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Rationally designed approaches to augment CAR-T therapy for solid tumor treatment

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ABSTRACT

Chimeric antigen receptor T cell denoted as CAR-T therapy has realized incredible therapeutic advancements for B cell malignancy treatment. However, its therapeutic validity has yet to be successfully achieved in solid tumors. Different from hematological cancers, solid tumors are characterized by dysregulated blood vessels, dense extracellular matrix, and filled with immunosuppressive signals, which together result in CAR-T cells' insufficient infiltration and rapid dysfunction. The insufficient recognition of tumor cells and tumor heterogeneity eventually causes cancer reoccurrences. In addition, CAR-T therapy also raises safety concerns, including potential cytokine release storm, on-target/off-tumor toxicities, and neuro-system side effects. Here we comprehensively review various targeting aspects, including CAR-T cell design, tumor modulation, and delivery strategy. We believe it is essential to rationally design a combinatory CAR-T therapy *via* constructing optimized CAR-T cells, directly manipulating tumor tissue microenvironments, and selecting the most suitable delivery strategy to achieve the optimal outcome in both safety and efficacy.

1. Introduction

Tumor-specific T cells play crucial roles in mediating tumor regression *via* antigen-dependent killing programs [1]. The recent decade has observed the rapid advancement in chimeric antigen receptor T (CAR-T) cell therapeutic modality for cancer treatment, in which nearly 90 % of complete remission is achieved in treating acute lymphoblastic leukemia [2,3]. The "living therapeutics", CAR-T cells, can recognize the specific antigen on tumor cell membranes, exert tumoricidal effects, and proliferate *in vivo*, which usually only requires "one shot" to fulfill their therapeutic potency [4].

Current CAR-T products are all for hematological cancer treatment,

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including Abecma, Breyanzi, Kymriah, Tecartus, Yescarta, and Carvykti, while none have been approved for solid tumor management [5–7]. Compared with hematological cancers, solid tumors are featured by dysregulated blood vessels and tumor-dense extracellular matrix impeding circulating CAR-T cells' tumor infiltration, and robust immunosuppressive signals that lead CAR-T cells into an exhausted and dysfunctional state [8]. Recently, Straathof et al. reported disialoganglioside GD2 CAR-T therapy for treating relapsed neuroblastoma patients [9]. According to the report, no patients exhibited objective clinical responses. Three out of twelve enrolled patients revealed regression of soft tissue. Two presented grade 2 to 3 cytokine release syndrome.

Narayan et al. recently reported a phase I study on prostate-specific membrane antigen (PSMA)-targeted tumor growth factor (TGF)-β-insensitive type of CAR-T cells for treating prostate cancer with metastatic castration-resistant type [10]. They discovered five of the total thirteen patients exhibited grade $\geq$2 cytokine release syndrome with no partial responses occurring. Three patients achieved a sharp decrease in prostate-specific antigen biomarkers of $\geq$30 % but were accompanied by CAR-T cell failure induced by the elevated expression of tumor-resident immune inhibitory molecules. Besides, even for current CD19 CAR-T therapy for treating B cell cancers, 50 % of patients had disease progression or relapse due to tumor antigen loss and heterogeneity [11].

With this regard, we conclude the four aspects that need to be considered in designing the next-generation CAR-T therapy for treating solid tumors: (1) how to strengthen the CAR-T cells' infiltration capability, (2) how to preserve their biological function in harsh tumor immunosuppressive environment, (3) how to surmount tumor antigen escape and heterogeneity, and (4) how to design a safer CAR-T therapy with lower susceptibility to side effects [12].

In this review, we first elucidate the basic CAR constructions and therapeutic mechanisms of CAR-T therapy. Then we summarize the recent preclinical studies that target the abovementioned problems. We discuss these problems from three aspects including (1) CAR-T design, (2) tumor modulation, and (3) delivery strategy. We believe it is essential to rationally design a combinatory CAR-T therapy by constructing optimized CAR-T cells, directly modulating the tumor microenvironment, and selecting the appropriate delivery routes to adapt to the complex and patient-specific tumor burden (Fig. 1). Finally, we conclude the necessary yet unelucidated problems, including tumor target antigen selection, cell source, manufacture, and *in vivo* tracing and monitoring.

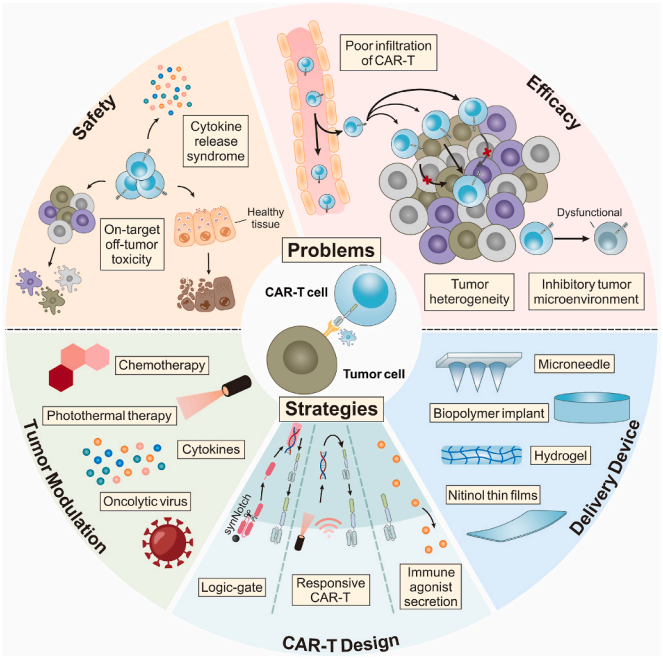

Fig. 1. Schematic illustration of rationally designed combinatory CAR-T therapy for treating solid tumors. Safety issues including on-target/off-tumor risks and cytokine storm, plus efficacy problems consisting of poor tumor infiltration, tumor immune suppression, and tumor heterogeneity impact CAR-T therapeutic effectiveness. Targeting these roadblocks, CAR-T cells' construction (producing immunostimulatory therapeutics including antibodies and cytokines, logic circuit innovation, on/off switches, and stimuli-responsive CAR-T cells), tumor modulation (cytokines, chemotherapy, photothermal therapy, and oncolytic therapy), and delivery strategies (microneedle, biopolymer implant, hydrogel, and nitinol thin films) are applied to achieve an optimized and safer therapeutic outcome.

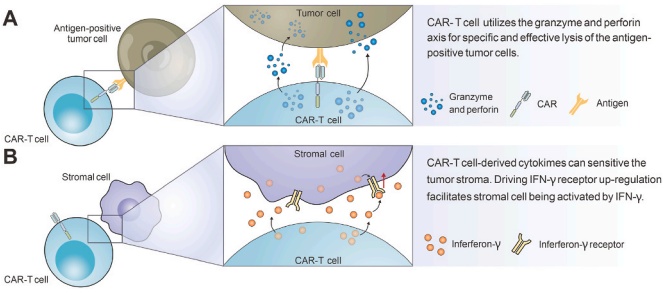

Fig. 2. The therapeutic mechanisms of CAR-T therapy. CAR-T cells directly recognize antigen-positive tumor cells and release granzyme and perforin for specific killing. In addition, they can produce immunomodulatory cytokines for tumor stroma sensitization. Adapted with permission [35].

2. The construction and therapeutic mechanisms

The CAR-T therapy includes five procedures: (1) extracting patients' cells, (2) isolating T cells, (3) *in vitro* genetic programming T cells to express the CAR constructs, (4) expanding CAR-T cells, and (5) reinfusing back to patients [13,14]. A CAR construct includes an antigen-binding domain, spacer, transmembrane region, co-stimulatory region, and activation domain [15].

In 1989, Zelig Eshhar and coworkers designed the initial type of CAR-T cells, the constructs of which were composed of T cell receptor constant (C) domains integrated into anti-2,4,6-trinitrophenyl antibody's variable (V) domains. Such designed CAR-T cells could recognize and eliminate target tumor cells through the major histocompatibility complex (MHC)-independent way [16]. In 1994, Moritz et al. grafted mouse cytotoxic T lymphocytes with single chain fragment variable (scFv)/hinge/ζ constructs *via* retroviral gene transferring and discovered their specific but moderate cytotoxicity toward target tumor cells.

However, the first-generation CAR-T cells are insufficient in signaling strength and durability due to a lack of the co-stimulatory domains [17]. In physiological T cell response, both activation stimulus (TCR-CD3 complex) and costimulation stimulus (costimulatory receptors like CD28 and 4-1BB for augmenting signal 1) are required to meet satisfactory activation and persistent state [18]. In 2002, Maher et al. generated human primary T cells producing fusion proteins (CARs) that contained PSMA-targeted scFv, TCRζ, and CD28 signaling domain. Upon stimulation with cell surface PSMA, these CAR-T cells expanded more than 2 logs in three weeks and released quantities of interleukin-2 [19].

Later, in 2004, Imai et al. incorporated the 4-1BB domain into the CAR construct, forming the second-generation CAR. This modification of the CAR constructs significantly reinforced the tumoricidal efficacy of CAR-T cells toward $CD19^+$ leukemia cells [20]. The third- or fourth-generation CAR incorporates two or three co-stimulation regions, respectively [21]. In 2014, Chmielewski et al. put forward TRUCK T cells (T-cells redirected for universal cytokine killing) [22]. TRUCK T cells can realize the local production of immunomodulatory cytokines,

shedding light on remodeling tumor immunosuppressive microenvironment. Apart from CAR construction, in recent years, the phenotype of CAR-T cells, that is exhaustion status or stem-like states, has received increasing attention due to its crucial role in mediating more potent and long-lasting immune memory and protective effect against cancer [23]. Meyran et al. discovered the poor tumor control efficacy of conventional CAR-T products (mainly exhibiting effector memory type markered by $CD45RA^{-}$ $CD62L^{-}$) targeting Lewis Y (NCT03851146) due to limited persistence of CAR-T cells *in vivo* [24]. They optimized CAR-T cell manufacture protocols, produced stem cell memory T cells (T_{stem}), and discovered their superior proliferation capacity and cytokine secretion capability. When combined with immune checkpoint blockade therapy, such CAR-T_{stem} therapy achieved tumor eradication in the subcutaneous OVCAR-3 tumor model.

2.1. CAR constructions

The CAR construction determines the eventual therapeutic performance (Table 1). First, it is essential to select an appropriate binding domain to target the tumor antigen. CARs targeting tumor-associated antigens (TAA) could induce on-target/off-tumor consequences [25]. CARs that target tumor-specific antigens (TSA), which are absent in normal tissues, can avoid such side effects. However, it remains a roadblock to determining the absence of the chosen antigen from normal tissues [26]. Even for the most successful CD19 CAR-T therapy in dealing with hematological cancer treatment, CD19 is not a tumor-specific antigen. Conversely, CD19 is uniquely and highly presented on all matured B cells' surfaces [15]. Regardless that nonmalignant B cells could be eliminated during the therapeutic procedure, antibody supplements can mitigate the potential infections that are induced by the loss of healthy B cells [27].

The hinge domain and transmembrane region that connect the antigen-targeting domain and signal transduction regions influence CAR-T cells' performances. The hinge domain offers the flexibility of the binding region to facilitate tumor antigen targeting. The length and composition of the hinge region are associated with the target antigen binding and downstream signal transduction [28]. For example, Yi et al. proved that for EphA2-specific CARs, substituting the CH2CH3 spacer with an IgG1-derived spacer could increase therapeutic efficacy against glioma by 20 folds [29].

The transmembrane region tethers the whole CAR construct on the cell membrane, which influences its stability and multimerization. The transmembrane domain sourced from CD28 is more stably bound on the cell membrane than that obtained from CD3ζ [30]. However, the transmembrane domain derived from CD3ζ can mediate CAR dimerization and facilitate CAR-T cells' activation [31]. Besides, researchers put great effort into optimizing signaling domains. The CD3ζ-based signaling activates CAR-T cells through tyrosine-based motifs [32]. Early studies discovered that CD3ζ-based activation signaling alone is inadequate in triggering T-cell mediated response. Researchers further added co-stimulatory domains to optimize their bio-function and metabolic fitness [33]. For example, the CD28 domain assisted in quicker and more potent CAR-T cell tumoricidal activity while the 4-1BB signaling domain could reinforce the persistence and expand the lifespan of CAR-T cells [34].

2.2. Therapeutic mechanisms

CAR-T cells specifically eliminate tumor cells in dual ways: (1) perforin and granzyme, and (2) cytokine secretion [35]. Perforin and granzyme is a cytotoxic program utilized by $CD8^{+}$ T cells. Upon the engagement between the CAR binding domain and tumor target antigen, the granules that contain perforin and granzyme can be transported to the T cell/tumor cell interface, fuse with the cell membrane, and then release the inner cargo towards the tumor cell (Fig. 2A) [36]. The perforin can induce pore formation on tumor cell membranes and enable

Table 1
Representative revolution engineering strategies for enhanced CAR-T therapy.

Time Author	Target Antigen	CAR constructions	Scientific meaning
1989 Eshhar *et al.*	2,4,6-trinitrophenyl (TNP)	TNP antibody (SP6) spliced to TCR chains	*In vivo* test of the antigen recognition and IL-2 production.
1994 Moritz *et al.*	ERBB2 receptor	scFv, targeting ERBB2 receptor, hinge region, and ζ chain of TCR.	Transduced T cells located at tumor tissues and retard NIH 3T3 tumor growth.
2002 Maher *et al.*	PSMA	PSMA-targeted scFv, TCRζ, and CD28 signaling domain.	CAR construction intergrated with CD28 co-stimulatory signal.
2004 Imai *et al.*	CD19	Hinge region and transmembrane domain derived from CD8α, signaling domain of CD3ζ, and CD137 (4-1BB).	CAR construction intergrated with 4-1BB co-stimulatory signal.
2018 Yi *et al.*	EphA2	Shorter IgG1-derived spacer, CD28 and 4-1BB costimulator	Revealing the impact of spacer length to the killing efficacy of CAR-T cells.
2010 Bridgeman *et al.*	CEA CD19	/	Transmembrane domain derived from CD3ζ mediated CAR dimerization and facilitate activation.
2014 Textor *et al.*	Her2	9-28-ζ-CAR	CAR-T cell secreting IFN-γ to polarize tumor resident M2 macrophages into M1.
2021 Cadilha *et al.*	EpCAM	CAR-T cells were engineered to express CCR8 and TGF-β dominant negative receptor	Additional receptors expressing of CAR-T cells for improving infiltration and overcoming tumor immunosuppression.
2018 Adachi et al.	hCD20	CAR-T cells were engineered to expresss IL-7 and CCL19.	CAR-T cells express chemokines and cytokines to improve infiltration.
2015 Caruana *et al.*	GD2	CAR-T cells engineered to produce HPSE.	CAR-T cells release enzymes to degrade tumor matrix for enhanced infiltration.
2018 Rafiq *et al.*	CD19 MUC16	CAR-T cells engineered to produce PD-1 blocking scFv.	Integration of cell therapy with immune checkpoint blockade therapy.
2021 Giuffrida *et al.*	Her2	A_{2A} receptor were depleted in CAR-T cells with CRISPR/Cas9 technology.	Knocking out receptors on CAR-T cells promote their better survival in tumor environment.
2021 Choe *et al.*	EGFRvIII EphA2 IL13Rα2	SynNotch system were intergrated on CAR-T cells for multi-antigen targeting.	SynNotch system promises spatial activation on targeted tumor sites.
2020 Lai *et al.*	Her2	CAR-T cells were engineered to secrete DC growth factor Flt3L.	Combined tumor killing and DC activation initiate tumor *in situ* vaccination.
2021 Gardner *et al.*	CD19	CAR-T cells were engineered to express enzymes that activate systematically injected small molecule prodrugs.	Integration of specific killing of CAR-T cells with targeted chemotherapy eliminate tumor antigen negative tumor cells.
2022 Zhang *et al.*	CD19	CAR cassettes were integrated into PD1 locus to blockade immune suppression.	Non-viral CRISPR/Cas9 technology to delete PD1 expression on CAR-T cells for clinic trials.

Abbreviations: CEA carcinoembryonic antigen; Her2 human epidermal growth factor receptor 2; EpCAM epithelial cellular adhesion molecule; EGFRvIII epidermal growth factor receptor variant type III; EphA2 Ephrin type-A receptor 2. HPSE heparanase.

the entry of granzymes [37]. CAR-T cells can also release cytokines for tumor stroma sensitization (Fig. 2Fig. 2B). It has been reported that the cytokines produced by Her2-targeted CAR-T cells increased IFN-γ receptor content of tumor stroma cells and facilitate the M1 polarization of tumor-resident macrophages [38].

3. Rational designs of CAR-T therapy for solid tumor treatment

3.1. Efficacy issues

Solid tumors are wrapped with dense extracellular matrices. When intravenously administrated, the initial step for CAR-T cells is circulating to the disease site and migrating across the compact tumor physical barriers [39]. In addition, tumor cells apply anaerobic respiration glycolysis ("Warburg effect") to meet their energy needs, which not only deprives T cell-required energy but also produces lactic acid that can impair the function of cytotoxic T lymphocytes by inhibiting their lactic acid exporting and promote the immunosuppressive functions of regulatory T cells [40,41]. The acidic environment and high frequency of immunosuppressive signals like PD-1/PD-L1 can downregulate the activation and proliferation of CAR-T cells [42]. Besides, antigen-negative tumor cells or those downregulating the expression of target tumor antigens can evade the immune surveillance by CAR-T cells, which eventually cause cancer reoccurrences [43].

Tremendous efforts have been concentrated on the solid tumor application of CAR-T therapy (Table 2). In this section, we conclude three approaches to improve the therapeutic efficacy including (1)

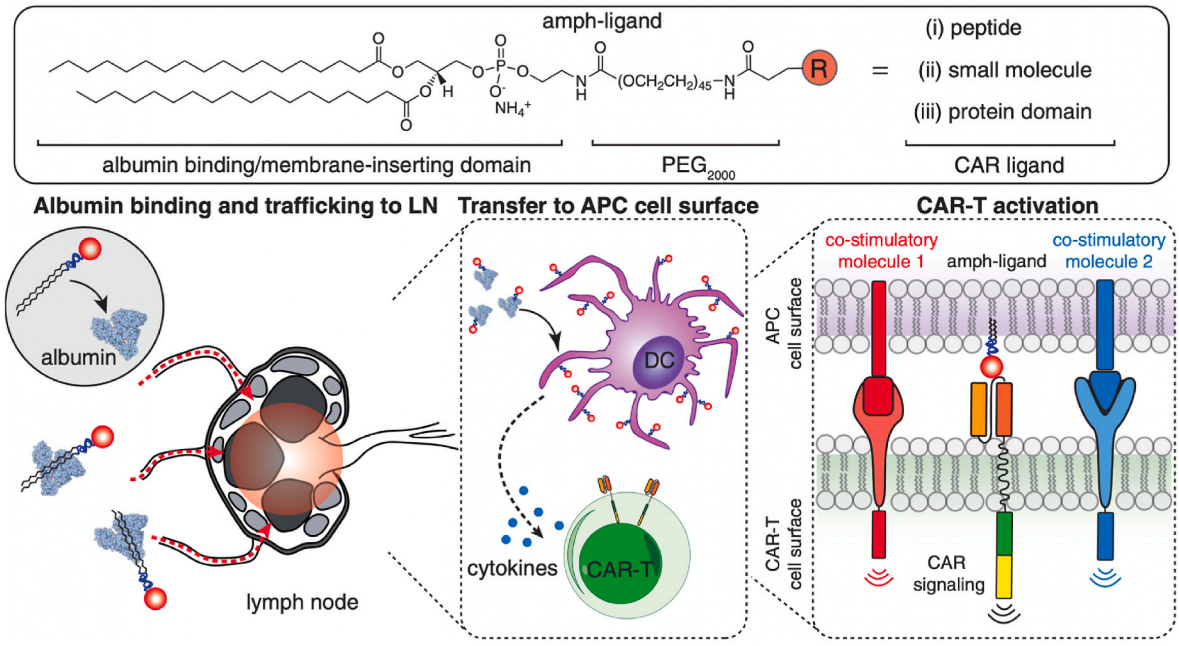

Fig. 3. Amph-ligand vaccine as an adjuvant strategy for CAR-T therapy. Lipid, PEG, and CAR ligands are included in the amph-ligand. The long-chain lipid moiety interacts and binds to serum albumin which augments amph-ligand being drained into peripheral lymph nodes. Amph-ligand mobilizes on dendritic cells' surface and facilitates CAR-T cells' expansion. Reproduced with permission [53].

Table 2
Representative ongoing clinic trials of CAR-T therapy for treating solid tumors.

Target Antigen	Disease	CAR Generation	Delivery Route	Clinical Phase	Clinical Trials Identifier
CEA	Liver metastasis	Unspecified	Hepatic Artery	I	NCT02416466
		Unspecified	Hepatic Artery	I	NCT02850536
		Second	Hepatic Artery	I	NCT03818165
CEA	Malignant ascites	Unspecified	Intraperitoneal	I	NCT03682744
LeY	Advanced cancer	Second	Intravenous	I	NCT03851146
EGFRvIII	Glioblastoma	Second	Intracerebroventricular	I	NCT05063682
CCT301	Renal cell carcinoma	Unspecified	Intravenous	II	NCT03393936
Glypican-3	Advanced hepatocellular carcinoma	Fourth	Intravenous	I	NCT03980288
		Unspecified	Unspecified	I	NCT02395250
GD2	Sarcoma	Third	Intravenous	I	NCT02107963
HER2	Sarcoma	Second	Intravenous	I	NCT00902044
PSMA	Metastatic castration-resistant prostate cancer	Unspecified	Intravenous	I	NCT04227275
MUC1	Metastatic breast cancer	Second	Intravenous	I	NCT04020575
ROR1	ROR1^{+} neoplasm	Third	Intravenous	I	NCT02706392
Mesothelin	Pancreatic adenocarcinoma	Unspecified	Intravenous, Intraperitoneal, and Intrahepatic infusion	I	NCT03323944
Claudin 18.2	Advanced gastric adenocarcinoma	Second	Intravenous	I	NCT03159819

improving CAR-T cells' infiltration into solid tumors, (2) surmounting tumor immunosuppressive microenvironment, and (3) overcoming tumor antigen escape and heterogeneity. For each subsection, we comprehensively review recent efforts from the aspects including CAR-T cell design, direct tumor modulation, and delivery strategy.

3.1.1. Improving tumor infiltration

For directly upregulating their moving tendency toward tumor tissues, researchers have placed chemokine ligands on CAR-T cells that respond to the attractions of chemokines derived from tumors. For example, L. Cadilha et al. added CCR8 and dominant-negative TGF-β receptor 2 (DNR), which is termed CCR8-DNR-CAR T cells, to strengthen tumor-targeting capability and prolong the CAR-T cells' persistence [44]. They identified that activated T cell-derived CCL1 presented positive feedback on $CCR8^+$ T cell recruitment into tumor tissues. In this way, augmented tumor infiltration of CAR-T cells could collaborate with DNR to overcome tumor-derived immunosuppressive signals. CCR8-DNR-CAR T cells that targeted the murine EpCAM achieved 3 out of 7 tumor rejection rates in the murine pancreatic solid tumor model.

In addition, they can also be manipulated to express chemoattractants for augmented tumor infiltration. Adachi et al. engineered CAR-T cells to produce interleukin-7 and CCL19, which were vital for the maintenance of T cell zones [45]. Immunohistochemical analysis showed that such CAR-T cells augmented tumor infiltration of both the recipient and donor T cells. As a result, they realized the complete elimination of the established P815-hCD20 tumors, and all mice were kept alive for 140 days.

Apart from directly upregulating the responsiveness toward tumor-derived cytokines, they can be modified to actively destroy solid tumor physical barriers. For example, Coruana et al. attempted to degrade the tumor extracellular matrix to promote CAR-T cells' penetration [46]. In their work, they identified that long-term expanded T cells *in vitro* lacked the heparanase (HPSE) production capability that could degrade heparan sulfate proteoglycans. With this regard, they generated CAR-T cells to produce HPSE, and a survival rate of 55 % was achieved upon day 50 in the $HPSE^+$ CAR-T group, vastly outperforming the $HPSE^-$ CAR-T group (10 %).

In addition, direct tumor modulation *via* chemotherapy can also facilitate CAR-T cells' infiltration. Deng and coworkers designed a strategy that utilized vascular disrupting agent combretastatin A-4 phosphate (CA4P) to promote infiltration [47]. In their study, they found that CA4P hindered microtubule polymerization of vascular endothelial cells and triggered their morphological deformation, destroying intratumoral vascular networks and assisting tumor infiltration and proliferation of Her2-targeted CAR-T cells.

Tumor-resident immune cells can be triggered to release chemokines that can facilitate CAR-T cells' infiltration. Srivastava et al. innovated a combinatory measurement by applying oxaliplatin to overcome the infiltration hurdle [48]. Mechanistically, oxaliplatin activated tumor-resident macrophage to express chemokines including CXCL9 and CXCL10 for recruiting circulating CAR-T cells, which further remodeled the tumor immune microenvironment *via* secreting IFN-γ.

Gu and coworkers reported a combinatory strategy that applied photothermal therapy in combination with CAR-T therapy [49]. Chen et al. intravenously injected chondroitin sulfate proteoglycan 4 (CSPG4) CAR-T cells targeting WM115 human melanoma cells along with intratumoral administration of indocyanine green (ICG, a NIR dye that can achieve light-heat energy conversion)-loaded PLGA nanoparticles. Mild heating of the tumor partially destroyed tumor cells and extracellular matrix which further promoted $CSPG4^+$ CAR-T cells' intratumoral infiltration and accumulation. As a result, two out of six mice that received combinatory treatment achieved tumor eradication.

Living organisms like bacteria exhibit innate capabilities in modulating tumor microenvironment for augmenting CAR-T infiltration [50,51]. In 2022, Guo et al. leveraged attenuated bacterial strain (*Brucella melitensis 16 M ΔvjbR*) as an adjuvant therapy for enhancing CAR-T treatment [52]. In the study, they discovered intravenous injection of bacteria could polarize macrophages into M1 phenotype and promote T cells' activation markers (perforin and granzyme B) *in vitro*. Single intravenous injection of bacteria leads to increased CAR-T cells' infiltration from 8 % to 34 % among tumor-resident CD8 T cells. As a result, the combination of CAR-T therapy and bacteria treatment achieved 100 % host survival at day 30 post tumor inoculation, although not completely eradicating the established tumor.

Apart from direct tumor modulation, vaccines can also adjuvant their bio-activity. For example, Ma et al. designed amphiphile CAR-T ligands (amph-ligands), which could flow into lymph nodes and decorate the cell surface of APCs to prime CAR-T cells (Fig. 3) [53]. Amph-ligand induced extensive expansion of CAR-T cells, improved donor cell polyfunctionality, and augmented therapeutic outcomes in multiple types of immunocompetent mouse tumor models. Reinhard et al. demonstrated a type of RNA vaccine, which was designated for the delivery of the CAR-antigen into immune organs [54]. The presentation of CAR-targeted antigen on the APCs selectively expanded adoptive transferred CAR-T cells.

Delivery strategies can also augment CAR-T cells' tumor accumulation by generating a direct interface between CAR-T cells and solid tumors, the simplest form of which is regional injection [55]. S. Adusumilli et al. compared the therapeutic difference between two administration routes (intravenous and intrapleural) [56]. They discovered that intrapleural-injected mesothelin-targeted CAR-T cells facilitated earlier and more sustained accumulation in tumors, vastly outperforming systematic administration (Fig. 4A and B). As a result, intrapleural injection reduced 30-fold fewer doses of CAR-T cells for realizing complete tumor remissions. This study supports the idea that selecting an optimal delivery route can tremendously augment CAR-T therapy.

Inspired by this, various delivery devices have been tested to optimize their *in vivo* delivery. For example, A. Ogunnaike et al. recently used *in situ*-formed fibrin gel to locally deliver CAR-T cells within the surgical cavity for post-surgical supplementary treatments against glioblastoma, which not only avoided blood-brain barrier impediment but also achieved the gradual release of CAR-T therapeutics (Fig. 4C) [57]. E. Coon et al. designed micropatterned nickel-titanium thin films as a CAR-T cell delivery platform (Fig. 4D) [58]. The nickel-titanium thin film was coated with fibrins that allowed CAR-T cells to freely migrate through the film and antibodies including anti-CD3/CD28/CD137 antibodies to promote CAR-T cell expansion. Nitinol thin films could be flexibly conformed to tumor tissues, augment CAR-T cells' proliferation, and directly deliver a high concentration of CAR-T cells to tumor tissues. They further verified its therapeutic superiority in the ovarian cancer model, achieving cancer eradication in 7 out of 10 treated mice (Fig. 4E).

Recently, Gu and coworkers exploited microneedle patches for CAR-T cell delivery [59]. Li et al. generated a porous microneedle patch (PMN) *via* the acid etching method to locally deliver CSPG4 CAR-T cells for melanoma treatment and B7–H3 CAR-T cells for orthotopic pancreatic tumor therapy ((Fig. 4F). This delivery technique led to the multipoint scattered seeding pattern which augmented the possibilities of cell-cell interactions between CAR-T cells and tumor cells.

Taken together, to strengthen the infiltration capabilities of CAR-T therapeutics, three main aspects could be aimed at, CAR-T cells, the tumor itself, and delivery methods. First, they can be armed to display certain chemokine receptors that can respond to the tumor-derived chemokines or they can secrete tumor matrix-degrading enzymes in order to penetrate into the tumor. Second, various direct tumor modulation measurements can be combined to immunomodulate tumor tissues, such as chemotherapeutic agents that induce tumor-resident macrophages to secrete chemokines that recruit circulating CAR-T cells; vascular disruption agents that facilitate CAR-T cells' penetration; and photothermal therapy which destroys tumor matrix for the convenient entry of CAR-T cells. Third, regional administration outperforms systematic injection. Innovative delivery devices, including fibrin gel,

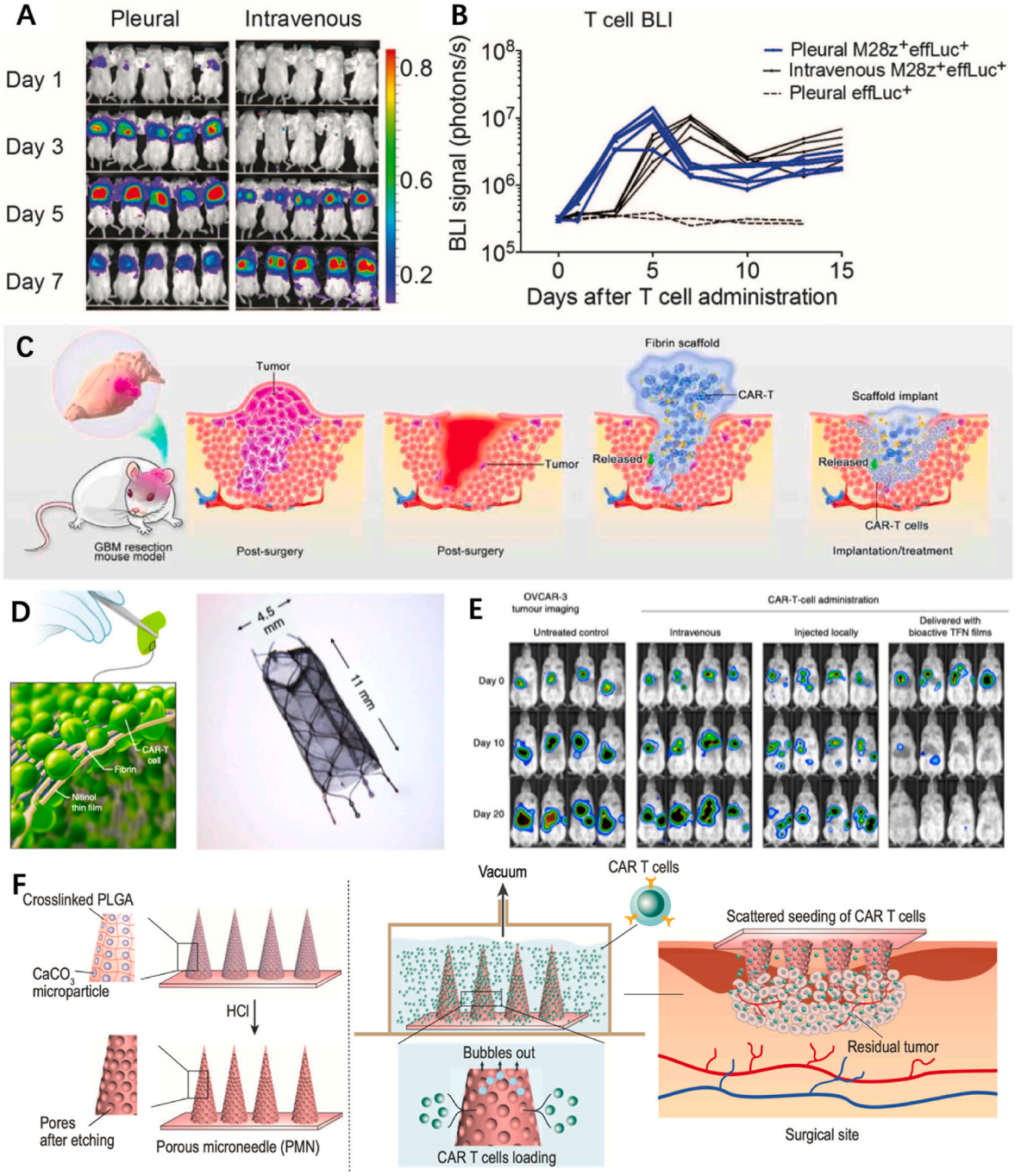

Fig. 4. Delivery technique for strengthening CAR-T cells' infiltration. (A) Serial mesothelin-targeted CAR-T cell bioluminescence imaging. (B) Average bioluminescence intensities of T cells. Reproduced with permission [56]. (C) Exploiting fibrin gel for CAR-T cells' delivery for glioblastoma post-surgical treatment. The fibrin gel can serve as the delivery depot, realizing the sustained release of CAR-T cells. Reproduced with permission [57]. (D) Single-layer TFN stent surface coated with fibrin and immunomodulatory antibodies for delivering CAR-T cells. (E) OVCAR-3-Luc tumors serial bioluminescence imaging. Reproduced with permission [58]. (F) Illustration of PMN construction, cell loading, and implantation of PMN in the surgical tumor bed. Reproduced with permission [59].

nitinol thin films, and microneedle, further augment CAR-T therapy by directly delivering high concentrations of cell therapeutics into tumor lesions and can combine with immunomodulatory adjuvants to promote their persistence and proliferation *in vivo*.

3.1.2. *Surmounting tumor immunosuppressive microenvironment*

For a specific CAR, the structure of the signaling region is associated with the persistence and tumoricidal potency [34]. Signal transduction domains normally consist of two parts, one for activation like CD3ζ, and the other one for co-stimulation like CD28 [60]. To obtain the optimal construct in facing tumor immunosuppressive microenvironment, researchers screened a large number of signaling domains and compared their performance *in vivo*.

For the activation signal, Wu et al. screened the CD3 subunits, including CD3ε, δ, γ, and ζ [61]. They found that these four types of CD3 chains exhibited function diversities. They discovered that CAR-T cells that incorporated the CD3ε domain reduced inflammatory cytokine release and augmented persistence, which not only alleviated the potential cytokine release syndrome but also augmented their tumor control capability. For the co-stimulatory domain, I. Philipson et al.

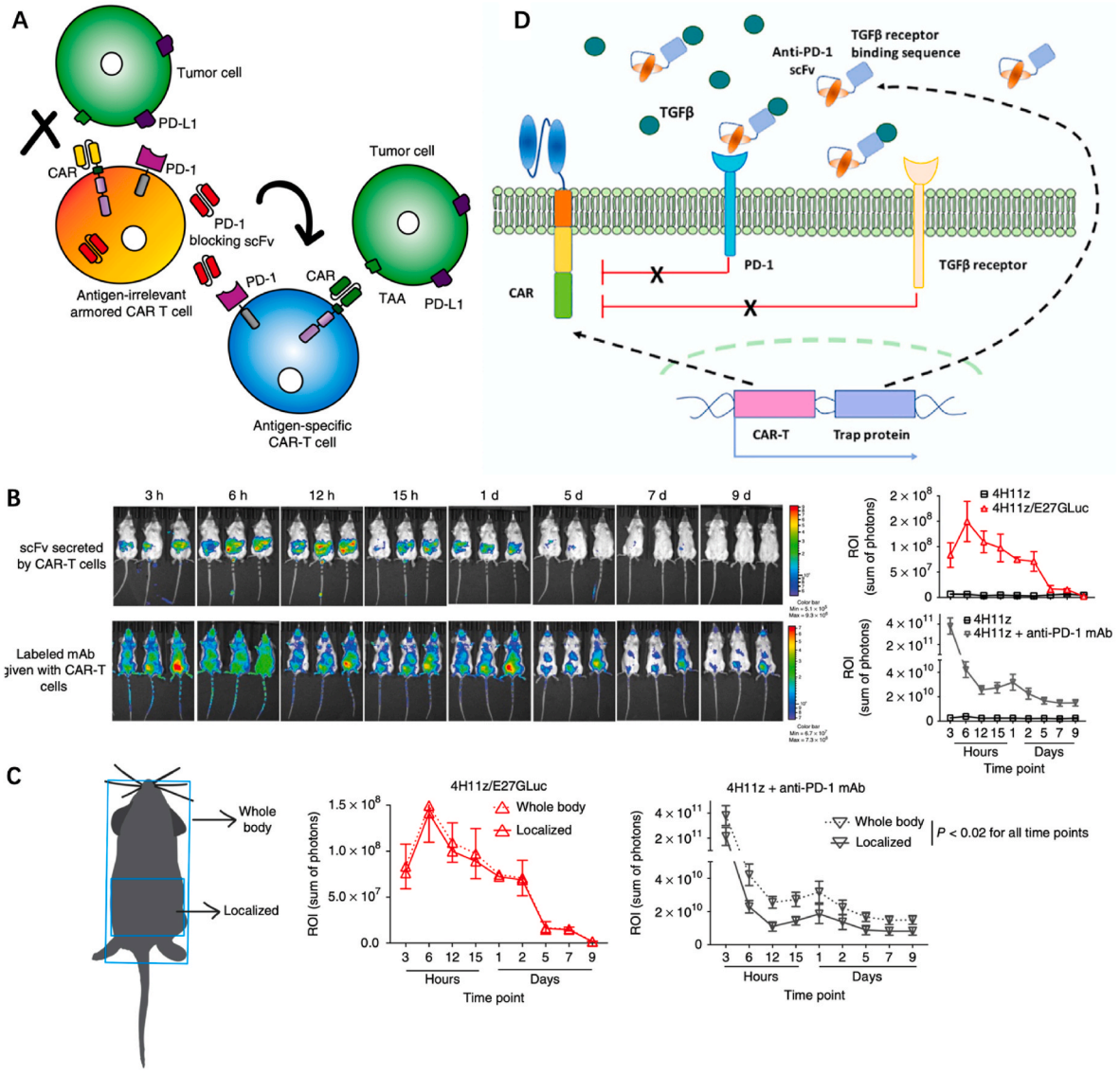

Fig. 5. Secretion CAR-T for overcoming immunosuppressive tumor microenvironment. (A) The impact of ICI scFv-secreting CAR-T cells on bystander tumor-relevant CAR-T cells. (B) Imaging and quantification of anti-human PD-1 mAb or scFv. (C) Quantification of systematic and local delivery of scFv or antibody. Reproduced with permission [66]. (D) Functional CAR-T cells secreting trap protein in tumor tissues. Reproduced with permission [67].

studied the clinical outcomes of CAR-T cells with 4-1BB co-stimulated or CD28 co-stimulated [62]. They revealed that the 4-1BB region could trigger ncNF-κB signaling which facilitated their expansion and survival. As a result, CAR-T cells that 4-1BB co-stimulated exhibited better persistence than those CD28 co-stimulated, pointing out the ongoing avenue of CAR optimization for solid tumor treatment.

Tumor inhibition capability depends on both CAR-T cells' persistence and tumoricidal cytotoxicity. The abovementioned CAR-T therapeutics only incorporate one co-stimulatory region. Those that incorporate two, for example, both CD28 and 4-1BB signaling, are expected to exhibit improved therapeutic capabilities, termed the third-generation CAR-T therapy [63]. For example, Zhong et al. investigated the therapeutic ability of PSMA-targeted CAR-T cells that CD28 and 4-1BB co-stimulated [64]. Mechanistically, they discovered their superiorities in comparison with the conventional version that only incorporates one co-stimulatory region from the aspects including cytokine-producing, T cell survival, and tumor inhibition capability.

However, integrating such two co-stimulatory domains into one CAR construct may hinder therapeutic performance since these signal transduction domains can only be activated simultaneously upon the engagement of the binding domain. In this regard, Zhang et al. generated novel CAR-T cells, in which a co-stimulatory domain could be activated individually by CAR activation [65]. In the study, they screened 12 co-stimulatory receptors and found OX40 was the most effective signaling enhancer, which boosted the tumoricidal efficacy and assisted in maintaining the biological function in the immune-suppressed environment.

Apart from optimal CAR construction, researchers also endowed CAR-T cells with the capability to secret immunomodulatory proteins or the capability to ignore tumor-derived immunosuppressive signals. In one study, Rafiq et al. engineered a new type of CAR-T cells that could self-produce PD-1-blocking scFv to overcome tumor immune suppression [66]. This construction strengthened the tumoricidal capability of bystander tumor-specific T cells (Fig. 5A). As a result, such therapy not only achieved similar or enhanced therapeutic efficacy but also realized tumor local delivery of PD-1 antibodies to mitigate potential side effects aroused by the systematic exposure of immune checkpoint inhibitors (ICI) (Fig. 5B and C).

Taking a further step, Chen et al. engineered a type of CAR-T cell that could produce bispecific trap protein co-targeting PD-1 and TGF-β

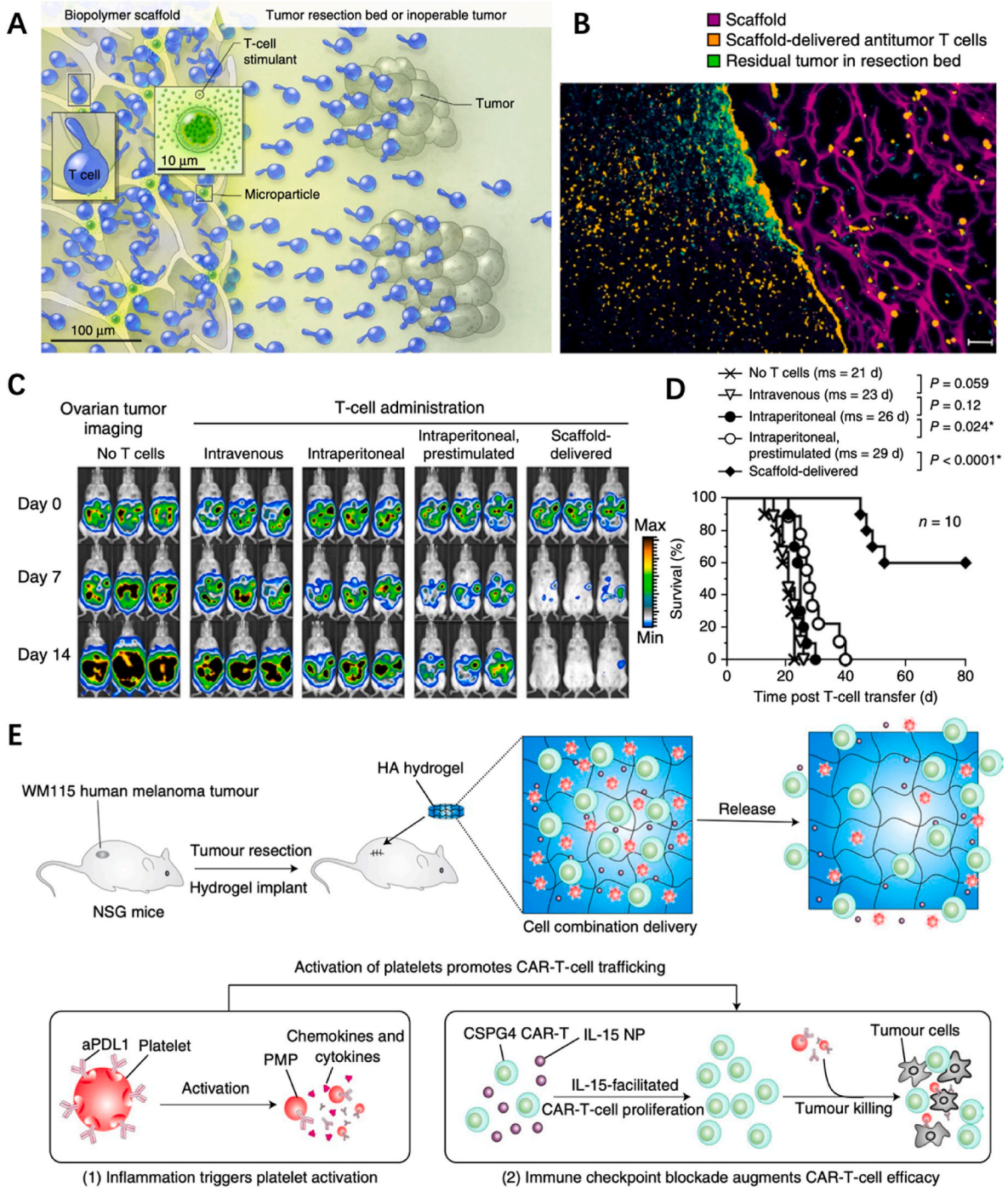

Fig. 6. Delivery strategies targeting tumor immunosuppressive microenvironment. (A) T cell-loaded scaffold implanted at tumor surgical resection cavity. Immunomodulatory microparticles integrated into the scaffold induced CAR-T cells' expansion and promoted their migration into surrounding tumor tissues. (B) Fluorescently labeled T cells, scaffold, and tumor cells. Scale bar, 100 μm. (C) Serial ID8-VEGF-Luc tumors bioluminescence imaging. (D) Survival curves of ovarian cancer-bearing mice after treatment. Reproduced with permission [84]. (E) Implantation of the hyaluronic acid hydrogel that was loaded with cell-based therapeutics for cancer treatment. Platelets decorated by *anti*-PD-L1 antibodies were activated in the surgical inflammation environment and released *anti*-PD-L1 antibodies in the form of platelet-derived microparticles. Reproduced with permission [85].

(Fig. 5D) [67]. The co-targeting trap protein could neutralize immune inhibitors PD-L1 and immunosuppressive cytokine TGF-β, which collectively protected CAR-T cells from tumor immune suppression. In another aspect, small molecules also contribute to the tumor immunosuppressive microenvironment. Adenosine is an immunosuppressive molecule that suppresses T cells' activities *via* engaging with the adenosine A_{2A} receptor ($A_{2A}R$) [68,69]. Based on these, Giuffrida et al. leveraged CRISPR/Cas9 technology to deplete adenosine $A_{2A}R$ on CAR-T [70]. As a result, such generated T cells were capable in surviving adenosine-mediated transcriptional changes, which induced enhanced cytokine production like IFN-γ.

In addition to engineering CAR-T cells to express immune checkpoint inhibitors, they can also be rendered PD-1 deficient to overcome inhibitory signals. In 2021, Wang et al. reported a phase I study involving CAR-T cells with disruptions to both PD-1 and TCR using CRISPR/Cas9 technology [71]. In this study, they found that

TCR-positive T cells became the predominant cell fraction in patients after infusion, indicating the impact of TCR presence on T cell persistence *in vivo*. However, single PD-1 knockout does not seem to be sufficient for effectively managing solid tumors. In 2022, the Huang group conducted phase I clinical trials on the use of non-viral, gene-specific targeted integration of the CAR cassette into the PD-1 locus [72]. When compared to integration into the AAVS1 safe harbor locus, CAR-T cells that integrated into the PD-1 locus outperformed in the treatment of Raji cells both *in vitro* and *in vivo*. When used for the treatment of relapsed/refractory B-cell Non-Hodgkin lymphoma (B-NHL), this product achieved an 87.5 % complete remission rate, with no severe CRS observed.

Direct tumor modulation can also mitigate the impact of tumor immune suppression on CAR-T cells, for example, intratumoral administration of immunomodulatory cytokines to reform the tumor microenvironment [73]. In one study, Agliardi et al. intratumorally injected IL-12 with systematic administration of CAR-T cells [74]. Local injection of IL-12 augmented the tumoricidal efficacy of CAR-T cells and immunomodulated the tumor microenvironment to increase the tendency of $CD4^+$ T cells along with a decreased regulatory T cell content. As a result, such a strategy achieved durable antitumor responses, in which CAR-T therapy alone failed to control the established glioblastoma multiforme.

However, local injection of immunomodulatory cytokines is inefficient for metastasis tumors, and systematic administration may result in severe dose-associated side effects [75]. Recently, Zhang and Aspuria back-to-back reported two types of orthogonal CAR-T cells, which specifically respond to amino acid-substituted human orthogonal IL-2. Mechanistically, artificially-engineered IL-2 could only activate the cognate-engineered IL-2 receptors on CAR-T cells while leaving wild-type T cells uninfluenced. Such a strategy not only avoided cytokine storm but also augmented the therapeutic efficacy in rescuing suboptimal dose-dependent anti-leukemic response and the clearance of bulky lymphoma [76,77]. In addition, tumor cells display aberrant glycosylation, which can negatively regulate the immune response of CAR-T therapy *via* masking neo-epitopes or interfering with immune cell functions. According to this, Greco et al. recently utilized 2-deoxy--D-glucose to disrupt *N*-glycan cover on tumor cells, which promoted CAR-T cells' proper immunological synapse formation, transcriptional initiation, and cytokine secretion for xenograft pancreatic adenocarcinoma treatment [78].

The oncolytic virus can also be applied to overcome tumor immunosuppressive microenvironment. Oncolytic virus-mediated therapy takes effect by directly infecting tumor cells and destroying tumor matrix and vasculatures [79–81]. In one study, the Suzuki group intratumorally injected oncolytic viruses that encoded PD-L1 blocking antibody and IL-12, followed by systemic administration of Her2-target CAR-T cells [82]. Such a combinatory strategy achieved both primary and metastatic tumor control. However, local injection of oncolytic virus sometimes can be unrealizable due to the unsatisfactory tumor location. Barriers remain in the systematic delivery of oncolytic viruses into solid tumors due to innate-immune-mediated antibody neutralization. In this regard, McKenna et al. designed a strategy that first systematic administration of mesenchymal stromal cells (MSCs) that encapsulated two types of adenoviruses (one for directly mediating cancer cell lysis, the other for expressing IL-12 and immune checkpoint PD-L1 blocker) with following CAR-T cells intravenous infusion [83]. This strategy improved CAR-T antitumor activity by producing IL-12 and PD-L1 blockers for overcoming tumor immunosuppressive microenvironment while boosting CAR-T cells' infiltration simultaneously.

The delivery device can serve as a cell reservoir, which both realizes local delivery of cell therapeutics and helps sustain CAR-T cell activity by creating a cell-preferable environment in tumor tissues. Stephan et al. described a bioactive polymer-based cell-loaded implant, which can deliver, expand, and disperse T lymphocytes [84]. In their work, the bioengineered polymer matrices acted as cell reservoirs, from which the transplanted CAR-T cells were released while the implant degraded (Fig. 6A and B). In addition, they incorporated lipid-coated silica microspheres with anti-CD3/CD28/CD137 antibodies and IL-15/IL-15 Rα which could serve as surrogates for antigen-presenting cells. As a result, a 22-fold boost of T cell proliferation was achieved after adding these immunomodulatory microparticles. The survival benefit was significant, with no mice receiving scaffold-delivered NKG2D-targeted T cells experiencing tumor reoccurrences in the breast cancer resection model (Fig. 6C and D).

Gu also reported a hydrogel-based delivery strategy for post-surgical treatment to avoid cancer reoccurrences. In the study, they encapsulated CAR-T cells, *anti*-PD-L1 antibodies decorated platelets, and IL-15-loaded PLGA nanoparticles into a hyaluronic acid hydrogel (Fig. 6E) [85]. The hydrogel promoted CAR-T cell accumulation within the post-surgical bed. The local inflammation triggered platelet activation and further achieved the release of *anti*-PD-L1 antibodies.

Summarizing above, the same three aspects are targeted to surmount tumor immunosuppressive microenvironment. First, efforts have been concentrated on screening the optimal signaling domains to obtain the most persistent and potent type of cell. They can further be designed to secret immunomodulatory proteins to combat tumor-derived immunosuppressive signals. Direct tumor modulation by intratumoral injections of immunomodulatory agonists like IL-12 or oncolytic virus that encode immunomodulatory cytokines and proteins can promote the persistence and proliferation of CAR-T cells in the immunosuppressive solid tumor microenvironment. The delivery device can create an interface between "living drugs" and solid tumors, which not only augments the infiltration but also helps sustain their bio-activity by providing a relatively cell-preferable microenvironment.

3.1.3. Overcoming tumor antigen escape and heterogeneity

Tumor antigen escapes and heterogeneity pose great challenges in managing cancer reoccurrences [86]. For tumor cells absent of target antigen, they may escape the tumoricidal surveillance of CAR-T cells [87]. Therefore, generating a type of CAR-T cells that could recognize multiple targets may mitigate this phenomenon. For example, Spiegel et al. reported a CD19 and CD22 dual-targeted CAR-T therapy to treat recurrent B cell malignancies [88]. According to other clinic reports, approximately 50 % of patients receiving CD19 CAR-T therapy eventually faced disease progression [11,89]. In their study, 88 % of relapsed B-cell acute lymphoblastic lymphoma (B-ALL)-experienced patients responded to CD19 and CD22 dual-target therapy with minimal residual disease-negative complete remission.

However, targeting more tumor antigens accompanies on-target/off-tumor risks. Therefore, a stricter CAR-T cell activation system is required to mitigate them. SynNotch system represents the most typical design. A synNotch receptor can be locally activated by tumor-specific antigens (priming antigens) to express the CAR [90]. The expressed CAR construction can further recognize homogeneous but not tumor-specific antigens to trigger the tumor cytotoxic program. Such a logic circuit not only promises tumor regional activation but also helps avoid exhaustion and keep a higher level of stem-state T cells [91,92].

In one study, H. Choe et al. developed EGFRvIII synNotch CAR-T cells which could further recognize EphA2 or IL13Rα2 to kill tumor cells (Fig. 7A) [93]. EGFRvIII is one of the glioblastoma-specific antigens while EphA2 and IL13Rα2 are more homogeneous glioblastoma antigens. When CAR-T cells are primed upon engaging with EGFRvIII, they further express CARs that can recognize more homogeneous tumor antigens EphA2 and IL13Rα2 (Fig. 7B). After implanting two different tumors with different compositions at the brain and flank, synNotch CAR-T group exhibited significant tumor regression of brain tumor but showed nonsignificant improvement in treating flank tumor compared with the non-transduced T cell group, which verified the spatial activation capability of synNotch system (Fig. 7C and D).

Although multi-antigens can be targeted for overcoming tumor antigen escape and heterogeneity, tumor surface antigens are evolving as

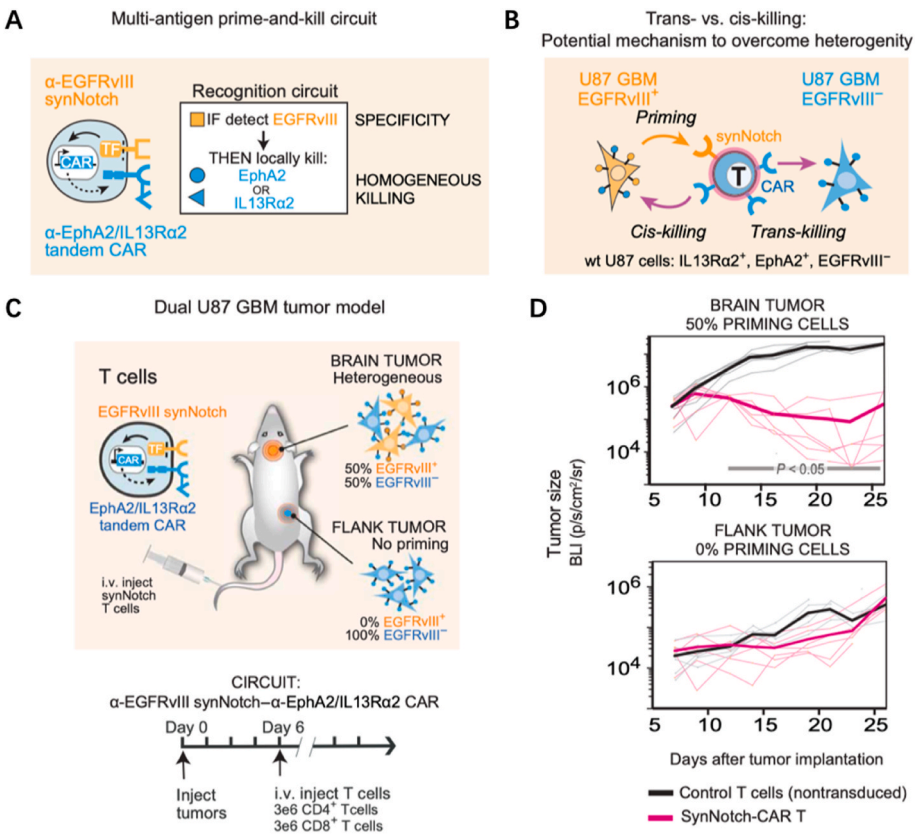

Fig. 7. SynNotch circuit in CAR-T designing. (A) SynNotch circuit that was first primed by EGFRvIII neoantigen and could eliminate EphA2^{+} or IL13Rα2^{+} tumor cells. (B) SynNotch CAR-T cells were primed by neoantigens and could overcome antigen heterogeneity when they executed *trans*-killing. (C) Schematic illustration of GBM tumor inoculation method. (D) Bioluminescence imaging of tumor size over time. Reproduced with permission [93].

tumors progress [43]. And owing to the individualized mutational variation of patients, strategies that trigger an endogenous adaptive immune response and yield a polyclonal antitumor immune response can potentially bring augmented therapeutic benefits [94]. The selection of the bioactive proteins that CAR-T cells secrete is vital for the quality of the elicited endogenous immune response.

Lai et al. reported a type of CAR-T cells that could produce dendritic cell growth factor Fms-like tyrosine kinase 3 ligands (Flt3L) [95]. They revealed that Flt3L-releasing CAR-T cells multiplied intratumoral type I conventional dendritic cells and tremendously promoted the activation of endogenous DC and T cells when agonist Poly (I: C) was co-supplemented. The Her2-targeted Flt3L-secreting CAR-T group significantly suppressed tumor growth in comparison with the non-secreting Her2-target CAR-T group.

Similarly, Jin et al. reported a type of CAR-T cell that could secrete bacterial virulence factor (neutrophil-activating protein, NAP) to elicit bystander responses [96]. NAP recruited innate immune cells, promoted the maturation and Th1-polarization of DCs, and produced an immune normalized microenvironment (Fig. 8A). As a result, NAP-secreting CAR-T cells induced epitope spreading that elicited cytotoxic T lymphocytes that targeted tumor antigens other than the CAR-target ligand, indicating their efficacy for overcoming tumor antigen heterogeneity.

As an alternative route to target antigen-negative tumor cells, Gardner et al. recently innovated enzyme-secreting CAR-T cells, which could activate the systemically administrated small-molecule prodrug in the tumor tissues, termed SEAKER cells (Fig. 8B) [97]. Such SEAKER cells could logarithmically expand at the disease site, which further expressed thousands of copies of prodrug-activation enzymes activating the prodrug to eliminate antigen-negative tumor cells (CD19^{-} Nalm6 cells).

Apart from directly strengthening CAR-T cells by grafting their multi-antigen targeting ability, logic circuit-controlled activation, and secretion capability, tumor cells can also be manipulated to present CAR-target antigens. Johnson et al. generated CAR-T cells that co-express peptide antigens with endogenous RNA RN7SL1 for overcoming tumor antigen loss and tumor immune suppression, respectively [99]. In their study, RN7SL1, as an immune agonist, boosted the proliferation and effector-memory differentiation of CAR-T cells. In addition, the co-expressed OVA peptide (SIINFEKL) of CAR-T cells could induce MHC I-SIINFEKL presentation on tumor cells through extracellular vesicle transfer. As a result, this strategy exhibited superior therapeutic efficacy in mixing antigen-heterogenous tumor models in comparison with other groups.

Besides, the oncolytic virus can also be leveraged to mediate tumor cells to present certain target antigens. For example, Park et al. engineered non-signaling truncated CD19 protein encoded oncolytic virus [100]. Upon intratumoral injection of CD19-encoding oncolytic virus, they infected tumor cells and generated *de novo* CD19 on the tumor cell membrane surface. They co-cultured CD19-target CAR-T cells with oncolytic virus-infected tumor cells and revealed upregulated cytokine levels. They further proved its feasibility in MC38 tumor-bearing C57BL/6j mice. The oncolytic virus alone only achieved 22 % complete regression, whereas the combinatory therapy reached 60 %. Such a method could well mitigate the current limitation of both tumor-uniquely and homogeneously presented tumor antigens. Leveraging the ideal target antigen like CD19 to decorate different tumor cells can be a universal therapeutic strategy. However, direct intratumoral injection remains unrealistic for metastatic and advanced tumors. Future efforts may concentrate on facilitating antigen-encoding virus to be precisely delivered to the tumor site throughout the body.

Engineered microbes like bacteria can produce functional proteins at targeted tumor sites [101,102]. Vincent et al. engineered *E. coli* Nissel

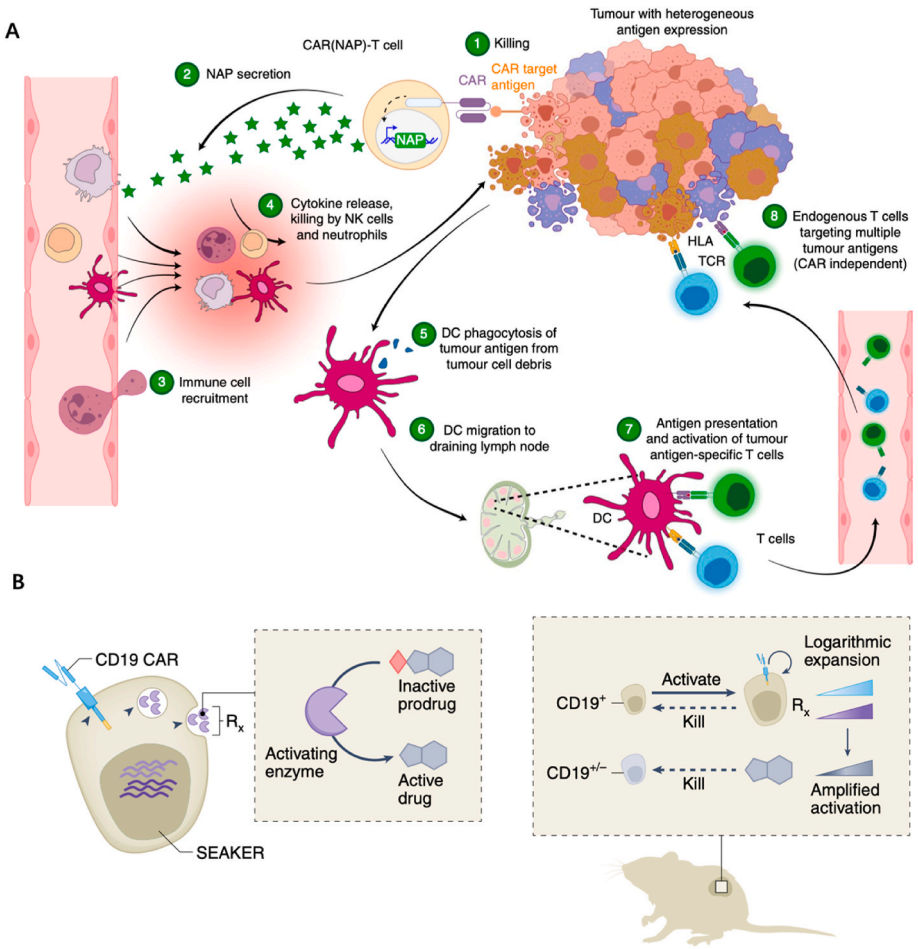

Fig. 8. Secretion CAR-T cells for overcoming tumor antigen heterogeneity. (A) The killing process of NAP-secretion CAR-T cells. NAP induces infiltration of endogenous immune cells, which further produces immunomodulatory cytokines to create an inflammatory microenvironment. Dendritic cells take up tumor cell debris, migrate to lymph nodes, and initiate tumor-specific T cells. Endogenous T cells circulate and migrate into solid tumors to exert cytotoxicity toward tumor cells. Reproduced with permission [96]. (B) Schematic illustration of SEAKER cells which can locally activate chemotherapeutic prodrugs to eliminate antigen-negative tumor cells. Reproduced with permission [98].

1917 (EcN) to synchronize lysis and produce CAR tags, which are composed of PIGF-2123-144 (heparin-binding domain), linker, and sfGFP (CAR target) [103]. These secreted CAR targets can easily decorate on tumor matrix and tumor cell membrane surface and expose the CAR target sfGFP exterior. The tumor-homing ability of EcN facilitated the CAR tag to be concentrated at tumor local tissues for alleviating potential toxicity to normal tissues to a large extent, providing a feasible and easy way for diverse tumor targeting of CAR-T cells.

3.2. Safety issues

The safety issues related to CAR-T therapy mainly consist of on-target/off-tumor risks, cytokine storm, and neurotoxicity [104,105]. The occurrence of on-target/off-tumor risk is mainly attributed to the lack of ideal tumor antigens [106]. The cytokine storm happens due to the hyper-activation of CAR-T cells and the feedback loop between endogenous immune cells and the infused CAR-T cells [107]. However, the underlying mechanisms of neurotoxicity remain unspecified. Many studies speculated the reasons to be the target antigen present in brain tissues or the pro-inflammatory cytokine-induced brain vasculature disruption [108,109]. Here, we concentrate on the recent advances in preclinical CAR-T studies that target the on-target/off-tumor risks and cytokine storm.

3.2.1. On-target/off-tumor side effect

The selection of target antigens is essential for alleviating on-target/off-tumor risks [110]. Carbonic anhydrase IX-targeted CAR-T cells induced liver enzyme abnormalities when applied in treating renal cell carcinoma [111]. Such side effect was induced by CAR-T cells' infiltration into CAIX-positive bile duct epithelium. Therefore, a stricter CAR-T cell activation system is required to mitigate such a side effect. The current effort is mainly focused on designing logic-circuit CAR and stimuli-responsive CAR-T cells. Given that logic-circuit-based CAR-T therapy has been extensively summarized elsewhere [112,113], in this section, we highlight stimuli-responsive CAR-T therapy.

Ideally, CAR-T cells should recognize tumors from healthy tissues according to tumor antigen density [114]. In this regard, Lopez et al. generated a two-step feedback recognition circuit for allowing CAR-T cells to distinguish tumor antigens depending on a sigmoidal antigen-density threshold [115]. The low-affinity initial synNotch receptor targeting Her2 controlled the production of a higher affinity CAR construct that targets Her2. The low-affinity Her2 synNotch receptor acted as a filter for high Her2-displaying cells and the high-affinity CAR for Her2 controlled the tumoricidal efficacy and expansion capability of CAR-T cells. The higher Her2 antigens presented on cells promoted both CAR production and initiation, thus triggering a sigmoidal efficacy. As a result, such CAR-T cells exhibited sharp distinguishing capability between healthy cells presenting a normal amount of Her2 and tumor cells displaying 100-fold Her2.

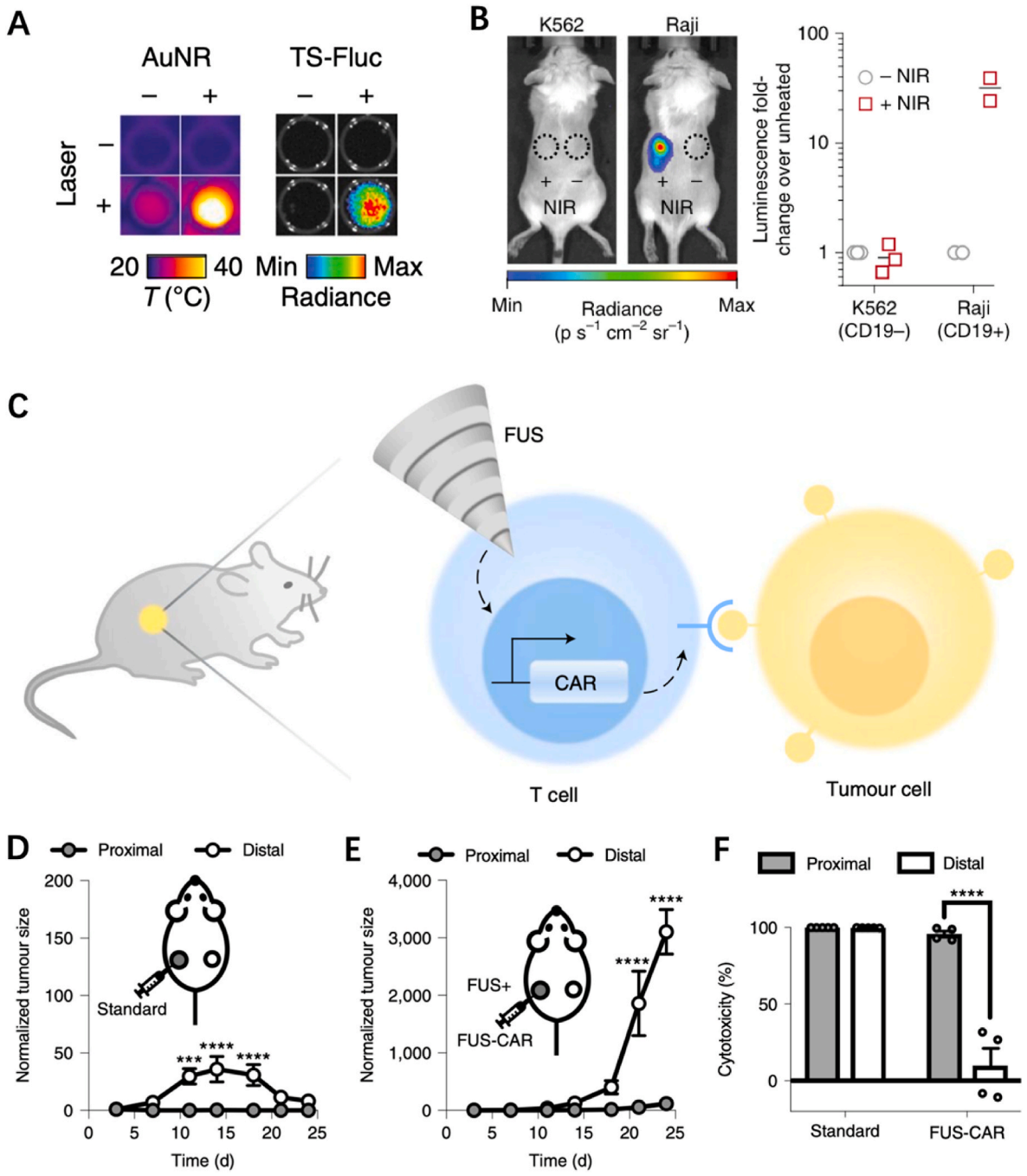

Fig. 9. Heat-responsive CAR-T therapy. (A) Thermal (left) and luminescence images (right) of wells loaded with TS-Fluc expressed T cells with or without gold nanorod after applying NIR laser irradiation. (B) Left: Bioluminescence images of mice implanted with different tumors and implemented with different treatments. The signal indicated luciferase activity of transferred TS-Fluc labeled CAR-T cells. Right: bioluminescence intensity ratio of tumor site *versus* unheated tumor in the same mice. Reproduced with permission [116]. (C) The mechanisms of FUS-CAR-T-cell treatment. (D) Proximal and distal tumor growth curves in mice treated by conventional CD19 CAR-T cells. (E) Proximal and distal tumor growth curves in mice applied with FUS-inducible CD19 CAR-T cells. (F) Quantified *in vivo* cytotoxicity comparison on conventional and FUS-CAR-T cells after tumor challenge. Reproduced with permission [117].

Stimuli-responsive CAR-T cells can be mainly divided into two categories, heat-induced gene activation, and light-induced gene activation CAR-T cells. For the former one, C. Miller et al. designed synthetic gene switches that could respond to mild temperature elevations (40–42 °C) [116]. In the study, they designed 7H-YB-based gene switches, which were upstream arrayed on the transcription site of the heat-shock protein and could be upregulated under thermal stress induced by the plasmonic gold nanorods (Fig. 9A). By using Raji ($CD19^{+}$) tumor model, they discovered a 30-fold luminescence increase in TS-Fluc expressed CD19 CAR-T cells that combined with photothermal treatment in comparison with unheated tumors in the same animal (Fig. 9B). In addition, $CD19^{-}$ K562 tumors exhibited no observable increased luminescence with or without light stimulation, which was attributed to the absence of the CD19 antigen and the migration of CAR-T cells out of the antigen-negative tumor.

Ultrasound can also be used for generating heat and spatiotemporally control the bio-activity of cell therapeutics. Wu et al. innovated CAR-T cells that could respond to the heat produced by focused ultrasound [117]. This flexibility was achieved by a CAR construction that can be activated by a promoter control for heat-shock protein

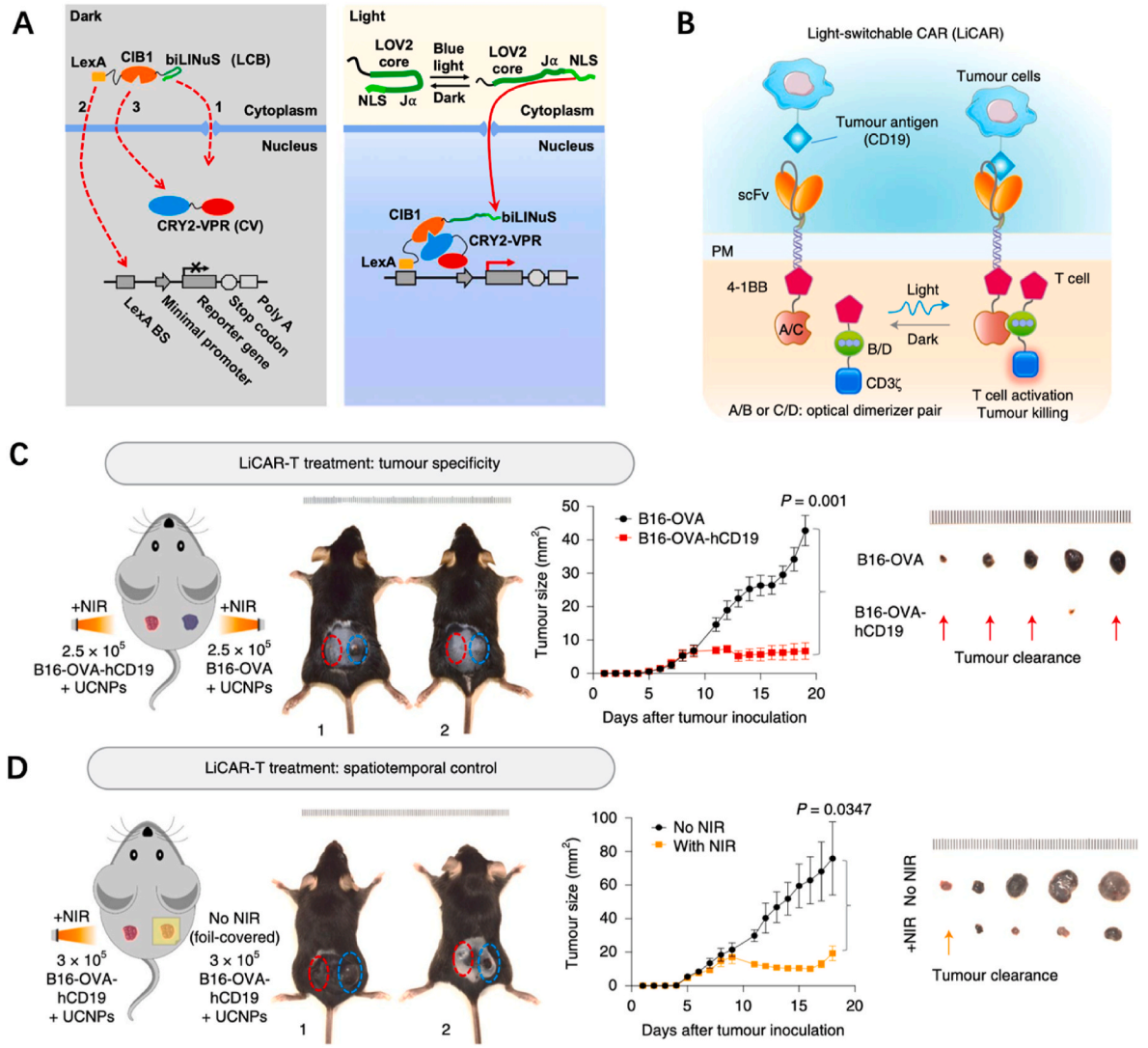

Fig. 10. Light-responsive CAR-T therapy. (A) Schematic illustration of the LINTAD system. Reproduced with permission [118]. (B) Schematic illustration of photo-activatable CAR-T cell. Their activation was under the control of CD19 and light. Such CAR-T cells could solely be activated in the dual presence of light and antigen-presenting tumor cells. (C) LiCAR $CD8^+$ T cells targeted and attacked $CD19^+$ melanoma when stimulated with NIR irradiation. (D) LiCAR T cells permitted NIR-induced elimination of B16-OVA-hCD19 melanoma. Reproduced with permission [119].

(FUS-CAR-T-cell therapy) (Fig. 9C). To test its safety, they used the $CD19^+$ Nalm-6 tumor model and intratumoral injected the same dose of conventional CD19 CAR-T cells or FUS-triggered CD19-CAR T cells at the proximal tumor (Fig. 9D and E). As a result, the conventional one induced proximal tumor remission and attacked the distal tumor. On the contrary, the FUS-inducible type dramatically inhibited the proximal tumor while sparing the distal tumor (Fig. 9F).

Light-triggered gene switches can be leveraged to confine the tumoricidal bio-activity of CAR-T cells within the confined area. For example, Huang et al. developed a light-inducible nuclear translocation and dimerization (LINTAD) system for realizing light-controlled initiation (Fig. 10A) [118]. In a dark environment, LexA-CIB1-biLINuS (LCB) stayed in the cell cytoplasm and CRY2PHR-VPR in the cell nucleus. When stimulated with blue light, the Jα helix of the LOV2 domain in bipartite light-inducible NLS displayed the NLS peptide, which led to LCB's nucleus translocation and targeted VPR to the minimal promoter region for the initiation of CAR-encoding reporter gene.

However, the penetration depth of NIR is down to a few centimeters. To overcome such defects, Nguyen et al. recently used upconversion nanoparticles (UCNPs) to serve as deep-tissue photon transducers [119]. They generated a light-switchable CAR (LiCAR), which could only be activated when stimulated with deep-tissue-penetrable NIR upon engaging with antigen-bearing tumor cells (Fig. 10B). The authors further verified the antigen-specific killing effect and the effectiveness of spatiotemporal control. For each flank of the same mice, they implanted B16-OVA and B16-OVA-hCD19, followed by each flank intratumorally injected with the same amount of CD19 LiCAR T cells, UCNPs, and NIR treatment. As a result, CD19-targeted LiCAR T therapy significantly impeded B16-OVA-hCD19 tumor growth while leaving B16-OVA largely unharmed (Fig. 10C). They further proved that only together with NIR could LiCAR T cells exert cytotoxicity toward $CD19^+$ tumor cells (Fig. 10D).

3.2.2. *Cytokine release syndrome*

Although logic circuits can mitigate on-target/off-tumor risks, their bio-activity is difficult to control once they are administrated into the body. Especially for high tumor burden patients, cytokine release syndrome may occur due to the redundant cytokines produced by the overactivated CAR-T cells and the body's intrinsic immune cells [109]. Although clinical management including supporting care, tocilizumab, and corticosteroids exhibit effectiveness, certain toxicities have stalled out of clinical reach [2,120]. Current efforts are mainly put into

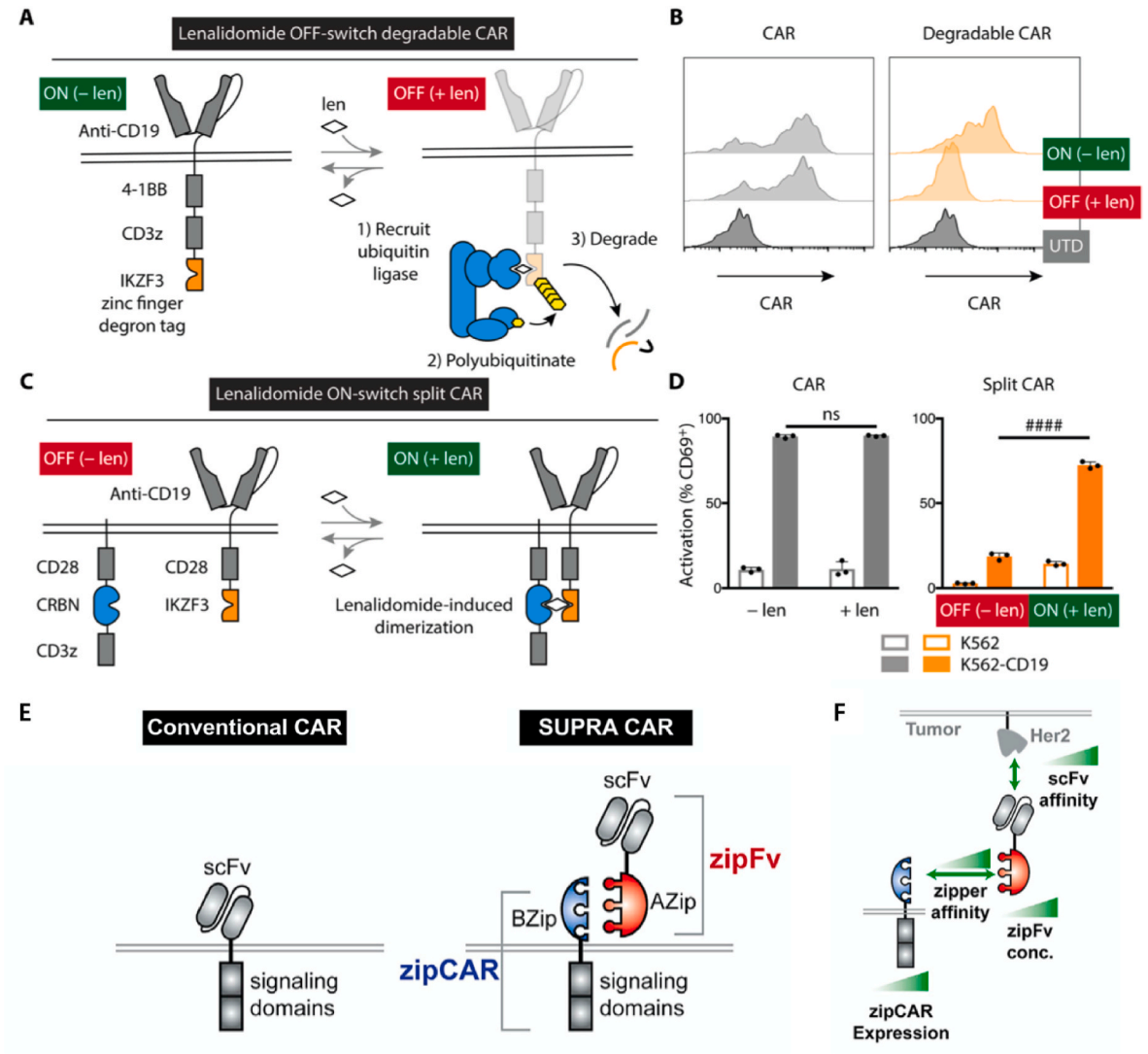

Fig. 11. CAR-T design for alleviating cytokine release syndrome. (A) Illustration of the off-switch CAR-T cells. (B) Surface CAR abundance characterization of anti-CD19 expressed Jurkat cells incubated with 1 μM lenalidomide or vehicle control. UTD stands for the untransduced T cells. (C) Illustration of on-switch CAR-T cells. (D) Conventional CAR-T and on-switch Split CAR-T were incubated with K562 cells engineered to either display CD19 or not, and vehicle control or 1 μM lenalidomide. Flow cytometry quantification of CD69, an early activation marker. Reproduced with permission [123]. (E) The design of conventional CAR and SUPRA CAR. (F) The therapeutic performance of the SUPRA CAR system could be tuned by zipCAR expression, zipper affinity, zipFv concentration, and scFv affinity. Reproduced with permission [125].

designing suicide gene-based CAR-T therapy, on/off switch-based CAR-T therapy, and modular CAR-T therapy.

For suicide gene-based CAR-T therapy, Amatya et al. generated SLAM Family Member 7 (SLAMF7)-target CAR-T cells with an inclusion of a suicide gene encoding a dimerization domain that was integrated into a caspase-9 domain [121]. Such CAR-T cells could be eliminated under artificial control by introducing a dimerizing agent (rimiducid) into the body. However, this method is irreversible. And on/off switches could provide enhanced flexibility.

In one study, Mestermann et al. tried to use tyrosine kinase inhibitor dasatinib to artificially switch their activation state *in vivo* [122]. They demonstrated that dasatinib could interfere with lymphocyte-specific protein kinase (LCK), inhibiting the phosphorylation of CD3ζ and ζ-chain of ZAP70. Therefore, the signal transduction domains were ablated and the tumoricidal activities of CAR-T cells were switched off. As a result, dasatinib could halt the cytolytic and expansion activity of CAR-T cells, hindering their cytokine production. Besides, this inhibitory effects can be rapidly and completely reversed upon discontinuation of dasatinib. Together, such a designed on/off switch system exhibited effectiveness in controlling *in vivo* CAR-T cells' bio-activity for adapting to the patient's disease states.

Jan et al. also engineered a molecule glue-based on/off switch CAR-T program [123]. In their study, thalidomide analog induced $CRL4^{CRBN}$-mediated ubiquitination and proteasome degradation of the degron-tagged CAR. When lenalidomide's concentration fell, translation of CAR further restored CAR-T cell activity and therefore achieved a reversible off switch (Fig. 11A). The off-switch CAR was decreased by 14-fold from baseline after being co-incubated with 1 μM lenalidomide (Fig. 11B). For on switch, two subunits were required. One construct consisted of the CD19 binding domain, CD28 transmembrane region, signaling domain, and lenalidomide-dependent zinc finger domain from IKZF3 (Fig. 11C). The other included the CD8α hinge region, a mutated CRBN, and CD3ζ signaling region. This construct achieved five-fold increased activation when CD19 and lenalidomide dually engaged in comparison with CD19 alone (Fig. 11D). Together, they provided a flexible and reversible program to control CAR-T cells' activity and mitigate the potential inflammatory cytokine release.

For modular CAR-T therapy, the T cells are redirected toward an

adaptor element. This adaptor element can bind to tumor antigens. This system allows controlled killing processes of CAR-T cells, as such an adaptor element can be on-demand administrated, providing augmented therapeutic flexibility and safety profile [124]. In 2018, Cho et al. reported a split, universal, and programmable (SUPRA) CAR system [125]. The BZip domain in the zipCAR construct could bind to the Azip domain in zipFv and the scFv domain further targeted the tumor cell surface antigens (Fig. 11E). The feature of the split CAR construct was that the CAR signaling strength could be flexibly tuned by modulating the zipCAR expression, zipper affinity, zipFv concentration, and scFv affinity (Fig. 11F). In this case, zipFv with higher affinity act as off switch *via* excluding the undesired zipFv, mitigating the potential cytokine storm.

4. Other roadblocks and prospects

Here, we mainly focus on constructing the optimal and innovative CAR-T cells, manipulating tumor cells and their niches, and selecting a delivery strategy. Dilemmas also exist in ideal tumor antigen identification [126], cell source selection, manufacture procedure [127], CAR-T cell activity monitoring, and clinical outcome prediction to achieve patient-individualized therapy and mitigate the potential toxicity [128].

First, the binding domain of CAR-T cells mainly recognizes tumor membrane proteins. However, membrane proteins only represent one-quarter of the proteome, and only a small part of these proteins is uniquely possessed by tumor cells rather than normal cells [129]. Relatively, intracellular proteins play a major role in the oncogenic transition. Numbers of tumor-specific antigens are located at the tumor cell cytoplasm and nucleus, which are recognized by the body's immune system only *via* displaying peptides on MHC. Generating CAR-T cells that target the peptide-MHC (pMHC) complex can greatly expand the immunotherapeutic target pool for solid tumor treatment. Recently, Mark et al. innovated a type of peptide-centric CAR-T cells (PC-CAR) [130], which recognized peptides on human leukocyte antigen (HLA) allotype. PC-CAR T cells could potently attack neuroblastoma cells that displayed these HLAs and achieved tumor remission in a mice tumor model.

Besides, current CAR-T cells are mostly generated from autologous patients' T cells. For late-disease-stage patients, it is relatively hard to acquire suitable autologous T cells due to previous clinical interventions like chemotherapy and radiotherapy [131]. In addition, the lengthy manufacturing process hinders in-time drugs taken by patients. A promising solution is to produce the off-the-shelf CAR-T cells, which extract T cells from healthy donors or induced pluripotent stem cells (iPSCs), mitigating the problem of inadequate T cell numbers, unqualified T cell state, and delayed treatments [132,133]. However, graft *versus* host disease (GvHD) may occur as a consequence of cross-recognition [134]. The current solution is to knock down MHC-I complex and T cell receptors in allogeneic T cells *via* genetic engineering tools like zinc finger nucleases [135], transcription activator-like effector nuclease [136], and CRISPR/Cas9 [137]. However, several studies have reported the potential risks of gene-editing technology including chromothripsis, DNA damage, and auto-immune responses [138]. Therefore, more effort should be concentrated on verifying the security of these gene-editing technologies and innovating next-generation gene-editing tools with enhanced accuracy and efficacy.

As an alternative route, *in situ* programming immune cells *via* gene-editing nanoparticles can reduce the therapeutic cost and avoid lengthy manufacturing time. Stephan's group took the lead in the field of *in situ* programming T cells and successfully translated it into CAR-T therapy [139]. In the study, they used nanoparticles that are surface conjugated with anti-CD3 antibody to target circulating T cells and encapsulated plasmid DNA that encoded the leukemia-specific 19/4-1BB/ζ CAR and hyperactive form of the transposase (iPB7). The transcripted iPB7 could efficiently integrate CAR expression cassette into chromosomes through a cut-and-paste mechanism *via* interaction with piggyBac inverted terminal repeats. As a result, five sequential injections of 3×10^{11} T cell-targeting nanoparticles that carried 19/4-1BB/ζ and iPB7-encoding transgenes eradicated tumors in seven out of ten mice, the therapeutic efficacy of which was comparable to clinical usage of *ex vivo* genetic engineered 19/4-1BB/ζ CAR-T cells.

Recently, Agarwalla et al. reported an implantable multifunctional alginate scaffold for T cell engineering and release (MASTER) for *in vivo* generation of CAR-T cells, which vastly shortened the manufacturing procedure of CAR-T cells from two weeks to one day [140]. This all-in-one program first isolated mononuclear cells from human peripheral blood and then put them together with CD19-encoding retroviral particles into the scaffold. As a result, MASTER allowed *in vivo* local transduction and expansion of CAR-T cells.

Apart from manufacturing, the characteristics of CAR-T cells' *in vivo* performance remain to be specified. Efforts have been put into engineering a type of CAR-T cell that can be traced. For example, Minn et al. tried imaging CAR-T cells with positron emission tomography (PET) [141]. Skovgard et al. realized dual tumor and T-cell imaging *via* bioluminescent reporter and PET [142]. The study revealed the relationship between T cell delivery method, activation state, and tumor heterogeneity with T-cell kinetics. We can use these technologies to predict therapeutic outcomes and potential side effects.

Although most novel CAR-T-based therapeutic strategies remain at the preclinical study stage, empirical sedimentation inspires us to take the essence and discard the gross of every component and procedure, including CAR-T cell design, tumor modulation, and delivery strategy, which motivates the exploration in applying preclinical knowledge into clinical products. For patients with specific tumor types and disease states, it is important to consider the abovementioned three aspects and rationally combine them to achieve optimal therapeutic efficacy and safety. For example, delivery devices can directly input high concentrations of CAR-T cells into tumor tissues and can be accompanied by diverse immunomodulatory adjuvants for therapeutic improvements, which is suitable for patients with localized solid tumors. For cold tumors that have low immune cell infiltration, using oncolytic viruses or photothermal therapy to destroy the tumor matrix can bring about augmented CAR-T cell infiltration [143]. Constructing a type of CAR-T cell that exhibits satisfactory tolerability or can even reverse the tumor immune suppression through secreting immunomodulatory cytokines and proteins can help. For low mutational tumors that are with low immunogenicity, the oncolytic virus can transfect tumor cells with neoantigens to overcome tumor antigen escape [144]. And for high tumor burden patients, the cytokine release syndrome should be taken into consideration [109]. In this case, optimizing CAR signaling domains that reduce cytokine production, and delivery devices that control the release and activation of cell therapeutics may mitigate such side effects [145].

However, the characteristics of patients' tumors are complex and diversified. Therefore, rationally designing a combinatory CAR-T therapy is therefore necessary for meeting clinical needs and shall be the embryonic form of next-generation CAR-T therapy for solid tumor treatment. Specifically, patient examples should first be derived and characterized with tumor antigen situation (TAA or TSA), and the type of tumor immune microenvironment. Based on the acquired information, the appropriate CAR-T product (the construction select: single target or multi-target; synNotch system or muti-display), adjuvant measurement (adjuvant injection routes; adjuvant categories; injection time point), and CAR-T delivery methods (direct injection or biomaterials implant) are selected to multi-target enhancing CAR-T treatment for solid tumors. Meanwhile, blood cytokine tests and imaging systems could help trace the bioactivity of CAR-T therapy *in vivo* in order to prompt decision-making to alleviate potential side effects.

Moreover, the convergence of CAR-T therapy with other cutting-edge biotechnologies may bring out-of-expectation advantages. For example, exosomes are naturally formed during cell culturation, and their biological function has been revealed and received increasing attention due to their inherent crosstalk with the host immune system

[146]. For example, DC-derived exosomes can naturally target lymphoid organs and elicit potent anticancer immune responses [147]. Such effects could also be leveraged as vaccination routes for promoting CAR-T cells' proliferation *in vivo* and exert more potent tumor-killing efficacy. Molecular understandings in $CD4^+$ T cell-mediated tumor killing could also possibly promote the emergence of innovative integration of therapeutic strategies targeting CAR $CD4^+$ T cells. Kruse et al. reported $CD4^+$ T cells could initiate remote inflammatory tumor cell death when encountered with MHC-II^+ $CD11c^+$ antigen-presenting cells, which could serve as complementary therapeutic strategies for overcoming tumor antigen loss faced by $CD8^+$ T cells [148]. Innovative biomaterials, for example, exosomes or microparticles, could be engineered to engage in this process to exploit the tumor-killing efficacy of CAR $CD4^+$ T cells.

With the advancement of synthetic biology, CAR technology has reached beyond CAR-T or cancer treatment [149]. The obstacles encountered by CAR-T therapy, as summarized here, bear similarities to those faced in the case of CAR-natural killer (CAR-NK) cells, specifically limited infiltration and tumor immune suppression [150]. Strategies, such as engineering CAR-NK cells to secrete functional cytokines, chemokines, enzymes, and immune checkpoint inhibitors, can be employed to overcome these challenges. It's essential to note that the selection of targeted expression proteins to activate CAR-cells should vary due to the inherent signaling differences between T cells and NK cells. Since the tumor-killing program of NK cells is antigen-independent, governed by activating and inhibitory receptors, there's a broader range of checkpoints that can be explored to enhance NK cell function when compared to CAR-T therapy [151]. This perspective also sheds light on the potential of utilizing oncolytic viruses or engineered bacteria for targeted protein delivery in enhancing CAR-NK therapy.

In conclusion, a diverse array of CAR constructions, adjuvant tumor manipulation techniques, and innovative delivery strategies have emerged, enhancing the potential of CAR-T therapy for solid tumor treatment. However, as more components are integrated into the treatment approach, there may be challenges related to reproducibility and stability. For instance, living therapies like oncolytic viruses and engineered bacteria may face issues with therapeutic efficacy due to considerations of viability and appropriate injection sites. Furthermore, it is of paramount importance to rigorously verify the biosafety of these approaches. It is worth noting that CAR-T therapy alone has the risk to induce severe cytokine release syndrome (CRS). Therefore, the incorporation of adjuvant strategies, such as the integration of oncolytic viruses or bacteria, should be approached with caution to prevent the exacerbation of cytokine storms. Despite these challenges and considerations, we anticipate that such a combinatory strategy holds the potential to shape the next-generation clinical paradigm of CAR-T therapy for solid tumor treatment.

Ethics approval and consent to participate

This review article does not require any ethical approval or allied consents for publication.

CRediT authorship contribution statement

Chaojie Zhu: Visualization, Conceptualization, Writing - Original Draft, Investigation. **Qing Wu:** Visualization, Conceptualization, Writing - Original Draft, Investigation. **Tao Sheng:** Visualization, Conceptualization, Writing - Original Draft, Investigation. **Jiaqi Shi:** Investigation, Resources, Visualization. **Xinyuan Shen:** Investigation, Resources, Visualization. **Jicheng Yu:** Investigation, Resources, Visualization. **Yang Du:** Investigation, Resources, Visualization. **Jie Sun:** Investigation, Resources, Visualization. **Tingxizi Liang:** Investigation, Resources, Visualization. **Kaixin He:** Investigation, Resources, Visualization. **Yuan Ding:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing - Original Draft, Writing - Review & Editing. **Hongjun Li:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing - Original Draft, Writing - Review & Editing. **Zhen Gu:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing - Original Draft, Writing - Review & Editing. **Weilin Wang:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing - Original Draft, Writing - Review & Editing.

Declaration of competing interest

Z.G. is the co-founder of Zenomics Inc., ZCapsule Inc., and μZen Pharma Co., Ltd., and the other authors declare no conflict of interest.

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