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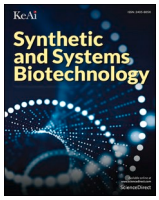

Review Article

Intelligent tunable CAR-T cell therapy leads the new trend

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ABSTRACT

Adoptive transfer of T cells engineered with chimeric antigen receptor (CAR) has been proved to have robust anti-tumor effects against hematological malignancies. However, problems about safety and efficacy, such as cytokine release syndrome (CRS), T cell exhaustion and antigen escape are still raised when patients are treated with CAR-T cells. Moreover, CAR-T therapy has limited applications in treating solid tumors, owing to inefficient infiltration and poor functional persistence of CAR-T cells and diverse immunosuppression in tumor microenvironment. In order to overcome these limitations and broad its applications, multiple controllable CAR-T technologies were exploited. In this article, we review the designs of intelligent controlled CAR-T technologies and the innovations that they bring about in recent years.

1. Introduction

Chimeric antigen receptor (CAR) T cell therapy has achieved unprecedented progress in relapsed or refractory hematological malignancies such as B-cell acute lymphoblastic leukemia (B-ALL), B-cell non-Hodgkin lymphoma (B-NHL), and multiple myeloma [1]. As a specific cell-based therapy, it can induce killing of target tumor cells through recognizing tumor-specific antigens, showing great promise. Up to now, six CAR-T cell products have been approved by FDA [2,3]. However, as a living drug, it is less likely to be controlled in vivo compared with other conventional molecule drugs. The clinical application of CAR-T therapy causes safety concerns such as cytokine release syndrome (CRS) and neurotoxicity [4]. Additionally, the expression of tumor antigens in normal tissues leads to the on-target off-tumor toxicity (CAR-T cells attack normal cells which express target antigens) [5]. On the other hand, CAR-T cells have limited efficacy in solid tumors in current clinical studies. The reasons include that the tumor microenvironment (TME) barriers the access of CAR-T cells, and long-time exposure to antigens impairs T-cell functions and drives them into exhaustion [6–8]. Thus, development of innovative strategies to solve these problems is imperative.

Although several strategies have been applied to improve the safety of CAR-T cells, such as the administration of immunosuppressive drugs and installing suicide switches in T cells, they irreversibly switch off the anti-tumor activities of CAR-T cells [9]. It also becomes a concern that re-rejection of T cells is highly-costed and undesirable. Moreover, small molecules controlling CAR-T cells and other antibody-mediated methods eliminating CAR-T cells may mitigate the toxicity, but the efficacy is alleviated simultaneously [10–13]. Except for the strategies to improve the safety, several modifications were aimed at enhancing its efficacy [14–21]. Nevertheless, it still remains some limitations. For instance, the systematic injection of cytokines was applied to activate T cell functions, but this method caused severe systemic toxicities. Besides, the CAR-T technology with the co-expression of cytokines was developed. However, its clinical effectiveness and safety are still under investigation. Therefore, it is of great significance to develop a controllable CAR-T platform to reversibly regulate T cell activities without sacrificing them, thereby minimizing the severe side effects and optimizing its efficacy.

2. Reversible control over CAR-T cells through on and off system

The emerging inducible systems which are able to turn on and off the activation of CAR-T cells (on and off systems) achieve reversible controls over CAR-T cells and ensure their safety and efficacy. For instance, chemical inducer of dimerization (CID) was exploited to regulate CAR-T cell functions according to dose and time. A costimulatory molecule including MyD88/CD40 signaling domains stimulated by CID improved

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anti-tumor activities of CAR-T cells for solid tumors. It was demonstrated that inducible activation of MyD88/CD40 was required for optimal IL-2 production which recovered functions of CAR-T cells in TME [22]. Encouragingly, dietary molecule resveratrol (RES)-triggered regulation devices allowed precise controls over CAR-T cell activity via a RES-titratable mechanism [23]. The results revealed that a RES-inducible (RES_{ind}) device could regulate the expression of CAR protein through a resveratrol-dependent transrepressor. Meanwhile, a resveratrol-repressible transgene expression (RES_{rep}) device inhibited CAR-T cell activity effectively, while it retained the therapeutic efficacy upon resveratrol removal. Except for chemical molecule and dietary fiber, some physical factors such as light can be leveraged to design a regulatable CAR-T system. In a recent study [24], the light-induced proteins were respectively fused to the transcription activator and the DNA-binding domain. Thus, the light-inducible dimerization of light-based proteins brought the transcription activator to the promoter to activate CAR expression. In this way, the input of light could precisely modulate CAR-T cell activation and downstream signaling responses. This light-inducible nuclear translocation and dimerization (LINTAD) system was sensitive to blue light. It was shut off reversibly when lacking of light stimulation.

In order to eliminate the CRS when the toxicity begins to get out of control [25,26], a spilt CAR structure model shows great safety than the conventional strategy. In this model, the CAR structure is divided into two parts: the antigen recognizing domain and the signaling domain. In the anti-CD19 constructs, the antigen recognition subunit contains an N-terminal CD19-targeting scFv fused to the FK506-binding protein (FKPB12) and a CD4 transmembrane domain, and a signaling subunit includes FKBP-rapamycin binding (FRB*) domain fused to the CD8α transmembrane domain with a costimulatory domain such as 4-1BB [27]. The addition of rapamycin made the separated domains dimerized to switch on the CAR-T cell signaling. On the contrary, when rapamycin was removed, the activation of CAR-T cells was paused. Thus, this improved CAR architecture circumvents the constitutive antigen-dependent signaling of CAR-T cells, which prevents them from sustained stimulation and ensures the safety. Besides, an NS3 (non-structural protease in hepatitis C virus) inserted CAR framework using two-component split-polypeptide configuration was designed to develop VIPER CARs (versatile protease regulatable CARs) [28]. NS3 can proteolytically cleave the viral polyprotein at junction sites of non-structural proteins downstream of itself. The authors designed a split CAR structure, in which an NS3-binding domain was incorporated with the antigen recognition domain and a catalytically dead version of the NS3 domain (S139A) was inserted into the signaling domain. In the on state, the antigen recognition domain combines with the signaling domain through NS3 and its binding domain. The addition of the NS3 inhibitor turns it to an off state, which leads to a transient rest when CRS and neurotoxicity occur. T cell exhaustion is one of major barriers, which limits the effects of CAR-T cell therapy in solid tumors. Transient cessation of receptor signaling was demonstrated to restore the functions of T cells. It was observed that induction of T cell rest by down-regulating CAR proteins in the surface of T cells using kinase inhibitor dasatinib could redirect CAR-T cell fate [29]. The rest of CAR-T cells facilitates the formation of a memory-like phenotype, global transcriptional and epigenetic remodeling, which restores T cell activity in TME. In addition, CAR-T cells modified with a C-terminal destabilizing domain (DD) enables T cells in an off state until a stabilizing drug is added to combine to switch on the activities [30]. Applying these methods, the transient inhibition of CAR signaling can enhance T cell fitness and ensure the efficacy, which shows great potential in treating solid tumors.

3. Control of CAR-T cells through autologous universal CAR system

Conventional CAR designs can only respond to one antigen which constrains its broad applications. Besides, these CAR-T cells must be generated on a custom-made basis, which greatly hinders its large-scale clinical applications due to its costly and laborious production [31,32]. Therefore, universal allogenic T cells have been developed to produce the "off-the-shelf" CAR-T cells to meet diverse customer demands. They are generally prepared from allogenic healthy donors [32], by genetically disrupting the TCR gene and HLA class I loci to abolish graft-versus-host disease (GVHD) and immune rejection [33]. However, allogenic CAR-T technology still faces the problems of efficacy and persistence. In another way, separating the CAR binding module into two parts provides an effective universal approach for autologous treatment, by which combining a specific antigen binding module with a fixed CAR module makes CAR-T technology more flexible and versatile. A previous study exploited an anti-tag technology to eliminate diverse tumors and offered a convenient method to alter the targeting of CAR-T cells [34]. This design took advantage of FITC, a fluorochrome dye that can be easily conjugated with antibodies. The specific interaction between a FITC-labeled antibody and the anti-FITC CAR cassette enables CAR-T cells to target a large range of tumors, which broads its application scale. In addition, a spilt, universal, and programmable (SUPRA) CAR system was designed to enhance the flexibility and controllability of CAR T cells [35]. It has the ability to switch targets without re-engineering T-cells, which is useful to combat with relapse, mitigate over-activation, and enhance specificity. By achieving a three-input logic, this programmable system was verified functional in diverse adaptive and innate immune cells [36].

The SUPRA CAR includes a soluble antigen-binding portion, zipFv, and a universal signal transduction receptor, zipCAR. The zipFv has a leucine zipper and a variable single chain. The zipCAR has intracellular signaling domains and an extracellular cognate zipper that can specifically binds to the zipper on the zipFv. The connection between zippers bridges the binding between the target antigens and the zipCAR-expressing T cells [35]. It not only enables ON/OFF switching, but also takes advantage of AND logic gates to finely tune T-cell activation.

The unique feature of the SUPRA CAR system allows modifications of CAR in such aspects: (1) the affinity between leucine zipper pairs, (2) the affinity between tumor antigen and scFv, (3) the concentration of zipFv, and (4) the expression level of zipCAR. By investigating the effects of the tumor-antigen affinity, the leucine zipper affinity in cytokine secretion and target cell killing efficiency, the authors observed that the SUPRA CAR exhibited its ability to mitigate toxicities and developed a tunable platform [35]. The on-target off-tumor toxicity could be avoided by introducing α-Axl-SYN2 zipFv that competitively binds to α-Her2-EE zipFv through a complementary zipper on each zipFv. Based on this design, Axl acts as an "safety marker", by which the SUPRA CAR system kills target cells that only express Her2 and protects Her2+/Axl+ cells meanwhile. Moreover, the addition of competitive zipFV not targeting specific antigens can be used to tune down the activation of the zipCAR T cells to minimize or terminate the toxicities.

Another attractive application of the SUPRA CAR system is its capability of responding to combinatorial antigens, which enhances the tumor targeting specificity [37]. A tunable AND-gate logic circuit was designed to include the FOS zipCAR (binding to α-Her2-SYN9 zipFv) and the RR zipCAR (binding to α-Axl-EE zipFv) that contained either the CD3ζ or co-stimulatory (CD28 or 4-1BB) domain, respectively. The addition of two zipFvs enabled CAR-T cells to kill target cells. In addition, the SUPRA CAR system shows the potential to solve the problem of antigen escape (tumor cells without target antigen expression are resistant to the elimination by CAR-T cells), by targeting multiple tumor antigens simultaneously without further genetic manipulation. For instance, one study indicated that CAR-T cells expressing RR zipCAR were capable to eliminate tumor cells with either Her2+ or Axl+ expression, when both α-Axl zipFv and α-Her2 zipFv were added, thus expanding the targeting range [35].

4. Modulation of CAR-T cells through SynNotch CAR system

The lack of tumor specific antigens increases the risks of CAR-T therapy. Diverse potential targets for CAR-T cells are also expressed in normal tissues which may lead to severe off-target toxicities [38]. An effective strategy to address this concern is to engineer a combinatorial strategy that activates CAR-T cells when more than one antigen exists [39]. Recently, the dual-receptor AND-gate T cells, which takes advantage of synthetic Notch (SynNotch) circuit, opens door to the recognition of a broad range of solid tumors [40]. SynNotch includes an extracellular ScFv, aimed to recognize tumor antigens, once it combines with target antigens. Ligand engagement triggers the self-cleavage of receptors to release the transcriptional activators to specifically bind to the promoter, which can induce the expression of defined target genes such as CAR [41]. In this design, a synthetic Notch receptor against one antigen induces the expression of CAR that targets a second antigen.

As cells could integrate multiple inputs to modulate their signaling pathways, exploiting such a combinatorial receptor displays an effective method to discriminate different targets in vivo when presented in normal and tumor cells respectively. For example, ROR1-targeted CAR-T cells with synthetic Notch receptors for EpCAM or B7-H3 can specifically eliminate tumor cells positive for dual antigens, accordingly preventing elimination of normal stromal cells [42]. In the treatment of glioblastoma (GBM), T cells are primed by a SynNotch receptor that recognizes a tumor-specific antigen EGFRvIII, thereby increasing the safety of CAR-T cells targeting tumor-associated antigens, such as EphA2 or IL13R [43]. Taken together, this provides a powerful tool to discriminate tumors cells and normal tissues, which greatly improves the safety of CAR-T therapy.

Supplement of inflammatory cytokines to augment T cell activity is an effective strategy to remove tumor cells [44]. However, systematic administration of IL-2 was proved to cause serve toxicities including capillary leak syndrome and eventually end-organ dysfunction [45]. Engineering T cells to bear a SynNotch receptor primed by a tumor-specific antigen to activate the expression of IL-2 offers a potential way to locally overcome tumor suppression while minimizing toxicities caused by IL-2. The autocrine production of IL-2, by which the same T cells co-express the CAR and SynNotch IL-2 circuit was proved to be more efficient than paracrine delivery of IL-2, owing to the existence of competing IL-2 consumer cells (both native T cells and T regulatory cells) [46]. As a result, driving SynNotch IL-2 circuit to overcome the tumor immune suppression makes it more effective for CAR-T therapy to treat solid tumors.

Previous studies have indicated that the tonic signaling caused by constitutively expressed CARs leads to premature CAR T cell differentiation and exhaustion, which impairs T cell persistence in vivo [47]. Temporary pause of CAR signaling or receptor redesigns have been proposed to enhance antitumor efficiency. SynNotch-CAR T cells showed lower expression of CD39 and transcriptional activity of NF-kB when the CAR was under the control of the SynNotch [48]. This phenomenon suggests that SynNotch regulation could circumvent unfavorable tonic signaling, which enables CAR-T cells to maintain a long-lived memory and non-exhausted T cell phenotype.

5. Discussion

In summary, increasing tools were exploited to achieve tunable controls over CAR-T cells and showed the potential of overcoming current limitations (Table 1). The on and off systems can reversibly control CAR-T cells with the addition or removal of stimulation materials such as small molecules and light. Not only do they relieve CRS and neurotoxicity through off switches, but also enhance the efficacy through on switches. But they are unable to solve problems such as on-target off-tumor toxicity or antigen escape. For the synNotch CAR device, it provides a novel way to modulate CAR-T cell activity by recognizing dual antigens, which makes it an ingenious method to prevent the on-target off-tumor toxicity. Moreover, synNotch circuits offer the possibility to drive the tumor-localized production of genetically encoded therapeutic payloads such as cytokines, chemokines and antibodies along with CARs [49,50], which enhances intrinsic CAR-T cell activity and improves immunosuppressive TME. Also, the synNotch strategy can ameliorate the limited anti-tumor function of CAR-T cells through diminishing the tonic signaling. However, the safety concern still remains if CRS or neurotoxicity occurs, because it is not a reversible system. The autologous universal CAR system takes advantage of the design of a fixed CAR module and a flexible antigen-binding scFv, which enables it to target diverse tumors without reengineering CAR-T cells. Besides its convenience to provide the prompt and flexible tuning of CAR-T cell signaling, it can solve the problem of antigen escape by adding various antibody modules to increase the tumor targeting range in a comparatively simple manner. Thus, this system enhances the safety of CAR-T therapy. Although these tunable CAR-T therapies show great promise, more clinical trials are demanded to testify the safety and efficacy, and some of them are under way. Based on the learning of current methods, further development of tunable platforms to intelligently modulate CAR-T cells will be anticipated in the future to break through the limitations.

Table 1
Intelligent tunable CAR-T systems overcome current limitations.

Systems	Problems to solve			
	CRS and neurotoxicity	On-target off-tumor toxicity	Antigen escape	Poor CAR-T cell function
On and off system [22–24,27–30]	✓	×	×	✓
Autologous universal CAR system [34–37]	✓	✓	✓	×
SynNotch CAR system [40–43,46,48]	×	✓	×	✓

Declaration of competing interest

The authors indicate that they have no conflicts of interest.

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