Current Pharmaceutical Design, 2018, *24*, 78-83

REVIEW ARTICLE

Safety Strategies of Genetically Engineered T Cells in Cancer Immunotherapy

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ARTICLE HISTORY

Received: October 17, 2017
Accepted: December 23, 2017

DOI:
10.2174/1381612824666171227222624

Abstract: T-cell therapy using genetically engineered T cells modified with either T cell receptor or chimeric antigen receptor holds great promise for cancer immunotherapy. The concerns about its toxicities still remain despite recent successes in clinical trials. Temporal and spatial control of the engineered therapeutic T cells may improve the safety profile of these treatment regimens. To achieve these goals, numerous approaches have been tested and utilized including the incorporation of a suicide gene, the switch-mediated activation, the combinatorial antigen recognition, *etc*. This review will summarize the toxicities caused by engineered T cells and novel strategies to overcome them.

Keywords: Immunotherapy, Genetically engineered T cells, Chimeric antigen receptor, Gene therapy, T-cell therapy, suicide gene.

1. INTRODUCTION

Recent years have witnessed significant progresses in immunotherapy and some innovative approaches are gradually becoming mainstays in clinical oncology. Adoptive cell therapy (ACT) through which T cell is genetically engineered by T cell receptor (TCR) or chimeric antigen receptor (CAR) is a novel and encouraging form of immunotherapy. The engineered T cells were originally designed to eradicate primary and metastatic tumor cells with enhanced efficacy and specificity [1]. Indeed, this novel cell-based therapy has already demonstrated robust antitumor effects in different clinical trials, especially in hematologic malignancies. Great promises are held with accumulation of phenomenal investigations in the fields of synthetic biology, gene editing, and gene transduction technology [2-6].

As the studies of genetically engineered T cells become intensive and extensive, some adverse effects are gradually recognized. These include cytokine release syndrome (CRS), on and off-target toxicities and so on [7]. While the CRS-related toxicities are manageable with use of the cytokine antagonists, pulse steroid therapy and supportive care in most cases, the fatality does exist. Although a number of strategies have been designed to avoid or alleviate these adverse events, they still remain the unmet medical needs and pose a tremendous challenge to oncologists and scientists. More comprehensive studies and in-depth understanding of this therapeutic approach are warranted for its widespread clinical application [8]. This review is aimed to summarize the recent progresses made in the field with a focus on suicide gene, switch-mediated activation, combinatorial antigen recognition, *etc*.

2. ADVANTAGES OF ENGINEERED T CELLS IN CANCER IMMUNOTHERAPY

Because of unsatisfactory efficacy of traditional cancer treatments, the notion of immunotherapy was proposed in order to enhance anti-tumor response through the modification of the immune system. T cells may migrate to tumor sites, where they proliferate and initiate cellular and humoral effects, causing cancer cell lysis and disease remission. Such inherent capability can be enhanced by redirecting T cells with advanced genetic engineering technologies. Two distinct strategies have been developed, which reprogram the activity of engineered T cells by either TCR or CAR to increase their tumor specific recognition, proliferation, and persistence [9].

Adoptive transfer of TCR-engineered tumor reactive T cells has been demonstrated to be a clinically effective therapeutic strategy in cancer immunotherapy [10]. Genetically modified T cells are generated by transferring α and β chains of TCRs derived from tumor reactive T cell clones, thus rendering T cells antigenic specificity through recognition of the antigens presented by major histocompatibility complex (MHC) molecules on the target cells. As such, TCR-engineered T cells can eradicate cancer cells from patients by the specific recognition between TCRs and epitopes from the processed intracellular proteins in cancer cells. The superiority of intracellular proteins used as molecular target by TCR not only expands the range of tumor-associated antigens (TAAs) amenable for T-cell therapy, but also allows TCR targeting of the cancer mutagenome [11]. From the structural perspective, TCRs are heterodimers and the mispairing with endogenous TCRs may reduce the desired specificity, even causing unpredicted and dangerous specificities.

The development of CAR targeting tumor antigen on the surface of cancer cells is a milestone in cancer immunotherapy [12, 13]. CAR is a synthetic recombinant receptor, e.g. an extracellular single chain Fragment variant (scFv) of a tumor antigen specific antibody linked to intracellular T cell activation domain of TCR CD3ζ and one or two endo-costimulatory domains. Equipped with these structures, CAR-T cells have the specificity as antibody, and the improved cytotoxicity and persistence as cytotoxic T lymphocytes (CTLs), thus enhancing antitumor activity [14]. CAR-T cells can recognize different types of cell surface targets on tumors such as proteins, carbohydrates and glycolipids, and bind target antigens independent of MHC molecule, therefore rendering more flexibility for precise and individualized treatment of different malignancies. Besides, CAR-T cells also overcome some limitations of the TCR modified T cells because they don't have the need for MHC molecule and costimulation in antigen specific recognition, activation and proliferation.

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1873-4286/18 $58.00+.00

3. APPLICATION OF ENGINEERED T CELLS IN CANCER IMMUNOTHERAPY

Encouraging results have been achieved to support broader clinical application of this novel method. In fact, a large number of studies have been carried out worldwide to test the effect of genetically engineered T cells. The engineered T cells hold great promise in that they can significantly increase therapeutic efficacy in certain types of cancers, especially in hematologic malignancies. Convincing outcomes have been obtained for the treatment of refractory B-cell acute/chronic lymphoblastic leukemia with CD19 CAR-T cells in multiple trials, and some of them had complete remission [15]. In a pioneering work done by Dr. June and colleagues, two out of three patients with refractory CLL receiving CD19 CAR-T cells therapy achieved complete response and the remaining one achieved partial response. In addition, the adoptive transferred CAR-T cells have demonstrated an excellent ability of cell engraftment (up to 3 log expansion) and tumor cell lysis [16]. These inspiring results motivated more clinical studies. This therapeutic approach has been used by many institutions to treat B cell malignancies including acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL). The emerging data have been very promising by targeting CD19, CD20, or CD30 [17-19]. The CD19-specific CAR-T cells in the B-ALL patients can achieve an average complete remission rate of 70~94%. Based on these solid findings, the US Food and Drug Administration approved the use of Kymriah for certain pediatric and young adult patients with ALL on August 30, 2017, paving a new way for cancer treatment.

In addition to hematologic malignancies, TCR- and CAR-based therapies have been used to treat solid tumors [20]. The first clinical trial was for patients with melanoma. T cells were transduced with a TCR directed against the melanoma-associated antigen, MART-1, cloned from tumor infiltrated lymphocytes (TILs) from excised melanoma tissue. Two out of fifteen patients showed partial tumor regression [18]. CAR-T therapy has also been conducted in solid tumors, including sarcoma and cancers from ovary, colorectum and prostate [21-25]. Although the efficacy of treatment was not satisfactory compared to that in hematological malignancies, some encouraging progresses have been made. A patient with recurrent multifocal glioblastoma received intracranial infusions of CAR-T targeting interleukin-13 receptor alpha 2 (IL13Rα2), regression of all intracranial and spinal tumors was observed and the clinical response lasted for 7.5 months [26]. In addition, the feasibility and safety profile have been confirmed in the CAR-T cells targeting the epidermal growth factor receptor variant III (EGFRvIII) intravenously delivered to patients with glioblastoma [27]. NY-ESO-1-specific TCR engineered T cells induced clinical responses in patients with advanced multiple myeloma and synovial sarcoma [28]. We expect that more investigations will be carried out to explore the important role of this approach in the treatment of solid malignancies.

4. TOXICITIES OF ENGINEERED T CELLS IN CANCER IMMUNOTHERAPY

Despite the striking results observed in clinical trials using the adoptive transfer of T cells modified with TCR or CAR, unexpected organ damage and fatality have been noticed following T-cell therapy [29]. It is likely that this approach has inherent defects that can damage normal cells due to its architectural features. In addition, poor control of the dose, location, and timing of T cell activity can cause severe, sometime life-threatening, adverse effects. The deleterious effects can be prolonged because of the sustained and aggravated effect of the engineered T cells over time. Hence, the concerns about its safety still remain before its widespread clinical application in cancer treatment and more studies are needed to overcome these unwanted effects.

Cytokine release syndrome (CRS), or a more severe condition known as a cytokine storm, is a common side effect following ACT infusions in hematological malignancies [30]. Because of an overshooting and highly activated immune system, a large amount of cytokines are produced and released, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interferon-γ (IFN-γ), *etc*. Clinical manifestations of CRS include fever, tachycardia, hypotension and hypoxia. In a number of CD19 CAR-T cell studies, CRS was observed due to large-scale T-cell activation upon the recognition of $CD19^+$ malignant cells [31]. IL-6 plays a pivotal role in CRS and tocilizumab, an IL-6 receptor antagonist mAb, has been approved for the treatment of CRS [32]. The severity of the toxicity was also correlated with serum levels of INF-γ, TNF-α, and tumor burden. A grading system to evaluate the severity of CRS has been published based on the clinical manifestations, and the predictive models of CRS based on cytokine profiles are under preparation [33]. Successful use of these models may avoid or lower the risk of CRS.

Another major obstacle for the development of effective adoptive T cell therapies is on-target, off-tumor toxicity. Such toxicity results from target-antigen recognition on normal tissues because of their expression, albeit low, of the targeted tumor-associated antigen (TAA) [34]. One example is immunoglobulin deficiency occurring in patients treated with the CD19 CAR-T cells due to CD19 expression on the B cells, although it can be effectively managed by regular intravenous immunoglobulin (IVIG) infusion [35]. Another example is the toxic effect on the melanocyte-rich tissues caused by T cells expressing a TCR specific for the melanocyte differentiation antigen MART-1 [36]. Furthermore, a patient with colorectal cancer with lung and liver metastases developed respiratory distress 15 min after infusion of human epidermal growth factor receptor 2 (HER2) specific CAR-T cells, and died from multiorgan failure 5 days later. The lethality was thought to be associated with the recognition of the low-level HER2 expressed on lung epithelium by HER2-specific CAR-T cell [37]. While it is clear that the same levels of expression of target-antigen in the essential tissues may have dreadful consequences, the adverse events may be manageable with the same level of expression of the target-antigen in nonessential tissues. Thus, it is crucial to perform a comprehensive analysis of target antigen expression on normal tissues when developing a CAR/TCR against a previously unrecognized TAA. At the same time, it is important to verify the effective susceptibility of normal cells killed by genetically targeted T cells.

While target-antigen recognition on normal tissues may cause on-target/off-tumor toxicity, off-target/off-tumor toxicity may result from the unexpected recognition on normal tissues. It may be induced by cross-reactive peptides on normal tissues or by the formation of mispairing TCRs generating unknown specificities. In one clinical study, two patients died of cardiogenic shock after the infusion of T cells with a TCR recognizing MAGE-A3 tumor antigen. Further investigation demonstrated that the MAGE-A3 TCR recognized a peptide from a normal myocardial protein called Tintin [38]. This type of off-target toxicity is difficult to predict and its fatal effect remains a major concern. Bioinformatic analysis of homology of target sequences may be a feasible approach to avoid this toxicity.

Of equal importance, the graft versus host disease (GVHD) hinders the clinical application of this approach because TCRs on allogeneic T cells may recognize the alloantigens of the recipient [39]. The potential immunogenicity of TCR/CAR derived from mouse mAbs may lead to severe immune reactions dampening its efficacy. Besides, anaphylaxis occurred in a patient after repeated CAR-T cell infusion, possibly due to an immunoglobulin E antibody response against the mouse scFv component of the CAR [40]. More comprehensive studies may lend some novel insights into the underlying mechanisms of immune-associated responses.

5. STRATEGIES TO IMPROVE THE SAFETY OF ENGINEERED T CELLS

5.1. Suicide Gene Switch

To control toxicities of the genetically engineered T cells, a suicide gene is a safe and efficient way to abrogate the adverse effects through conditional elimination of transferred T cells. The incorporation of a suicide gene into the engineered T cells enables them to convert specific harmless prodrugs into toxic antimetabolites causing conditional self-elimination of transferred T cells whenever the adverse effect occurs. The first suicide gene to be clinically tested is herpes simplex virus thymidine kinase (HSV-tk) which can convert ganciclovir into ganciclovir triphosphate, a toxic substance for dividing cells [41]. Previous studies have demonstrated that the donor T cells were ablated by this suicide gene, thus saving patients from lethal GVHD in the context of hematopoietic stem cell transplantation [42]. Of note, such cell-killing process may need some time as it removes the engineered T cells by blocking the DNA synthesis.

A suicide gene called inducible caspase 9 (iCasp9) was developed for clinical application because of its high efficacy and biocompatibility to human [43]. The iCasp9 is composed of the intracellular domain of the pro-apoptotic human caspase 9 protein and a human FK506 binding protein (FKBP), allowing inducible dimerization when treated with small molecules such as AP1903. The dimerization of iCasp9 then activates the intrinsic mitochondrial apoptotic pathway. T cells expressing low level of iCasp9 are also susceptible to dimerization-induced killing, albeit to a lesser extent. A study showed that iCasp9 suicide gene caused apoptosis of approximately 99% of transduced cells both *in vitro* and *in vivo* in the presence of AP1903. Activation of iCasp9 in patients who developed GVHD after genetically engineered T cell transplantation rapidly induced T cell apoptosis, therefore preventing the progression of the GVHD [44]. In the first-in-human study, the T cells transduced by a retrovirus encoding iCasp9 showed a good safety profile and effectively eliminated alloreactive T cells. Activating iCasp9 with a small synthetic molecule AP20187 resulted in prompt apoptosis in over >90% of the cells [45]. The iCasp9 system is an effective strategy to control early toxicities of engineered T cells because it is not only humanized but also can cause rapid apoptosis.

Different from destroying engineered T cells by suicide gene, another suicide switch approach is to co-express on the T cells a protein recognized by clinically approved monoclonal antibody in order to eliminate therapeutic T cells. The genetic modified T cells may be specifically ablated through an antibody or a complement-mediated cytotoxicity after the administration of the specific mAb. The protein could be CD20 and targeted by rituximab, or truncated epidermal growth factor receptor (EGFRt) by cetuximab [46, 47]. Furthermore, as the codon-optimization considerably improved CD20 expression, the codon-optimized CD20 has great potential to reduce toxicities [48]. The anti-CD20 mAb treatment after T cell infusion has been validated in preclinical models as a suicide gene strategy [49], while the EGFRt-mediated CAR-T cell elimination has been incorporated in several CAR-T cell clinical trials by targeting CD171 (NCT02311621), CD19 (NCT02028455) and CD123 (NCT02159495).

5.2. Synthetic Splitting Receptor

The abortion of infused T cells plays a critical role in the safety of the engineered T cells when dreadful toxicities occur. The disadvantage of this approach is that, all infused T cells will be removed permanently causing a premature termination of the treatment. Consequently, non-lethal control of engineered CAR-T cells was developed, which was an important advancement to improve the CAR-T cell safety. Recently, Lim and colleagues proposed a synthetic splitting receptor to redirect CAR-T cells [50]. In this system, the timing, location, and dosage of T cell activity can be precisely and remotely controlled by pharmacologic regulation. In the synthetic splitting receptors, antigen binding and intracellular signaling components are separated in the absence of small molecules and assembly starts after treatment with a heterodimerizing small molecule. The FK506 binding protein domain and the mutant of FK506 binding protein-rapamycin binding domain implement heterodimerization in the presence of the rapamycin analog AP21967, a component with less immunosuppressive activity than rapamycin. Strikingly, they confirmed that the CAR-T cells can be effectively controlled with this small molecule *in vivo* and the magnitude of responses such as target cell killing can be adjusted simply by changing the dosage of the small molecule. Another synthetic splitting receptor described by Juillerat *et al*. was that, the CAR architecture was directly activated at the hinge domain with the addition of a small dimerizing molecule drug [51]. The engineered CAR-T cell demonstrated a significant cell lysis activity after use of the molecule inducer with a dose-dependent manner. The development of these novel controlled CAR-T cells opens innovative and attractive ways to control potential adverse effects by improving the safety of CAR-T cell therapy in the clinic.

5.3. Combinatorial Target-Antigen Recognition

In the engineered T cell therapy, the simplest and direct scenario to improve its safety is to target an absolutely tumor specific antigen (TSA) on tumor cells. However, highly specific targets for the ACT are very rare. The vast majority of current targets have been TAAs, e.g. overexpressed on tumor cells but also shared by normal bystander cells, which may cause the on-target/off-tumor toxicities. An alternative strategy for enhancing the specificity of CAR is combinatorial antigen targeting instead of targeting a single antigen. Combinatorial target-antigen recognition identifies two different antigens simultaneously on the tumor cells by engineering T cells with two CAR molecules: one CAR only provides the activation signal while another one incites co-stimulation. The antigens are not necessarily to be truly tumor specific, as long as the tumor specificity is acquired after the combination. T cells engineered with two CARs may be fully activated when recognizing a set of antigens co-expressed on the tumor cells. Theoretically, normal cells expressing only one of these two different antigens will not activate T cells. Through this combinatorial antigen recognition strategy, the on-target/off tumor toxicity is minimized. Based on this rationale, T cells expressing two CARs were tested in different pre-clinical mouse models. Kloss *et al*. constructed two CARs specific for prostate stem cell antigen (PSCA) and prostate specific membrane antigen (PSMA) to modify T cells. The result showed that T cells expressing two CARs had greater activity against tissues expressing both PSCA and PSMA as compared to tissues expressing only PSMA [52]. Lanitis *et al*. redirected the T cells by two CARs targeting mesothelin and folate receptor, and the results showed that these CAR-T cells have less activity against single-positive tumors [53]. However, the report about the T cells transduced with a CAR specific for HER2 and a CAR specific for MUC1 was somewhat controversial. The results suggested that cytolytic activity of these T cells was only dependent on the engagement of HER2, irrespective of MUC1 [54]. Certainly, more research work needs to be conducted for the clinical application of the combinatorial target-antigen recognition to avoid damage of the normal tissue expressing single target-antigen.

5.4. Synthetic Notch Receptors

A recent paper published in *Cell* reported a new class of modular receptors called synthetic Notch (synNotch) receptors [55]. SynNotch receptors use an extracellular domain to recognize a target antigen. However, binding of the target antigen does not trigger T cell activation as seen in CARs. Instead, ligand engagement leads to the release of a transcriptional activation domain that can translocate to nucleus to upregulate the expression of the user-specified target genes. Roybal *et al*. generated the T cell circuits in which a

synNotch receptor for one antigen drives the inducible expression of a CAR targeting a second antigen [56]. Only when both antigens are present, T cells can be activated. In this study, human primary T cells were engineered with the GFP synNotch receptor and the corresponding response elements control CD19 CAR expression. These T cells were then exposed to K562 target cells expressing CD19, GFP, or both. The T cells only displayed expression of the CD19 CAR when stimulated with cells expressing the synNotch ligand, GFP. Moreover, these T cells can only be activated by exposing to target cells expressing both GFP and CD19 on their surface as evidenced by cytokine production. This system has a good flexibility in the regulation of specific signal-response cascades in different applications as it works orthogonally and does not require an intermediate signaling molecule. It remains elusive whether the non-human transcription factors are immunogenic.

5.5. Bispecific T Cell Engager

As CAR-T cell activity can be regulated by changing the dosage of a small molecule, an intermediate bifunctional molecule was proposed to redirect the engineered T cell to control its activity. These CAR-T cells cannot bind directly to any target antigen and are basically inactive in the absence of the bispecific molecule, e.g. the molecule specific for both the target antigen and the CAR. The interaction between the CAR-T cell and target cancer cell mediated by such molecule can destroy cancer cells through activation of the CAR-T cells. Bispecific T cell engagers, such as anti-CD20/CD3 and anti-FR/folate-FITC, were designed based on the theory mentioned above [57, 58]. A recent paper published in *PNAS* demonstrated the feasibility of the peptide-specific switchable CAR-T (sCAR-T) cells [59]. The bifunctional molecule is a recombinant containing a tumor antigen specific Fab molecule incorporated with a peptide neo-epitope (PNE), which exclusively binds to the peptide-specific sCAR-T cells. The antibody-based recombinant redirects the activity of the sCAR-T cell through the selective formation of immunological synapses, in which the sCAR-T cells, bispecific molecule, and target cells interact in a structurally defined and temporally controlled manner. They showed that the bispecific molecule specific for CD19 and PNE controls the activity, tissue-homing, cytokine release, and phenotype of switchable CAR-T cell in a dose-dependent manner in the xenograft mouse models of B-cell leukemia. Furthermore, this approach can easily target CD20 on cancer cells using the same sCAR-T cell, and therefore can be widely used in heterogeneous and resistant malignancies. Although this approach is to harness, not to abolish the therapeutic cells, it has potential to alleviate the toxicities in a safer way.

5.6. Inhibitory Chimeric Antigen Receptor

The cytotoxicity of CAR-T cell may be mitigated through the inhibitory strategy. The inhibitory chimeric antigen receptor (iCAR) consists of an antigen recognition domain specific to the antigens expressed exclusively on normal tissue, and an inhibitory signaling domain to abrogate T cell activity despite ongoing engagement. The combination of inhibitory receptors specific for the antigen present on normal but not on tumor cells would protect the normal tissues from a CAR-T cell-mediated attack because of the negative signaling conferred by iCAR. Normal cells that express the target of iCAR, even in the presence of the activating CAR antigen do not activate T cells. Pioneering work from Fedorov and colleagues made use of anti-PSMA iCAR carrying intracellular tails of CTLA-4 or PD-1 to test their ability to block TCR- or CAR-driven T cell functionality both *in vitro* and *in vivo* [60]. This proof-of-concept experiment demonstrated that the iCAR did inhibit the response mediated by either TCR or CAR in an antigen-restricted mechanism. Furthermore, this inhibition showed a temporary and reversible manner as evidenced by sequential T cell stimulation by target and off-target cell experiments. Such property is of critical importance in that previous engagement of iCAR by most T cells can preserve their function although it is likely that a small part of those T cells may be anergized over time. In this way, off-tumor toxicities are prevented by a dynamic, self-regulating safety switch without affecting antitumor activity of the engineered T cells.

5.7. Other Available Strategies to Reduce the Toxicities

Besides the optimal structure design of CAR to improve the safety of CAR-T therapy, different medical precautions have also been taken into considerations including applying differential conditioning regimens, risk-adapted dosing using patient stratification based on tumor burden, splitting the initial dose of CAR-modified T cells over different time periods, strict monitoring of vital parameters after infusion and early detection of clinical and laboratory signs [61]. High-dose corticosteroids and tocilizumab can also alleviate the immunotoxicity of the therapeutic T cells [62]. A local route including intratumoral (IT) and intraperitoneal (IP) administration of engineered T cells has demonstrated improved safety and feasibility in clinical studies [63]. It is also important to test therapies in an appropriate mouse model to rule out unwanted severe side effects before initiating a clinical trial [64].

CONCLUSION

Great progresses have been made in the clinical applications of immunotherapy in the recent years. Adoptive cellular therapy utilizing the CAR/TCR engineered T cells is an attractive approach for cancer treatment and encouraging results have been achieved. While satisfactory efficacy has been noticed in patients with hematological malignancies treated with CD19 CAR-T cells, the development of more potent T cells with minimal toxicities remains an unmet medical need. Innovative strategies and the related medical managements have been studied in order to overcome these problems and make such therapy a safer procedure. Equipped with powerful tools such as sophisticated synthetic biology and genetic engineering techniques, we expect an improved safety profile, enhanced potency and widespread application of the engineered T cell therapies for curbing different cancers.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This work was supported by National Natural Science Foundation of China (No. 81372405).

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