CAR T Cell On-Target, Off-Tumor Toxicity in Solid Tumor Treatment: Generation and Management

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Abstract. CAR-T cells involve incorporating T cells with a synthetic receptor to target tumor-specific molecules and this therapy enhances the patients' own immune system to combat tumors. CAR-T cells are successful to elicit an enduring response to hematological malignancies but remains unsatisfactory during their application in solid tumors. Mostly, OTOT is a main safety concern during trials of CAR-T cells, which has led to destruction of healthy tissues and their normal functions, potentially threatening the patients' life if damage were done on essential organs. OTOT arises from the CAR-T activation upon normal cells due to presence of tumor-like molecules on their surfaces, accompanied by potential cytokine release syndrome (CRS), which further lyses normal tissues. In this review, the structure and mechanisms of CAR-T cells will be described. More importantly, we will discuss the OTOT generation in CAR-T trials targeting solid tumors and available methods to mitigate this problem for advancing CAR-T application.

Keywords: CAR T cells, on-target off-tumor toxicity (OTOT), CRS.

1. Introduction

T cells act as the key mediators of adaptive immune system, which is responsible for their anti-tumor cytotoxicity. To better combat cancer, T cells have been infused into patients by researchers to enhance the anti-tumor effects. With the patients' endogenous T cells with synthetic receptors targeting tumor cell membrane molecules, CAR-T cell therapy is one of the promising applications of T cell [1]. These engineered cells are cultured ex vivo until infusing back to the patients [1]. Upon recognition of tumor molecules by the recombined receptors, T cells are activated and they help to clear the tumor cells [1]. Nowadays, six FDA-approved CAR-T products are presenting to treat B cell-related malignancies, and this proves the success this therapy has reached in hematological cancer [2]. However, CAR-T cells remains unsatisfactory in treating solid tumors with known risk factors, among which OTOT is the major safety concern [3]. OTOT generation is due to the expression of biomarkers that resemble tumor-like molecules present on non-malignant tissues, which bind to the synthetic receptors and induce T cell cytotoxicity on normal cells [3]. In this review, we described CAR-T cell structures and their mechanism of function. OTOT generation is also explored and discussed, along with current strategies to mitigate OTOT, including improvement on CAR-T cell structure and logic engineerings.

2. CAR-T cell structures and mechanisms

CAR-T cells are engineered T cells transfecting with a recombinant receptor for better recognizion of tumor cells. One single CAR contains an extracellular monoclonal antibody derived scFv to recognize tumor surface molecules [3]. The scFV usually consists of one heavy (V_H) and one light (V_L) chain of antibody variable regions, which are responsible for identifying a specific protein on tumor cell surfaces in an MHC-independent way [3]. Within the six FDA-approved CAR-T cell therapies for hematological malignancies, four of them recognize CD19 and the rest two bind to B-cell maturation antigens (BCMA) [4]. Both CD19 and BCMA are relatively perfect biomarkers that are exclusively expressed in B cells involved in most B-cell related malignancies [4]. Selection of appropriate antigens for solid tumors is more difficult because usually the targeted antigens are not

constrained to tumors, but also on non-malignant tissues [4]. Improvements in the scFV needs to be investigated to reduce the incidence of CAR T activation on non-malignant cells.

The scFV is fused with intracellular signaling domains bridged by a transmembrane domain (TM) [3]. While the TM is necessary for fixing the CAR on the T cell, it also contributes to the interaction with targets on the tumor cell surface [5]. The intracellular domains are crucial for transduction of signals for CAR-T activation. First generation CAR-T designs with a single cytoplasmic CD3ζ chain [3]. When the scFV recognizes tumor-like molecules, the CD3ζ chain transmits a primary signal or 'signal 1' for CAR-T activation mediated by ITAM [5]. The ITAMs are phosphorylated that activate downstream molecules to elicit a chain of responses, and the number of ITAMs are shown to affect the intensity of CAR T activation [5]. While the primary signal can induce T cell activation, it alone leads to limited CAR T activation and low persistency after infusion [5]. Therefore, later generations fuse the CD3ζ with co-stimulatory domains such as CD28 or 4-1BB (CD137) [5]. Antigen sensitivity has been shown to associated with the type of co-stimulatory domains incorporated, and evidence has suggested that 4-1BB domain leads to slower and less intense signaling [4]. These together indicate the possibility of adjusting the level of responses when applying CAR-T cells in solid tumors.

T cell anti-tumor cytotoxicity is mainly mediated by releasing cytolytic particles: perforin and granzymes [6]. Upon CAR-antigen recognition, intracellular vesicles within T cells containing the cytolytic particles migrate to and fuse with CAR-T membrane [6]. This results in the release of perforin into the synaptic cleft to form pores on the tumor cell membrane and allow granzyme entrance [6]. Granzymes promote apoptosis of the tumor by cleaving the substrates [6]. Furthermore, the Fas and Fas ligand (FasL) mediated pathway is also utilized by CAR T cells as an alternative killing mechanism [6]. CAR T cells can up regulate FasL expression on their surface to be recognized by Fas receptors on tumor cells [3]. This pathway induces apoptosis via activation of caspases to cleave a number of subsequent substrates in a antigen-independent way [6]. Apart from direct CAR T cell-tumor interactions, cytokines such as IFNγ and TNF produced by CAR T cells are also crucial for secondary pathways to lyse tumors [6]. These cytokines are involved in re-polarization of other immune effectors such as macrophages into an anti-tumor state, while they also contribute to off-tumor toxicities on non-malignant tissues [6].

3. OTOT as a safety concern for CAR-T cell use in solid tumors

OTOT presents as one main limitation of CAR-T cell application in solid tumors, and it originates from recognition of target antigens expressed on non-malignant tissues to activate CAR-T cells [3] (Table. 1). Lysis of normal cells lead to destruction of non-malignant tissues, which lead to disruption of their functions and potential organ failure [3]. While OTOT has been widely documented in CD-19 targeted CAR-T cells caused by CD-19 presence on normal B cells, it presents as a even more challenging issue when applying this treatment cells in solid tumors [3]. This is because of less appropriate selection of antigens available for solid tumors. The optimal candidates of tumor biomarkers are tumor-specific antigens (TSA), which are constricted to tumor cells but absent on normal cells [7]. Unfortunately, surface expressions of TSAs in solid tumors are rare and the majority of TSAs discovered so far exist as intracellular proteins [7]. The only examples of current available TSAs are EGFRvIII and EGFR806 that are found in glioblastomas [7]. Therefore, researchers have focused on targeting TAA on both tumor and non-malignant cells, with examples such as HER2, B7-H3, and GD2 [3].

Despite the feasibility of targeting TAAs, it increases the risk of OTOT occurrence in patients due to low TAA expression on normal cells. Among the available TAAs for solid tumors under investigation, a number of them show body wide expression on non-malignant tissues, such as B7-H3, c-MET, FAP [3]. Nevertheless, there are some TAAs that are more specifically expressed, including CLDN18 that is commonly upregulated in lung and pancreatic cancers and MART1 for melanoma [3]. OTOT generation is affected by several factors and one example is the use of HER2 as a TAA, which gave different levels of OTOT during clinical trials for solid tumor treatments [3].

In one report, a fatal case was documented due to HER-2 expression in lung tissues, while less severe symptoms such as skin irritations and gastric hemorrhage were observed in another trial [3]. By contrast, from a phase I/II study to treat sarcoma, all patients were well tolerated with HER-2 targeted T cells without evident dose-limiting toxicity [3]. In conclusion, using HER2 as a target molecule gave various toxicity profiles based on differences in CAR-T designs and doses, and tumor locations, which indicates the need to adjust CAR-T structures to reduce OTOT incidence [3].

Cytokine release syndrome (CRS), a common type of on-target, on tumor toxicity, is characterized by excessive cytokine release by bystander cells after lysis of large quantities of tumor cells [8]. The CRS can also contribute to OTOT because lysis of normal cells caused by CAR-T off-tumor cytotoxicity induces further cytokine release (Table. 1). Current explanation on CRS pathophysiology believes that the release of IFN-γ and TNF by activated CAR-T cells triggers activation of effectors of the innate immune system, including macrophages and endothelial cells that further secrete cytokines such as IL-6, IL-10 and TNF-α [8]. IL-6 is the key cytokine involved in CRS, and it is associated with many of the characteristic symptoms such as vascular leakage, complement activation as well as cardiomyopathy [8]. There are two IL-6 mediated pathways. The classical signaling pathway is based on IL-6 binding to IL-6 receptors (IL-6R) constricted to cell membranes, while the trans-signaling pathway involves IL-6 binding to soluble IL-6Rs that are cleaved from the cell surface [8]. The existence of such soluble IL-6Rs allows activation of cells that lack surface IL-6R expression, giving an even broader range of CRS [8].

4. Strategies to overcome OTOT caused by TAA expression on normal cells

4.1. Affinity tuning of CAR-T cells

The principle of adjusting CAR-T affinity to overcome OTOT stems from the difference in antigen densities between cancerous and non-malignant cells [9]. Evidence has suggested that the expression levels of some TAAs are commonly unregulated in tumor cells, while exceptions still exist and normal cells can express TAAs at high levels [9]. The scFv segments are responsible for antigen recognition and they can have different affinities to the same TAA depending on the designs [9]. CAR-T cells with high affinity scFv can induce higher cytotoxicity in tumor cells with lower TAA expression, but they are also more likely to be activated upon non-malignant cells [9]. By contrast, low affinity CAR-T cells can discriminate cancerous cell with higher TAA densities compared to normal cells [9]. Thus, reducing scFv affinity can theoretically enhance CAR-T specificity and limit OTOT incidence (Table. 1). However, this increase in specificity can lead to reduction in the anti-tumor potency against tumor cells with lower TAA expression [9] (Table. 1). In conclusion, to design the appropriate scFv, a balance between specificity and potency needs to be determined based on the TAA density on the surface of targeted tumor cells.

Apart from tuning the scFv, modifications to the co-stimulatory domains as well as the number of ITAMs can influence the threshold of TAA density necessary to activate CAR-T cells. For instance, among hematological malignancy treatments, CD28-based CARs require less antigen density for activation compared to those use 4-1BB as the co-stimulatory domain [4]. Moreover, decreasing or deleting number of ITAMs can increase TAA threshold for CAR-T cell activation, hence reducing OTOT [4]. Similar to modification to reduce scFv affinity, the problem of decreased sensitivity against tumors with lower TAA expression also exist.

4.2. Dual CAR-T cell designs

To solve OTOT caused by TAA expression on non-malignant cells, dual CAR-T cells have been designed based on expression of two CARs recognizing distinct TAAs [3] (Table. 1). Each CAR is fused to either one single CD3ζ chain or one co-stimulatory segment [3]. Dual CAR-T can reduce OTOT in theory because two TAAs are required simultaneously to induce anti-tumor cytotoxicity, while a single TAA expressed on non-malignant cells would lead to incomplete signaling and inactivation of CAR-T cells. This was validated by one study in which dual CAR-T cells targeting

carcino-embryonic antigen (CEA) and mesothelia (MSLN) required simultaneous presence of both TAAs to be activated in the pancreatic cancer cell lines [3].

One potential problem associated with dual CAR-T cell use is leakiness, which can also occur in conventional CAR-T therapy targeting myeloid malignancy [10] (Table. 1). For example, patients with B-cell acute lymphoblastic leukemia (B-ALL) show relapse tumor cells that lack CD19 expression after CAR-T treatment [10]. This can remain as a significant issue for dual CAR-T use in solid tumors because mutations of either TAA can render the CAR-T cells ineffective. By contrast, some clinical trials provided the evidence that dual targeting CAR-T cell can reduce antigen escape [10]. Nevertheless, further improvements such as discovering more broadly expressed TAAs might reduce the risk of antigen escape, while it is expected to raise the incidence of OTOT [10]. The key for future dual CAR T development is to balance the specificity and scope of the chosen TAAs.

4.3. TME-targeting dual CAR-T cells

Another approach is to target the TME, which is a shared feature of tumor cells. The TME is characterized by dysregulated vasculature, unregulated levels of immunosuppressive cytokines as well as altered metabolism, which differentiates tumor cells from the non-malignant cells [2]. Incorporation of a CAR targeting the TME and another CAR targeting a single TAA can theoretically reduce OTT incidence with lower possibilities for antigen escape (Table. 1).

Researchers have made it possible to design a dual CAR-T system that targets immunosuppressive cytokines such as TGF-β and IL-4 to combat pancreatic cancers [3] The anti-PSCA CAR-T cells were incorporated by a second CAR that recognizes immunosuppressive cytokines and invert the signals into co-stimulatory ones [3]. Similarly, the characteristic hypoxic environment of TME has also been targeted utilizing the subdomains of transcription factor hypoxia-inducible-1 factor alpha (HIF1α) [3]. The HIF1α subdomain was fused to the CAR-T C-terminus to allow hypoxia-induced CAR transcription [3]. This modification allows selective CAR-T activation under stringent oxygen deficiency (0.1% oxygen) only to reduce OTOT generation in normal tissues [3]. Reduced pH (pH6.2-6.8 compared to pH7 in normal tissue) caused by the "Warburg effect" can be another target. Researchers have combined the scFv of AXL receptor tyrosine kinase (RTK) and RTK-like orphan receptor 2 (ROR2) that were shown to bind their antigens in a higher affinity under acidic conditions (pH6.7) than under physiological pH [3]. These recombined CARs required both antigens and acidic TME conditions for activation, hence reducing OTOT [3].

4.4. Strategies to overcome OTOT caused by CRS

Conventional ways to mitigate CRS includes targeting IL-6 that is the key driver for CRS while IL-6 has little impact on cytotoxicity to clear tumors [9] (Table. 1). Tocilizumab, the monoclonal antibodies against both membrane-bound and soluble IL-6Rs, is used to interfere with both pathways of IL-6 signaling and is approved by FDA to treat CRS [9]. Corticosteroids can be used as a second line treatment when patients experience refractory CRS after tocilizumab or they experience severe neurotoxicity, but the duration of corticosteroid use must be monitored to reduce its impact on CAR T cell effectiveness [9]. In cases where both treatments mentioned are ineffective, other immunosuppressants such as siltuximab that blocks IL-6 can be used [9].

Apart from using immunosuppressant after CAR-T infusion, improvement in CAR-T design can also help to prevent CRS incidence (Table. 1). Evidence suggested that affinity modulation of CAR-T cells can influence cytokine secretion after activation, and this can be further explored to reduce the amount of IFN-γ and TNF release within the safety scope [9]. Another approach to reduce CRS is to incorporate a suicide gene into CAR-T designs to reverse cytokine over-production. One example of the safety switch mechanisms is the caspase 9/AP1903 suicide system.

Table 1. Summary on OTOT mechanisms, current strategies to overcome them, and potential challenges with these methods

Mechanism of OTOT generation	Strategies under research	Potential challenges regarding current strategies
TAA expression on non-malignant cells	Affinity tuning of the scFv/co-stimulatory domains/ITAM multiplicity to reduce CAR T activation on cells with low antigen density	Reduction in CAR T cell affinities impairs effectiveness against tumor cells with lower antigen expression; Balancing CAR sensitivity and potency requires further research
	Dual CAR T systems requiring recognition of two distinct TAAs for full activation	Tumor escape is more likely to occur in dual CAR T system due to stringent requirement for co-expression of TAAs on tumor cells
	Dual CAR T systems with one CAR targeting features of the TME	Existence of physiological environment resembling TME (e.g., hypoxia, low pH) can lead to CAR T activation upon normal cells
CRS due to CAR T activation and lysis of normal cells	Affinity tuning to reduce cytokine release	IFN-γ is the key cytokine for CAR T cytotoxicity, so reduced release can impair anti-tumor effect
	Combinational therapy with immunosuppressants to mitigate CRS symptoms	Resistance to the immunosuppressants can be a potential problem

5. Summary

The effectiveness of CAR-T therapy in hematological cancers has attracted researchers to translate its success in solid tumors. However, use of TAAs as target molecules for CARs has led to generation of OTOT because of TAA presence on non-malignant tissues. This OTOT is further exacerbated by excessive cytokine release after destruction of non-malignant tissues. Intensive researchers have focused on solving OTOT based on affinity tuning of the scFv and co-stimulatory domains to reduce CAR-T activation upon normal tissues. Dual CAR T systems have been explored to more specifically target tumor cells, and characteristic features such as TME have been targeted as well. CRS is managed via affinity tuning and combinational therapies with immunosuppressants. Managing OTOT is crucial for safer use of CAR-T cells in future developments to combat solid tumors.

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