cytokine release syndrome: non-antigen-specific toxicity that occurs due to high level of immune activation than that occurs under natural settings often associated with immune therapies.

Neoepitopes: Peptides generated by somatic mutations distinguished as non-self.

Desmoplastic stroma: Dense fibrosis that surrounds neoplasm creating a barrier to entry of T cells.

Checkpoint: immune checkpoints are proteins that are regulators of the immune response by either stimulating or inhibiting an ongoing response.

tryptophan[89–91]. Solid tumors are also surrounded by a desmoplastic stroma with high levels of extracellular matrix elements that limit T-cell entry into tumors[92].

CAR-T cell approaches incorporating systemic or cell-intrinsic PD-1 blockade show enhanced anti-tumor activity in preclinical models and are being actively pursued in the clinic[93–95]. Further, armored CAR-T cells secreting the proinflammatory cytokine interleukin12 (IL-12) reverse the immunesuppressive environment to a pro-inflammatory Th1 phenotype[96]. CAR-T cells incorporating chimeric switch receptors with the extracellular domain of the inhibitory receptor PD-1 with the endodomain of CD28 have been tested for their ability to convert T-cell inhibitory signals to costimulatory signals[97]. CAR-T cells targeting the fibroblast activation protein (FAP) expressed in cancer stroma or targeting tumor vasculature are being tested in addition to targeting of tumor cells[92, 98]. Numerous approaches are being pursued to build CAR-T cells to resist the immune environment and combinations often show synergistic increases in anti-tumor activity[99]. Engagement of the endogenous T-cell response may also be beneficial in solid tumors especially in the case of antigen escape and the ability of CAR-T cells to promote cross-presentation and facilitate endogenous T-cell infiltration in this setting needs to be evaluated[100]. The early clinical trials in solid tumors will help guide development of future combination or gene-editing strategies.

8 Safety Switches

The Yin of a powerful CAR-T cell always has to be balanced with the Yang of normal tissues toxicities due to low expression of the CAR target. A couple of strategies have been built into CAR-T cells to enhance their safety, which range from ablation of CAR-T cells to development of synthetic circuits to enhance selectivity to tumor cells (Fig. 2). To ablate CAR-T cells in case of normal tissues toxicity, researchers have incorporated suicide genes such as drug-inducible caspase 9 or tags such as truncated EGFR in CAR-T cells that can be depleted with antibodies targeting the tag (Fig. 2a)[101, 102].

Synthetic approaches to enhance tumor selectivity mainly are built on AND logic gate approaches where the presences of two antigens increase the activity or turns on the expression of the CAR molecule on T cells. The activation of the CD3ζ domain of the CAR is driven by the recognition of one target while the costimulation CD28 domain is turned on by the presence of the second antigen[103]. The presence of both antigens is required for maximal function of the CAR-T cells; therefore, normal tissues expressing just one of the target antigens are spared from CAR-T cell toxicity (Fig. 2b). Further, inhibitory circuits have been developed where the recognition of the second antigen dampens the activity of the CAR when the recognition domain is connected to the endodomain of an inhibitory checkpoint moiety such as PD-1 or CTLA-4 (Fig. 2c)[104].

Another method to enhance CAR selectivity to tumor tissues is through the development of synthetic Notch receptors, where the expression of one tumor antigen drives cleavage of the Notch endodomain causing transcriptional activation and expression of the CAR, which recognizes a different tumor target (Fig. 2d)[105]. Drug-inducible CARs where the presence of a heterodimerizing small molecule is required for assembly of the recognition and signaling domains of the CAR allow for precise control of timing and titratable activity of the CAR-T cell[106].

9 Future Opportunities

The field of adoptive CAR-T cell therapy has a list of formidable tools under its belt to combat advanced malignancies. For durable anti-tumor responses, CAR-T cell will have to infiltrate tumors, resist the inhospitable environment and a multitude of immunesuppressive mechanisms, and tackle antigen loss variants while not causing significant off-tumor toxicities. This will require the development of custom circuits depending on dominant suppressive mechanisms at play in solid or hematological malignancies. Further, the development of universal CAR-T cells with gene editing from healthy donors or the targeting of a universal tag combined with antibody-based molecular switches provide the exciting opportunity of 'off-the-shelf" CAR-T cells[107, 108]. The CAR-T cell field would also benefit from more mechanistic insights into signaling cascades turned on by CAR engagement compared to physiological TCR signaling. Further, the development of bioinformatics tools to predict CAR design instead of empirical testing would enable a more rational design of the next generation of CAR T cells. The testing of CAR-T cells in relevant preclinical tumor models representative of the human tumor microenvironment will also help predict the factors essential for clinical success[109]. CAR-T cell therapy initially introduced in the United States is clearly spreading to other

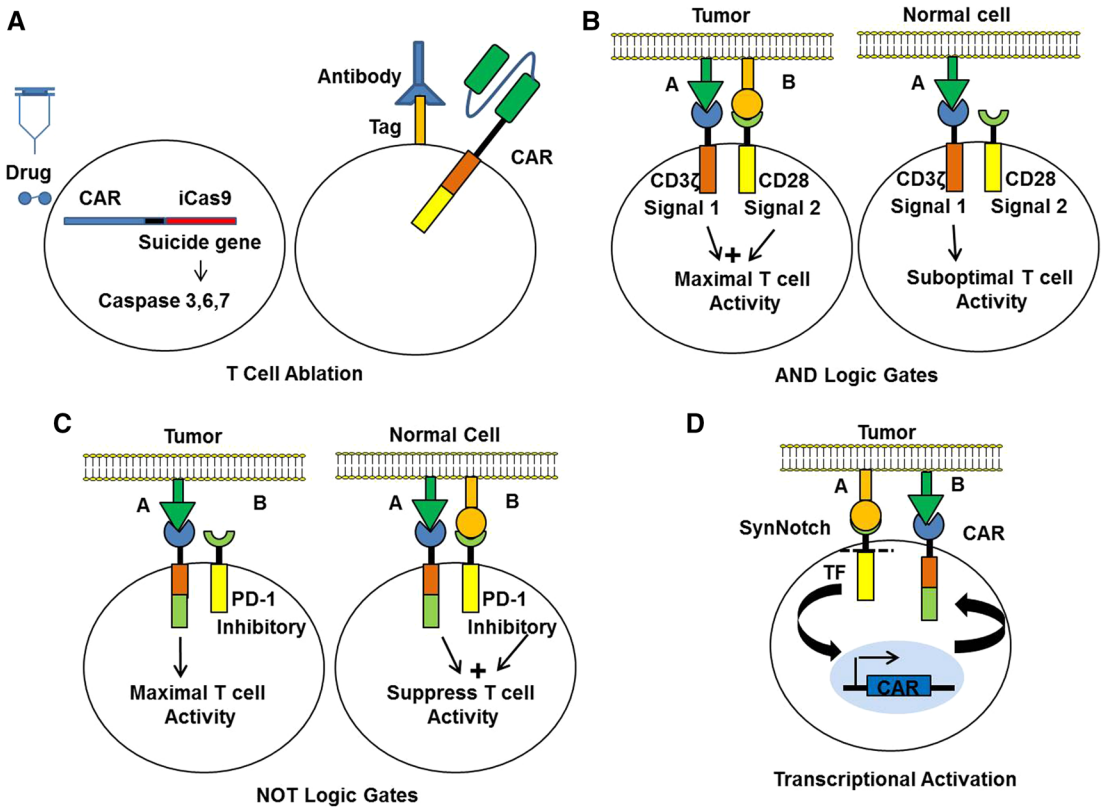

Figure 2: Safety circuits in CAR-T cells include **a** targeted ablation of CAR-T cells. **b** Spilt receptor signaling to confer maximal activity on expression of two antigens. **c** Split receptor to dampen signal in the presence of two antigens. **d** Transcriptional activation/expression of CAR driven by independent antigen.

countries in the world with a large number of clinical trials being conducted in China followed by Europe[110]. Improvements towards the ease of manufacturing cellular therapies will help facilitate translation of CAR-T cell therapy on a global scale.

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