Review Article

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Recent progress in improving the safety and efficacy of chimeric antigen receptor T cell therapy

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Abstract

Chimeric antigen receptor (CAR) T cell therapy has demonstrated unprecedented feat in a variety of malignancies, providing a transformative approach to treating patients with hematological malignancies and solid tumors. Although CAR-T cell therapy has shown remarkable anti-tumor activity, toxicities and tumor antigen escape have largely compromised its efficacy. In the case of solid-tumor management, it has shown modest results to date, likely due to heterogeneous antigen expression, an inhibitory tumor microenvironment, and other immunosuppressive factors. The predominant goal for this field now is to achieve more precise tumor recognition and design CARs with more robust proliferative ability. To this end, a multitude of novel CARs and new immunomodulatory antibodies are being developed and tested clinically. Intense efforts are underway to improve the engineering of synthetic immunotherapies and combine these strategies with other agents to amplify immune responses. In this review, we will discuss the current landscape of CAR-T cell therapy, with an emphasis on primary challenges that need to be addressed urgently. In addition, we characterize some newly designed CARs proposed for improving specificity and proliferation of CAR-T cells, offering new insights into improving safety and efficacy of CAR-T cell therapy.

Key words: chimeric antigen receptor, CAR T cell design, immunomodulator, checkpoint blockade

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Introduction

T cells modified to express chimeric antigen receptors (CARs) using transfer gene techniques have provided a compelling salvage treatment option for some lethal hematological diseases that have no alternative therapy. CARs are synthetic fusion proteins that are capable of specifically recognizing cell surface antigens. The most commonly used CARs typically consist of a variable portion of an antibody, known as an scFv (single-chain variable fragment) and a trans-membrane region that connects the extracellular domain to the cytoplasmic signaling domains[1-3]. The scFv-antigen interaction leads to activation of cytoplasmic signaling domains, resulting in T cell activation and proliferation. Subsequently, the expanded T cells may trigger cytolysis and secretion of cytokines to eliminate the target cells in a manner independent of major histocompatibility complex. Along with the emergence and development of CAR-T cell therapies, the designs of the CARs have undergone a long history of evolution, which has played a significant role in the development of CAR-T therapies. The first generation of CAR constructs consisted of the signaling molecule CD3z, which was essential to induce T-cell activation. However, the T cells activated by this initial design showed limited efficacy, leading to the development of the more potent second and third generations of CARs, which are further engineered to express multiple costimulatory domains[3-8]. A number of clinical trials have demonstrated significant antitumor activity of CAR-T cell therapy in the treatment of hematological malignancies. Despite efficacy in hematological diseases, however, the reported results of clinical experiments using this approach in solid tumors to date is not so encouraging[9-12]. Additionally, the safe clinical employment of CAR-T cell therapy has been largely limited by a number of signifi-

cant safety concerns. In this mini-review, we review the current challenges encountered in the clinical application of CAR-T cell therapy, and highlight some feasible strategies aiming to overcome these emerging problems to achieve more effective CAR-T cell therapies.

Modified CAR-T cell therapies for B Cell malignancies

The past decade has witnessed a tremendous advancement of CAR-T cell therapy in the treatment of many advanced B cell malignancies. Clinical trials have demonstrated unprecedented clinical efficacy of CAR T cells against many aggressive B cell malignancies including acute lymphoblastic leukemia（B-ALL）, chronic lymphocytic leukemia[13-17]and B cell lymphomas[18,19]by targeting CD19[1], CD20[20], CD22[21], or some other antigens. Among all of these antigens, the most compelling success has been achieved in CD19-targeted CAR-T cells for B-ALL, with complete remission（CR）rates as high as 90%[15,16,22-24]. In August 2017, the first CD19-targeted CAR-T cell product was approved by the US Food and Drug Administration（FDA）for the treatment of pediatric and young adult patients with relapsed and/or refractory B-ALL.

The exciting clinical outcome achieved by CD19-targeted CAR-T cells across multiple institutions against leukemia and lymphoma has inspired the application of CAR-T cell therapies in the treatment of multiple myeloma（MM）. Some of the potential targets for MM under investigation include CD138[25], CD38[26], SLAM7[27], κ light chain[28], and B cell maturation antigen（BCMA）[29]. Among them, some early clinical trials have demonstrated significant anti-myeloma effects of CAR-T cells targeting BCMA. BCMA is an antigen expressed on mature B cells, including plasma cells and myeloma cells. In the first in-human clinical trial of BCMA-targeted CAR-T cell therapy, 12 patients with MM were enrolled and treated with an escalating dose of reengineered T cells[29]. More recently, Fan et al. reported promising clinical results of BCMA CAR-T cells in a phase-1 clinical trial, achieving up to a 100% objective response rate（ORR）in refractory/relapsed myeloma patients. Among these patients enrolled, 18 out of 19 patients achieved CR after receiving anti-BCMA CAR-T cell therapy over a long-term follow-up[30]. Nevertheless, CAR-T cell therapies for MM are in the preliminary stages of development. Researchers are continuing to look for unique plasma cell antigens expressed exclusively and uniformly on malignant plasma cells but not normal B cells.

Challenges in solid tumors

More recently, the success of CAR-T cell therapy in B cell malignancies sparked a search for applying it in solid tumors. Although certain progress has been made in early phase clinical studies, the wide application of CARs in the purview of solid tumors has been impeded by the lack of unique tumor associated antigens, an immunosuppressive tumor microenvironment, and limited trafficking of effector T cells to tumor sites[31,32]. Thus far, the overall outcomes of clinical trials are disappointing[12,33]. Nearly all clinical trials reported cross-reactivity caused by T cells attacking normal cells expressing low level of target antigen[32], a mechanism similar to B cell aplasia and hypo-immunoglobulin in the case of the CD19-specific CAR-T cells[34]. While the B-cell aplasia could be counteracted by infusion of immunoglobulin, damages to pivotal organs cannot be reversed and may lead to death in the worst cases[35,36]. Hence, a primary challenge is identifying tumor-specific antigens that can provide sufficient discrimination. The ideal tumor-restricted antigen is required to be expressed broadly and exclusively in tumor cells but is undetectable in normal cells. However, limited validated antigens and heterogeneous antigen expression pattern of solid tumors make the identification of such tumor-associated antigens very challenging. In this scenario, great effort should be made to further broaden the available target antigens by identifying target antigens expressed at low levels in healthy tissues, where depletion could be well tolerated in order to achieve more precise tumor recognition and ameliorate CAR-based toxicity.

Translating CAR-T cell therapy to solid tumors necessitates overcoming the inefficient trafficking of CAR-T cells to tumor sites. Chemokines are secreted by tumor cells and play an important role in trafficking and migration of effector T cells. Potential reasons for insufficient effector T-cell infiltration include the production by many human tumors of low levels of chemokines, and effector T cells lacking appropriate chemokine receptors of tumor-derived chemokine, all of which damage the homing capabilities of adoptively transferred T cells. Accordingly, it affords us the opportunity to correct the deficiencies in T-cell chemotaxis by transducing CAR-T cells with chemokine receptors. The consequent overexpression of chemokine receptors which match with the chemokines secreted by tumor cells could redirect migration of T cells towards tumors sites[37]. Several recent studies have proved the feasibility of genetically modifying CAR-T cells with the chemokine genes such as CCR2b[38]and CCR4[39]. In addition, the method of CAR-T cell delivery also exerts great impact on the effectiveness of T cells to penetrate the tumor tissue and migrate to the site of the tumor. Various preclinical and clinical studies

have evaluated different ways of CAR-T cell delivery[40,41]. It has been proved that in contrast to conventional intravenous delivery, regional delivery of CAR-T cells promotes more efficient anti-tumor potency and T cell trafficking[42].

Provide a Bridge to allogenic-hematopoietic stem cell therapy (allo-HSCT) for patients with relapsed/refractory disease

Adoptively transferred T-cell therapy is not only a stand-alone treatment, but also a bridge therapy to subsequent allo-HSCT. CD19-targeted immunotherapies have largely changed the paradigm of treatment for relapsed/refractory B-ALL by providing an alternative salvage treatment option. Brentjens et al. first utilized CD19-targeted CAR-T cell therapy in patients with relapsed B-ALL who were thought to be ineligible for allo-HSCT[14]. The patients enrolled in this study who achieved MRD-negative CR after CD19-targeted CAR-T cell therapy were permitted to undergo subsequent allo-HSCT. In another phase-1 dose-escalation clinical trial involving children and young adults with B-ALL, all of the ten enrolled patients achieved MRD-negative complete response, allowing for subsequent HSCT[15]. All of these promising clinical results highlight the dramatic anti-tumor ability of CD19-targeted CAR-T cells to induce MRD negative CR in patients with relapsed/refractory B-ALL who are unable to undergo HSCT. This could significantly improve poor prognoses by providing a bridge to allo-HSCT under optimal conditions.

Concerns about CAR-T cell therapy

Toxicities of CAR-T cell therapy

While CAR-T cell therapy has achieved compelling efficacy in treating hematological diseases, wide scale application of CAR-T cell therapy is impeded by severe toxicities, among which cytokine release syndrome (CRS) has been described as the most predominant and severe complication. CRS is a potentially life-threatening systemic inflammatory response, characterized by rapid elevation of a wide variety of cytokines released by activated T cells, such as interleukin-6 (IL-6), interferon-g (IFNγ), IL-15, IL-8, and/or IL-10[14,34,43]. The major symptoms of CRS include high fever, hypotension, and hypoxia, with the most severe condition leading to widespread irreversible organ dysfunction and even death[22,44,45].

Moreover, damage of healthy tissues expressing low levels of tumor associated antigens (TAAs) by cross-reactive T cells accounts for the so called in-target/off-tumor effect. Ideally, the selected targeted antigen is supposed to be uniformly and specifically expressed on tumor cells while absent on healthy tissues. However, as demonstrated in many clinical trials, activation of T cells with engagement of normal tissue expressing low levels of TAAs has resulted in off-tumor effect, thus compromising the safety of CAR-T cell therapy. In the case of CD19-redirected CAR-T cell therapy, CD19 is not only expressed on malignant B cells, but also on normal B cells and other normal cells. Not unexpectedly, previous clinical data indicate CD19-redirected CAR-T cells attack both normal B cells and malignant B cells, resulting in profound B cell aplasia and hypo-immunoglobulin syndrome[34]. Similar off-tumor effects have also been reported in solid tumors, because most TAAs are simultaneously expressed on normal tissues, albeit at low levels[9,35]. Serious side effects can even led to death in the worst-case scenario. For example, fatal acute respiratory distress syndrome was reported in a phase-1 trial with infusion of anti-ERBB2 CAR-T cells. The death was attributed to the damage of lung epithelium expressing low levels of HER2 by CAR-mediated cross-reactivity[35]. Similarly, on-target/off-tumor toxicity has been observed in other adoptive immunotherapies. For instance, a serious adverse event induced by affinity-enhanced TCR-engineered T cells was reported in anti-MAGE-A3 TCR-T therapy[46]. In this case, effector T cells recognized the unexpected expression of MAGE-A3 in cardiac muscle and finally resulted in severe cardiovascular toxicity. The cross-reaction between activated T cells and normal tissues has dramatically raised safety concerns about the wider application of CAR-T cell therapy in solid tumors.

Antigen negative escape

Another main concern that has been raised is antigen-negative relapse, rendering CAR-T cells ineffective against the relapsed tumor cells[13,47]. In clinical trials of CD19-redirected immunotherapy, a subset of patients treated with CD19-specific CAR-T cells experienced relapse with CD19-negative leukemia after an initial response despite persistence of CAR-T cells[48,49]. It has been confirmed that a substantial proportion of relapse cases can be attribute to antigen mutation or down-regulation, which have emerged as a primary challenge compromising the clinical efficacy of CD19-redirected CAR-T cell therapy[50]. Immune escape has also been documented in other hematological diseases[51]and solid tumors[52]. Nevertheless, antigen-negative immune escape could involve multiple complex mechanisms, including alternative antigen splicing, missense mutations, lineage switch induced by persisting CAR T immune pressure, or conversion to a myeloid phenotype[50,53,54]. On this basis, it is suggested that future CARs should be designed to target more than one antigen to prevent an antigen-negative relapse, as will be discussed below.

Improving the safety and efficacy of CAR-T cell therapy

Given the safety concerns about adoptively transferred T cell therapy, improving safety has emerged as a major focus area of current research. Here, we outline some new developments in preclinical and clinical studies to reduce the risks associated with CAR-T cell therapies.

Bi-specific CARs

The concerns mentioned above prompted researchers to improve the design of CARs to further mitigate toxicities and prevent antigen-loss relapses. The presence of multiple specific TAAs on the surface of tumor cells provides opportunities for simultaneous targeting of multiple antigens using dual-specific CAR-T cells. More recently, the concept of targeting more than one antigen simultaneously has been actively investigated in the scientific community. While single-specificity CAR-T cells will result in immune escape and outgrowth of antigen negative tumor cell subpopulations[41], this does not seem to be the case for their counterparts of dual-specific T cells. In principle, there are several approaches that can be employed to target multiple antigens, and each one has its advantages and shortcomings. One strategy is to incorporate two binding domains into a single CAR structure, which is termed as bispecific CAR[55]. This bispecific CAR construct infuses two antigen-binding domains in tandem, showing a typical OR-gate signal recognition: it is activated by target cells that express either or both antigens. In comparison with their monospecific counterparts, bispecific CAR is superior in that they remain effective in the presence of a single antigen loss variant[55]. The effectiveness of tandem CD19/CD20 bi-specific CARs has been tested in several studies[56], in which a significant reduction of both CD19 and CD20 expression on B cells was detected after just a short-term of co-incubation. Besides CD20 and CD19, other pan-B cell markers such as CD123 and CD22 can also be potential candidates for dual-targeted CARs[57-59]. For example, Ruella et al. evaluated the expression of CD123 in the samples from B-ALL patients. The CD19-CD123+ populations were found to be preexisting in most B-ALL samples at baseline, and persisted in patients who relapsed after CART19 therapy. They speculated that these CD19-CD123+ cells were precursors of CD19-negative blasts, and were responsible for relapse after the administration of CD19-targeted T cells. Thus, investigators devised dual signaling CART123/CART19 T cells and tested them in a B-ALL xenografts model. As expected, dual CD19/CD123 targeting CAR-T cells provided more potent effector activity against B-ALL *in vivo* in comparison with monospecific CAR-T cells[57].

Surprisingly, compared with pooled administration of two different CAR-T cells, the bispecific construct was more efficacious with less toxicity[55]. Moreover, bispecific CAR-T cells exhibited synergistic effects when both antigens were simultaneously encountered, which could optimize the capacity for long term disease control[60]. In summary, targeting combination antigens by dual-signaling receptors has provided an effective preemptive approach to preventing relapses due to antigen escape.

Optimization of the affinity of CAR design to reduce off-tumor toxicities

Another approach for improving recognition specificity is to enhance CAR-T cell discrimination between tumor cells and normal cells based on antigen density. CAR-T cell therapy is an extremely sensitive treatment method, for which the threshold target antigen density required to induce activation of effector T cells and lysis of target tumor cells is considerably low[61]. Potential recognition of normal tissue expressing low density TAAs by effector T cells results in on-target/off-tumor effect, limiting a wide range of clinical application of CAR-T therapy. In order to improve its safety profiles, it is necessary to develop a strategy to reduce CAR sensitivity of normal tissues expressing relatively lower levels of target antigen, while retaining its antitumor activity. Indeed, the functional thresholds of antigen density became lower with the increasing level of the affinity of CARs for the ligand within a definite range[62]. Accordingly, affinity is of vital importance for adjusting the binding properties of T cells. CAR-T cells engineered with lower affinity CARs were confirmed to have comparable antitumor activity against tumor cells in comparison to their counterparts, but demonstrated impaired killing activity upon encountering healthy tissue expressing relatively lower level of TAAs[63]. These results suggest that a defined affinity window exists that strike an optimal balance between efficient T cell response and emergence of on-target/off-tumor autoimmunity[64]. It prompted us to ameliorate the safety profiles of CAR-based approaches by modulating CAR binding affinity for the target antigen, and generating CAR-T cells which are capable of preferentially recognizing the tumor cells expressing a high-density of target antigen. Thus far, the conception of tuning sensitivity of CAR-T cells to discriminate between tumor and normal cells has been practiced in several tumor models. The results have confirmed that it is feasible to improve CAR-T cell sensitivity according to antigen densities of tumor cells by tuning the CAR-TAA binding properties[63,65-68]. In a pre-clinical study, investigators proposed a strategy to generate new CD38 antibodies with different affinities to CD38 ranging from 10- to 1000- fold lower relative to the normal ones[66]. They observed rapid tumor eradication with affinity-tuned anti-CD38 CAR-T cells and the absence of systemic toxicity on healthy hemato-

poietic cells expressing CD38. Similar outcomes were observed in another study in which investigators tuned a CAR affinity based on the density of EGFR expression[52]. The results suggested that CARs with reduced affinity rendered T cells preferentially activated by high density EGFR on glioblastoma cells, but exhibited no apparent T-cell activation to lower densities of EGFR found on normal tissues[65]. All of these studies have shown that equipping CARs with an optimal affinity to the target antigen offers a solution to avert off-tumor side effects. However, the optimal affinity range appears to differ enormously when targeting different epitopes or antigens given the differences in each pathological situation[63]. To harness this feature, future studies are warranted to identify the optimal affinity window in a more context-dependent manner, and to search for antibodies that possess lower affinities but still maintain specificities.

Incorporation of inducible safety switches

In order to alleviate CRS and long-term B cell aplasia while maintaining potent antitumor activity, it is necessary to have control over CAR-T cell proliferation. Many safety switches have been designed to rapidly and selectively eliminate CAR-T cells in case of toxicities by incorporating a suicide gene. In principle, safety switches should meet several criteria: they should be expressed stably and efficiently in T cells without impairing the manufacturing process; the reagent turning on the gene should be well tolerated and be able to elicit T-cell dysfunction within a safe dose range. Safety switches that have been incorporated with CARs include iC9[69], herpes simplex thymidine kinase (HSV-TK), and epidermal growth factor receptor (EGFR)[70]. Among all these safety switches, iC-9 is one of the most appealing safety strategies to eradicate CAR-T cells without causing side effects. It has been extensively tested in multicenter clinical trials[71-73]. Apoptosis mediated by iC9 can be activated rapidly and irreversibly upon exposure to specific chemical inducer of dimerization (CID)[72,73]. Compared with other suicide genes, iC9 proved to be a more efficient and specific way to induce apoptosis of effector T cells. Importantly, the sensitivity of iC9-transduced T cells to CID persists over time, providing a permanent control to terminate the effects of CAR-T cells. Moreover, the suicide gene terminates transduced T cells in a dose-dependent manner[72]. Given this advantage, when required, iC9 can reverse the expansion capacity of CAR-T cells, allowing for the reconstruction of B cells by adjusting the dose of the reagent without completely abrogating antitumor activity.

Enhancement of proliferation and persistence of CAR-T cells

Armored CAR-T cells

The inhibitory tumor microenvironment is one of the major challenges compromising the persistence and proliferation of transferred T cells, which is especially true in solid tumors. The solid tumor microenvironment is extremely immunosuppressive, which involves many inhibitory factors including regulatory T cells (Tregs) and inhibitory cytokines[74]. In an effort to control the negative effects of the tumor microenvironment on CAR-T cells, additional regulatory modulators would be incorporated. As supported by preclinical data, IL-12 is a proinflammatory cytokine shown to modulate the tumor microenvironment through multiple mechanisms[75-78]. Preclinical studies have already demonstrated that infusion of IL-12 can mediate potent *in vivo* antitumor activity in mice[79]. The multiple roles of IL-12 in adaptive and innate immunity provide the foundation and rationale to further modify CAR-T cells to secrete IL-12 using transgenic technology, in order to enhance persistence and resistance to immunosuppression. This construct is known as an 'armored' CAR-T cell[80,81]. Transgenic expression of IL-12 endowed CAR-T cells have improved proliferation ability compared with non-IL-12 secreting CAR-T cells *in vitro* and *in vivo*[81]. In a phase I clinical trial, Koneru et al. utilized these modified CAR-T cells with IL-12 secretion ability and administered them in patients with ovarian cancer. They were able to favorably modulate the tumor microenvironment and the endogenous immune system[82].

Combination with checkpoint inhibitors

Despite encouraging results in preclinical and clinical trials, the existence of different immunosuppressive pathways can restrict the full potential of adoptive T-cell therapy. Many tumors are capable of expressing ligands binding to inhibitory receptors (IRs) on T cells, such as programmed death 1 (PD1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which lead to T cell dysfunction and exhaustion. The interaction between IRs and their ligands exerts profound immunosuppressive effects on T-cell function, which provides tumor cells an evasion mechanism from immunosurveillance[83]. As upregulation of inhibitory receptors is common among CAR-T cells, these pathways have been increasingly targeted in recent studies in an effort to neutralize their detrimental effects on T-cell function[84]. These agents are referred as immune checkpoint inhibitors such as PD-1 inhibitors which mediate unprecedented clinical benefits by targeting the PD-1/PD-L1 pathway[85,86]. Evidence from preclinical studies carried out in murine models indicated that anti-PD1 antibody could reverse T-cell

exhaustion and enhance antitumor activity, with the potential to produce durable clinical responses[87]. More recent clinical trials using anti-PD-1 antibody reported remarkable clinical response in a significant fraction of patients with melanoma, renal cancer, ovarian cancer, and other malignancies[88,89]. In this regard, checkpoint inhibitors seem to be an ideal partner of adoptive T cell therapies, and indeed, a synergistic effect has been observed in some initial clinical trials[90,91]. It is worth noting, however, that various degrees of graft-versus-host disease were detected in a subset of cases, which was highly correlated with the reactivation of autoimmune reactive T cells[86]. Hence, toxicity profiles of combined therapy have to be carefully evaluated before wider clinical application can be considered.

Furthermore, based on the critical role that PD-1/PD-L1 plays in T-cell exhaustion, it is appealing to divert the inhibitory signals into stimulatory ones. In pursuit of this objective, a study designed a costimulatory converter in the form of chimeric PD1/CD28 receptor. The T cells were transduced with both a CAR and a chimeric switch-receptor containing the extracellular domain of PD1 fused to the transmembrane domain[92]. In this way, when the PD1 portion of this switch-receptor engages its ligand, it will transmit an activating signal via the CD28 cytoplasmic domain, thus augmenting the clinical responses to CAR-T cell therapy[93].

Conclusion

In this mini-review, we have discussed the current advances in the development of CAR-T cell therapy. Despite the great therapeutic efficacy exhibited by CAR-T cell therapy, its wide-scale application is still hampered by concerns of its intrinsic safety and long-term disease control.

Considering the commonness of in-target/off-tumor effect and antigen-negative relapse, a variety of genetic engineering approaches are being studied to further endow CAR-T cells with superior attributes, in order to enhance potency and safety. Some strategies were proposed to overcome toxicities and optimize tumor recognition specificity, including introduction of safety switches and tuning the affinity of CARs to recognize differential expression of antigens.

In addition, the combination of CAR-T cell therapy with other therapeutic entities has shown the potential to enhance the efficacy of CAR-T cell therapy, paving the way for combination immunotherapy in a clinical setting. For example, combining CAR-T cell therapy with additional regulatory modulators or checkpoint inhibitors can prevent rapid exhaustion of CAR-T cells within the immunosuppressive tumor microenvironment, enhancing the proliferation and persistence of CAR-T cells. Further developments in this field continue to evolve, which may require multi-disciplinary management and multi-center cooperation.

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Author's contribution

Yingying Yang wrote the first draft of the paper; Jiasheng Wang and Yongxian Hu contributed revision of the manuscript; He Huang supervised the work and made final approval of the manuscript.

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Conflict of interest

The authors declare no conflicts of interest. Disclosure forms provided by the authors are available here.

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