# CAR T cell-induced systemic cytokine toxicity: current understanding and innovative designs

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**Abstract.** CARs engineering has emerged as a promising treatment for tumor. However, CAR T cell-induced systemic cytokine toxicity is one of the roadblocks limiting the widespread application of the therapy. Current study contributes to some toxicity-related monitoring and management guidelines. Researchers have further developed novel engineering strategies to produce inducible CARs and passively or autonomously control CAR T cells. This review described the recent bench and clinical outcomes of novel engineering CAR T-cell products and discuss their function, advantages, and limitations.

**Keywords:** CAR-T therapy, Mechanism, Toxicity.

## 1. Introduction

CARs are recombinant receptors that fuse an antigen recognition moiety and signaling domains [1]. CARs contribute to T lymphocytes redirecting and consequently lead to rapidly evolved tumor-targeted CAR T cell therapies. Although this research field has been catapulted into an era of the fast-paced and innovative stage, researchers are still fighting with many roadblocks that limit the broad application.

Even though CAR-T therapy is effective for treating cancers, sometimes, it could also contribute to toxicities in the patient. Toxicities are main barriers to limiting the applications of CAR T cell therapy. The fulminant HLH/MAS is a rare but deathly case of CRS. CAR T cells can also cause the ICANS. These symptoms are potentially generated by the cytokine storm synergistic reaction reflecting the robust activation and correlated host immune cells [2]. These toxicities are usually reversible and manageable in most patients, though some severe cases require immunosuppressive therapy. In general, pharmacological immunosuppression drugs tocilizumab, siltuximab, and corticosteroids can effectively mitigate the cellular toxicities and rapidly induce reversal of CRS and ICANS symptoms. Although the existing toxicity grading systems and treatment strategies can primarily prevent the risk of life-threatening complications, these approaches are imprecise. Hence, fundamental modifications of CAR design or CAR T cell engineering might be an improved method to ameliorate cytokine-related toxicities.

There are emerging four categories of modified CARs with "ON/OFF Switches", including adapter-mediated CAR, split-CAR, CAR with systemic T-cell inhibition, and CAR with protease inhibitor. These methods rely on co-administration of small-molecule agents [3]. Permanent CAR T-cell death can be induced by co-expression of "suicide genes" or elimination markers. In addition, the systems allow a reversible control of CAR expression on the transcriptional level.

This review focused on CAR T cell-induced systemic cytokine toxicity, including the pathophysiology, clinical presentation, management strategies, and engineering approaches.

## 2. Current understanding of CAR T cell-induced systemic cytokine toxicity

High systemic cytokine release generally reflects the robust expression of CAR T cells. This cytokine storm can lead to a vicious cycle that causes severe and sometimes lethal toxicities during the interactions of cells and immune cell cross-activation. Although the initial trigger of CAR T cell-induced cytokine disorder remains unknown, evidence has revealed an inflammatory circuit. The core

cytokines that are massively produced are IL-6, IL-10, and interferon (IFN)-γ [4], as do correlated immune cells, usually secrete TNF-α, IL-2, and IL-10.

Elevation of interleukin 6 (IL-6) level in the serum seems to be a key factor in systemic cytokine toxicities. IL-6 receptor (IL-6R) associating with gp130 and initiating the intracellular signaling via JAK/STAT pathway. Classical signaling could generate anti-inflammatory or regenerative activities at low levels of IL-6. Because the metalloproteinases ADAM17 mainly regulate the shedding of the IL-6R, it might be promising to manipulate this proteinase to reduce the systemic cytokine toxicity in CAR T cell therapies.

The cytokine storm leads to the activation of endothelial cells and consequently elevates ANG2 in the blood of patients associated with severe systemic and neurologic toxicity. The aberrant activation of endothelium results in some severe vascular dysfunction symptoms, *i.e.*, capillary leakage, hypotension, and consumptive coagulopathy (*i.e.*, disseminated intravascular coagulation).

Although neurologic toxicity pathogenesis is unknown, two potential explanations can be postulated. First, high serum levels of IL-6 and IL-15 were observed in CAR-T patients. Second, CAR T cells were detected in cerebrospinal fluid from neurotoxicity patients; thus, the trafficking and direct CAR T-cell toxicity may be partly responsible for the neurologic side effects. Models have been developed to predict severe systemic cytokine toxicities by tracking the serum cytokine levels [5]. These models may guide early intervention with immunosuppressive agents against toxicity in high-risk patients.

CRS was frequently reported as a severe adverse event after CAR T cell therapy [6]. Clinically, patients with CRS suffer various symptoms. Constitutional symptoms of CRS typically involve fever, hypotension, hypoxia, and organ toxicities. Studies demonstrated that high cell doses and the addition of lympho-depleting chemotherapy especially fludarabine, have been associated with developing severe CRS and ICANS. Nevertheless, clinical results proved that lympho-depleting chemotherapy enhances both response rates and activity of CAR T cells.

CAR T cell-induced HLH/MAS is rarely reported; however, it can be a severe, even fatal, systemic hyperinflammatory syndrome associated with robust immune activation [7]. HLH/MAS have similar clinical manifestations as CRS after CAR T cell therapy assembling with elevated serum levels of ferritin, liver enzymes, lactate dehydrogenase, and hemophagocytes but lowered serum levels of fibrinogen [8]. Although refractory HLH/MAS can be highly mortal if not treated promptly, the traditional diagnostic criteria could be confused by CRS symptoms.

Typical symptoms of ICANS are confusion, delirium, aphasia, ataxia, cognitive defects, nerve palsies, tremor, hallucinations, obtundation, somnolence, myoclonus. It often occurs with CRS, associated with the disrupted blood–brain barrier and increased cytokine levels. However, ICANS may also occur in patients before CRS, without CRS, or after CRS abatement. Based on these reports, ICANS may have independent mechanisms compared with CRS but have overlapping risk and causative factors. Although the severity of ICANS can deteriorate rapidly, it is usually reversible and causes rare fatal cases.

Management of the systemic cytokine toxicities is a critical step in CAR T cell therapies. Approaches to nonspecific immune suppression have been used widely across institutions currently. Pharmacological immunosuppression drugs are commonly effective in ameliorating cellular toxicities and rapidly inducing reversal of CRS and ICANS symptoms. Tocilizumab was approved by FDA in 2017, whereas siltuximab has been used off-label of CRS yet. Accordingly, clinical trials have used tocilizumab more commonly than siltuximab. Moreover, tocilizumab has poor central nervous system penetration, which limits its efficacy in remission of neurotoxicity. Even worse, a study observed that serum IL-6 levels increased after tocilizumab administration, probably because of the prevention of IL-6R-mediated uptake of IL-6 into peripheral tissues. Therefore, a theoretical concern has been concluded that tocilizumab may increase the risk of neurotoxicity by passive diffusion of IL-6 into the CNS. Systemic corticosteroids (*e.g.*, dexamethasone) can suppress inflammation and thus have been used to manage CRS, HLH/MAS, and ICAMS.

## 3. Engineering the CAR protein with "ON/OFF switches"

ON/OFF switches in CAR designs predicates on the administration of small-molecule agents, functionally controls the intensity and toxicity of CAR T-cell activities. An adaptor-mediated CAR can recognize bi-specific adapter molecules that bridge CAR T cells and cancer cells. For example, an adaptor molecule consists of a folic acid moiety and a fluorescein moiety (Figure 1a). The folic acid moiety binds to folate receptors on cancer cells, while the fluorescein moiety binds to anti-fluoresceine scFv CAR on CAR T cells. These bi-specific adapter molecules can subsequently be manipulated dynamically in order to prevent CRS [9]. In addition, Urbanska *et al.* designed a "universal" CAR. BBIR T cells can, in principle, recognize and bind exclusively to cancer cells that have been labeled with specific biotinylated molecules [10]. Two advantages of adapter-dependent CAR include 1). the ability to titratable and reversible control the ON/OFF state of the CAR T cells; 2). the ability to target tumor-associated antigens by changing the specificity of the adapter molecules. However, considerable differences in adapter-molecule kinetics have been reported, limiting the standard and optimized administration of adapters. Many factors may reflect those differences, including biodistribution of the adapter molecules and affinity differences between the adapter and both targets on CAR T cells and on tumor cells.

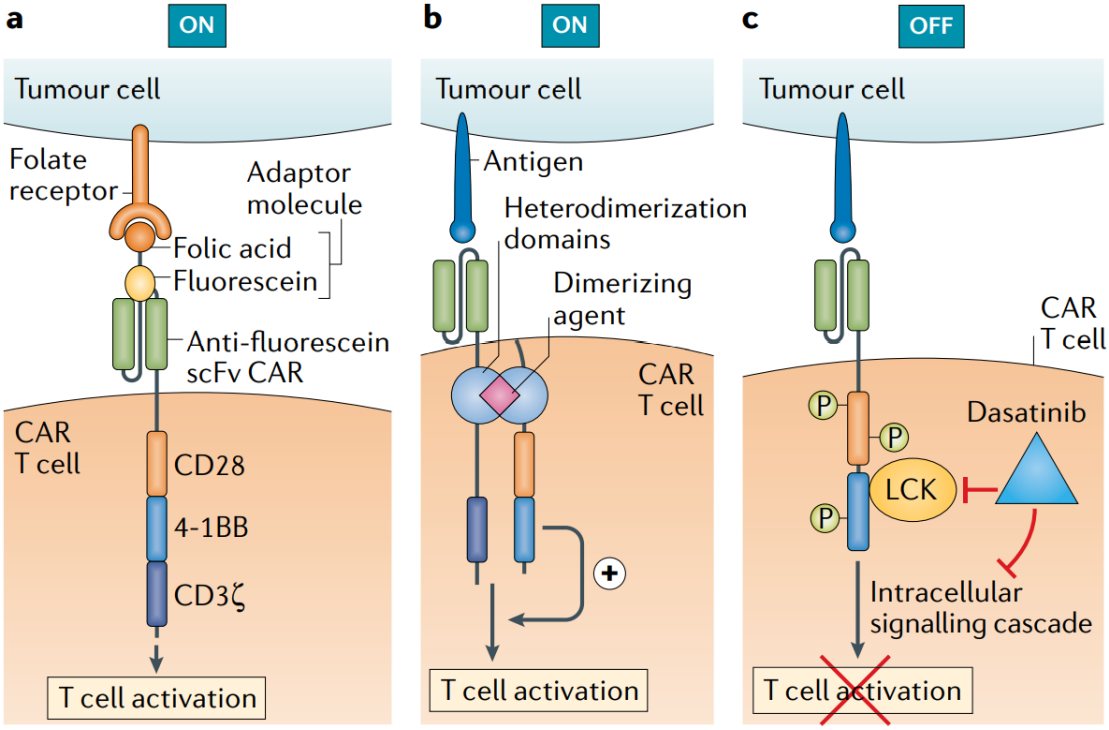



**Figure 1.** Small-molecule agents mediated ON/OFF switches [3]

CAR T-cells induced toxicities can be temporally and reversibly controlled through engineering a split intracellular signaling domain of CAR construct [11]. Conventionally, CAR has been designed into a single-linkage molecule, consisting of scFv, the main signaling motif, and co-stimulatory motifs. In 2015, Wu *et al.* developed a strategy for constructing CAR subunit heterodimerization domains and the complementary dimerizing agents (Figure 1b). They applied the FKBP/FRB* domain pair as a functional heterodimerizing agent in the presence of the rapalog. They also applied GID1-GAI system because this type of "ON-switch" CAR became activated only with gibberellin. However, the split-CAR cannot prevent on-target, off-tumor toxicities because it lacks control over the distribution of the drug.

"OFF-switch" strategies provide a means to deactivate CAR T cells in CAR construct engineering. In 2019, Mestermann *et al.* demonstrated that dasatinib act as a pharmacologic "OFF-switch" [12]. Dasatinib disrupts signaling by interfering with CAR activity by inhibiting LCK, thus inhibiting phosphorylation of CD3ζ (Figure 1c). Dasatinib can rapidly and reversibly prevent CAR T cell

activation without eradication. Moreover, dasatinib has a short half-life of 4 hours which gives it the advantage of acting as a quick ON/OFF switch. However, the usage of dasatinib is limited against acute CRS, because it cannot sufficiently inhibit already activated CAR T cells.

Another study in 2019 developed a protease-based SMASh-CAR, also known as SWIFF-CAR [13]. In this system, a CAR was designed following a protease target site, a protease, and a degron. In the 'on' state, the protease inhibitor is absent; thus, the degron is cleaved from CAR and eventually allows the expression of the scFv on the surface of the T-cells. Conversely, protease inhibitor leads to the CAR degradation via the proteolytic pathway (Figure 2). However, the virally derived NS3 protease may lead to immunogenicity and restrict CAR T-cell persistence.

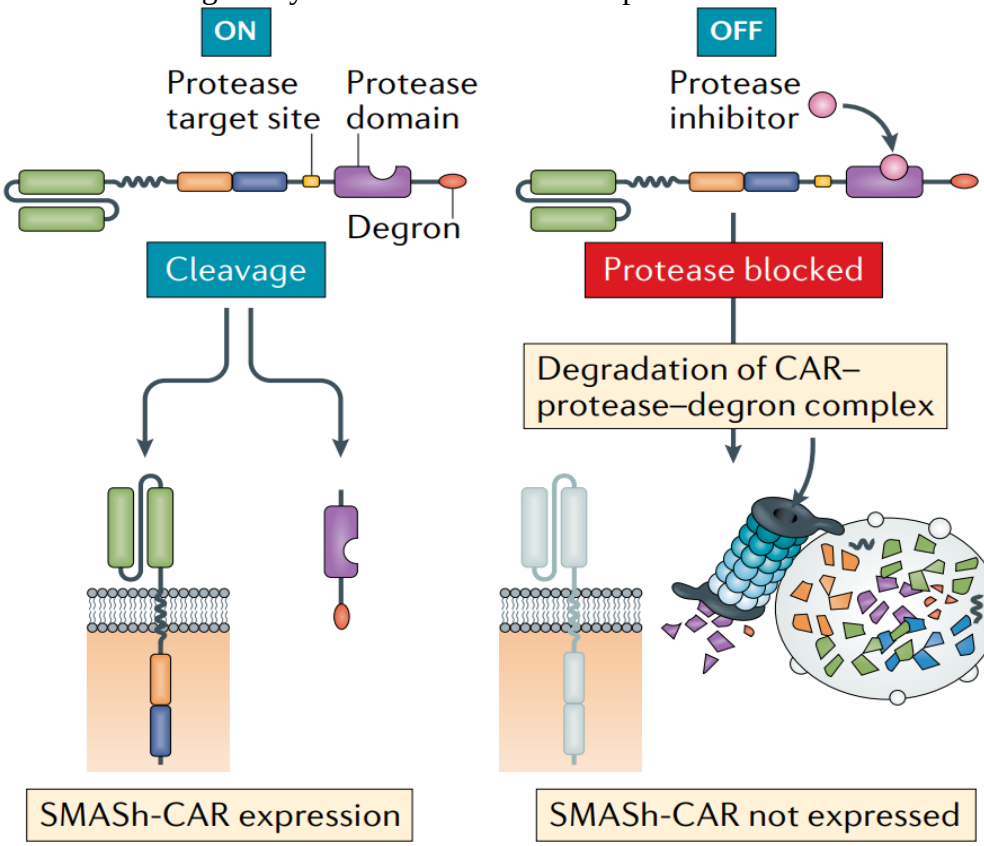



**Figure 2.** ON/OFF switches predicated on protease inhibitor [3].

## 4. Induction of CAR T-cell death by "suicide gene system"

Suicide genes that enable the depletion of CAR T cell via induction of apoptosis has been established [14]. In 2011, Stasi *et al.* developed a suicide gene iCasp9, which consists of the sequence human FKBP12-F36V connected to the gene encoding human caspase 9. AP1903, forms a dimeric bind between two FKBP12-F36V and dimerizes two iCasp9. These dimerized iCasp9 act as a peptidase that ultimately activates the intrinsic apoptosis pathway in CAR T cells (Figure 3a). Most of these iCasp9-modified donor T cells (>90%) can be rapidly eliminated after administering AP1903, which results in reversal of graft versus host disease. However, an *in vitro* study demonstrated that the iCasp9 dimerization might occur tonically without the small-molecule dimerizing agent [15]. Accordingly, the iCasp9 suicide gene system needs to be more precisely controlled to achieve widespread utility.

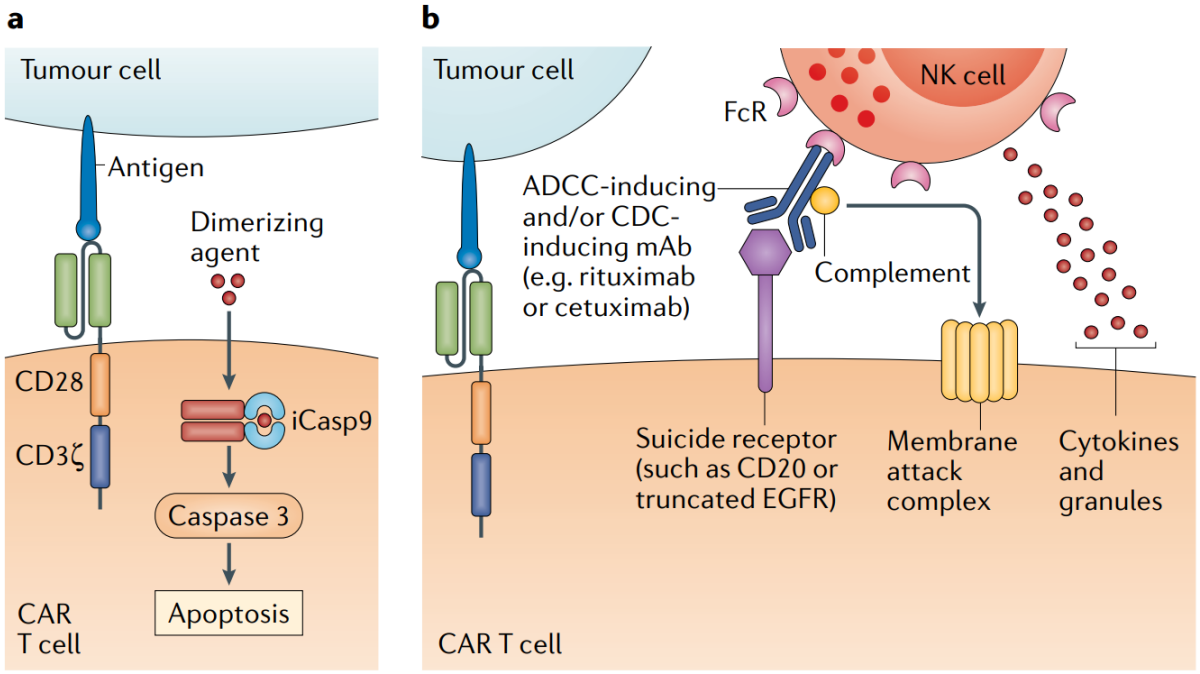



**Figure 3.** Induction of CAR T-cell death by "suicide gene system" or elimination markers [3]

## 5. Induction of CAR T-cell death by elimination markers

Other inducible CAR T-cell death approaches were based on co-expression of a CAR and an elimination marker, which commonly were human cell-surface antigens, such as CD20 or truncated EGFR (Figure 3b). Cell-surface-associated antigens enable the positive selection of CAR T cells in the manufacturing process. Additionally, since both monoclonal antibodies are FDA-approved, these engineered CAR T cells seems clinically acceptable. However, this methodology is restricted by on-target side effects inheriting to monoclonal antibodies binding to normal tissue. Another limitation is lack of biodistribution and tissue penetration of antibodies in poorly vascularized tumors.

## 6. Engineering CAR T cells with direct antagonism of systemic cytokines

Researchers engineered CAR T cells with the intrinsic ability to either secrete antagonists that neutralize relevant cytokines or direct knockout cytokine genes (Figure 4). Sterner et al. demonstrated that GM-CSF neutralization, using monoclonal antibody lenzilumab, increases anti-CD19-CAR T cell proliferation while decreasing the risk of CRS and neuroinflammation. Moreover, they generated GM-CSF–deficient anti-CD19-CAR T cells through CRISPR/Cas9 knockout of the GM-CSF gene. These GM-CSF-knockout CAR-T cells had enhanced antitumor activity as well as improved overall survival. These cells have significantly less GM-CSF expression without impairing the production of other key T-cell cytokines [16]. On the other hand, Giavridis et al. revealed that IL-1 released by macrophages leads to CAR T cell-associated CRS and ICANS [17]. They found that systemically administered IL-1 receptor antagonists (IL-1Ra), *e.g.*, Anakinra, ameliorated CRS and ICANS. Engineered CAR T cells can be designed to constitutively secrete IL-1Ra. Using IL-1Ra CAR T cells can potentially reduce the CAR T cell-related neurotoxicity because the IL-1 receptor antagonist has the ability to cross the blood-brain barrier.

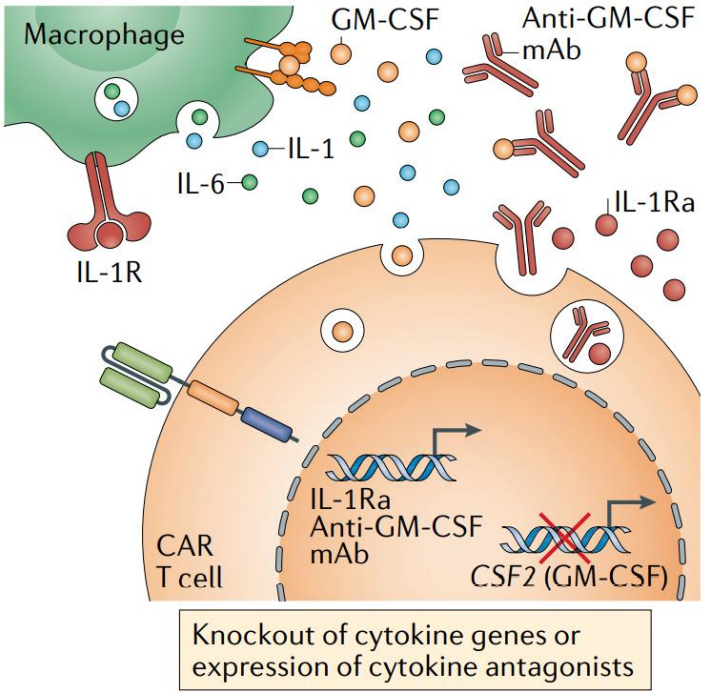



**Figure 4.** Engineering CAR T cells with direct antagonism of systemic cytokines [3]

## 7. Transcriptional regulation of CAR expression by "Tet-ON/OFF systems"

It has also been suggested that transcriptional regulation can provide another reversible control to improve CAR T cell safety. In 2016, Sakemura et al. developed an inducible CAR system by using Tet-ON system (Figure 5a) [18]. In the Tet-CD19CAR T cells, CAR expression is induced by the reverse tetracycline transactivator (rtTA) protein only in the presence of doxycycline (Dox). However, the TET-on system holds significant immunogenic potential with the risk of host-mediated elimination of the CAR T cells because it is derived from bacteria and virus. Another drawback is the lack of immediate control of life-threatening cytotoxicity due to the system acting on a transcriptional level.

Conversely, Mamonkin et al. designed a retroviral Tet-OFF expression system. In the absence of Dox, tTA activates the PTight promoter and drives CD5-CAR expression (Figure 5b). Instead, Dox acts as an ''OFF switch'' by abolishing the ability of tTA to activate the promoter and minimizes CD5-CAR transcription [19]. This system can reversibly inhibit deleterious CAR signaling and functionally prevent CD5-CAR T cell fratricide. Although removing Dox resulted in the restoration of CAR expression over 4 to 5 days, adding Dox to CD5-CAR T cells minimized CAR expression within 24 hours. These observations indicate that the Tet-OFF CAR expression system can either maintain functional CD5-CAR T cells without Dox and rapidly diminish the cytotoxicity.

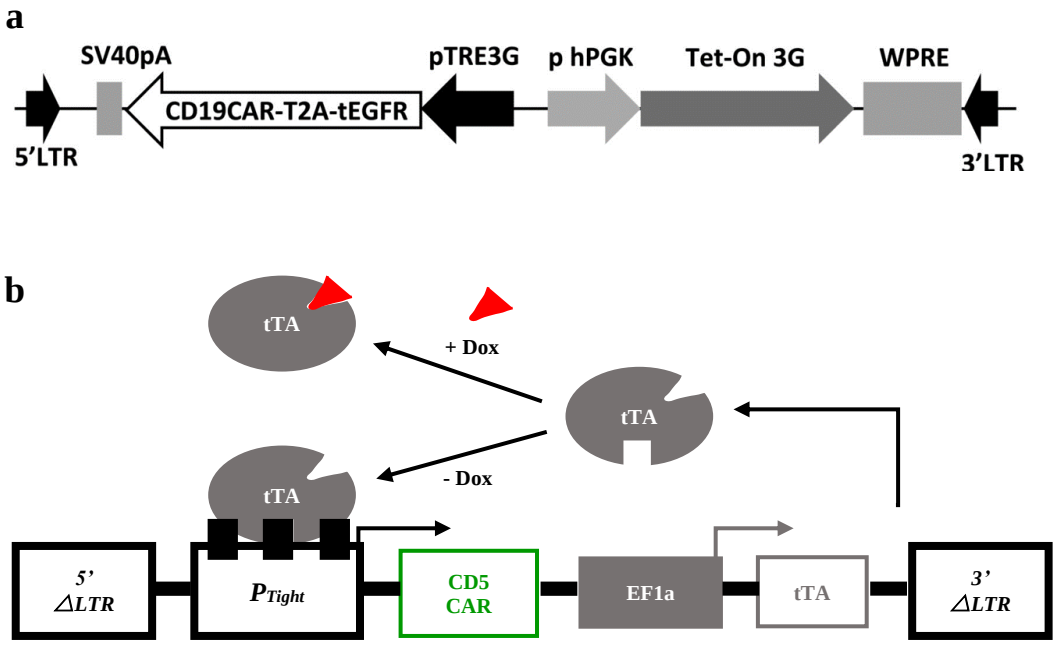



**Figure 5.** Transcriptional Regulation of CAR Expression by "Tet-ON/OFF systems"

## 8. Conclusion

The advance of CAR T cell therapy in clinical trials has revealed its great therapeutic potential. Clinicians have developed some universal grading scales for CRS, HLH/MAS, and ICANS and built generalizable guidelines for treating toxicity. Research priorities include mechanism of systemic cytokine toxicities and an optimal next-generation CAR or CAR T-cell design that intrinsically minimizes toxic effects.

Although CAR designs might improve safety, there are barriers to widespread applications. First, the starting time of on/off kinetics for each method can be variable from less than one hour to more than one day depending on different design. However, ideal management should be rapid reaction and effectively attenuated symptom [20]. Second, some methods that permanently eliminate CAR T cells can abolish the anti-tumor effect and cause costly re-administration. Therefore, researchers may aim at reversible control strategies that temporarily halt CAR T-cell activity following reawaken ability. Third, the transformation of T cells using gene-editing technique and viral transduction may lead to off-target disruption of genes or generate significant immunogenicity. Finally, the production of clinical-grade retroviruses add complexity and even higher cost to the CAR T cell manufacturing process[3]. The affordability of CAR T cell therapy needs to be fundamentally improved to facilitate its widespread application.

Emerging approaches are under development to improve the range of application, the efficient production, and the therapeutic efficacy of CAR T cells while avoiding toxicities. These promising modulating methods to optimize CAR T cell therapy will benefit patients along with the adoption and evolution of this technology.

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