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Review Article

Advancing Chimeric Antigen Receptor-Engineered T-Cell Immunotherapy Using Genome Editing Technologies: Challenges and Future Prospects

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Chimeric antigen receptor engineered-T (CAR-T) cells also named as living drugs, have been recently known as a breakthrough technology and were applied as an adoptive immunotherapy against different types of cancer. They also attracted widespread interest because of the success of B-cell malignancy therapy achieved by anti-CD19 CAR-T cells. Current genetic toolbox enabled the synthesis of CARs receptors which are targeted against tumor-specific antigens, and enabled to arbitrarily T-cells function reprogramming. Approximately all of CAR-T cell based studies apply autologous CAR-T cells in which, modified T-cells are engineered using patient's own T cells. Currently, four different generation of CAR-T cells have been developed, and the evolution of this kind of therapy illustrates an excellent example of the application of basic research into the clinical trial stage. However, development of allogenic CAR-T cells can be a turning point for CAR-T cells therapy. Appearance of the reliable gene editing approach, CRISPR/Cas9 system, provided a new hope for designing universal CAR-T cells which are off-the-shelf, and enable to use for treatment of any patient with any kind of tumor. This review outlined four different generations of CAR-T cells. Also, we discussed different types of genome editing systems especially CRISPR/Cas9 system, and their capabilities for generating engineered T-cells. Additionally, we tried to explain challenges faced in improving universally generated T-cells.

Keywords: CAR-T cells, cancer, therapy, CRISPR/Cas9 system

Despite the fast progress in the appearance of helpful medical technologies and therapeutic approaches, cancer is still an intractable problem of public health (1). Most commonly used conventional therapeutic strategies such as chemotherapy, surgery, and radiotherapy probably give short-term advantages but have irritating side effects because of their invasiveness and highly biotoxicity (2). Moreover, multidrug resistance remains a major challenge in the chemotherapy, and several side effects of radiotherapy depend on the type of cancer and the radiation therapy dose, limiting therefore their curative efficacies (3, 4). Correspondingly, novel therapeutic strategies that

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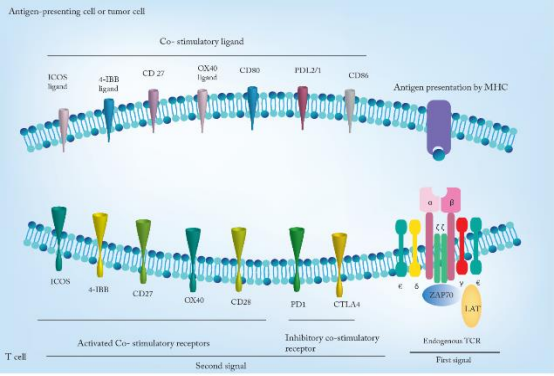

Figure 1. Natural occurring immune responses against tumor cells.

are highly specified for a targeted tumor and have a long-term beneficial are required to be developed.

According to the naturally occurring immune responses against tumor cells (figure 1), currently, several immune system based therapies such as usage of T cell receptor (TCR)-engineered T cells, tumor-infiltrating lymphocytes (TILs), and chimeric antigen receptor (CAR) –engineered T cells, have been developed for different human malignancies (5-7).

TILs are isolated and cultured from resected tumors, and sent back to patients. Infusion of these cells in patients with metastatic melanoma have provided encouraging results (8) , but this method showed some limitations in other solid tumors owing to the low efficiency of isolation and *in vitro* expansion (9).

However, re-engineering of the immune system, especially T cells, for targeting and killing tumor cells has been a primary aim of cancer biology for many years. Thereby, the idea that adoptively transferred T cells can act as a harness to cancer treatment was demonstrated after the seminal study of Medawar et al. (10). Along with, progresses in genetic engineering technology, for example retroviral transduction, has allowed researchers to insert artificial transgenes into primary T cells, and their subsequent expression caused the redirection of T cells against tumor-associated antigens (TAAs) (11).

Clinical trials of modified T cells in cancer therapy are based on the genetically modified T cells which are synthetic receptors that preserve the primary TCR structure, but are designed for specific antigens on tumor cells.

Rapoport et al. used New York esophageal squamous cell carcinoma 1 (NYESO 1) (a cancer-testis antigen) specific TCR engineered T-cells for multiple myeloma in phase I/II trial, and showed that *in vivo*–expanded genetically altered antigen-specific T cells can be well tolerated without remarkable safety concerns (12). However, like native TCRs, these kinds of engineered TCRs need MHC presentation, but many tumors down-regulate MHC class I and potentially disappear from these TCR-modified T cells.

Until now, CAR-modified T cell therapy has been best effective interesting research field in cancer therapy. So far, a high number of clinical trials have been directed to improve the efficiency and safety of CAR-T cells for cancer treatment (11). CARs are chimeric molecules that are typically composed of antigen recognition domain of the B-cell receptor, such as extracellular antibody single-chain variable fragment (scFv), a TCR-derived CD3ζ domain, and the co-stimulatory domains of the T cell receptor (13). Unlike early engineered T cells, CARs allow enhancing the specific targeting of antigen in an MHC-independent manner. Therefore, CARs enable the redirection of cytotoxic T cells toward any various kinds of protein or non-protein targets that are expressed on tumor cell surfaces. Accordingly, there is no requirement for antigen processing and presentation by MHC on cell surface, and is appropriate to non-classical targets such as carbohydrates and glycolipids structures (14).

Eliminating the MHC restriction limitations afford a promising opportunity to broaden the applicability of CAR-T cell-dependent immune-therapy in cancer. Additionally, progresses in *ex vivo* expansion enable to rapid development, and improve the capability of CAR-T cells. Therefore, construction of clinically appropriate doses of these

"living drugs" becomes possible. Nevertheless, CAR-T cell targets were restricted to cell surface antigens.

With the proof of concept established, CAR-T cells therapy have emerged as a probable approach to treat different kind of malignancies. For example, adoptive delivery of CD19 directed CAR-T (CART19) cells have created promising and resilient therapy in patients with relapsed B cell malignancies and refractory (15-17). Nevertheless, there are several challenges in adoptive T cell immunotherapy for cancer treatment, notably usage of CAR-T for solid tumors treatments, for example: target specificity, T cell exhaustion, and suppressive situation of tumor microenvironments. Mentioned challenges and limitation thoroughly will be discussed in this review. In addition, we will explain some of the latest and promising developments in adoptive T cell immunotherapy, with a specific attention to the latest gene-editing systems. Likewise, we will describe how they are being used to modified-T cell immunotherapy.

Chimeric antigen receptor structure

CARs are typically composed of four main regions: extracellular binding region (ectodomain), hinge or spacer domain, transmembrane domain and intracellular signaling domain (endodomain) (figure 2) (18). The ectodomain of CARs has conventionally provided the antigen binding domain of receptor, and single-chain variable fragments (scFvs) are the largest group of extracellular domain

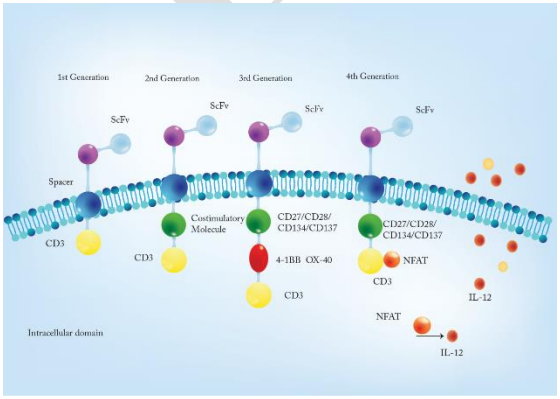

Figure 2. Four different CAR-T cell generations.

for CARs. ScFv affinity is the major determinant of CARs function and specificity (19, 20). As a result of this, Hudecek et al. used receptor tyrosine kinase–like orphan receptor 1(ROR1), as a tumor-associated molecule, to create ROR1-CAR-T cells with high affinity to ROR1 positive tumor cells. Thereby, ROR1-specific scFvs were able to enhance CAR-T cell effector function (19). While the ectodomain detain the CAR specificity, spacer region, as a binding domain between the ectodomain and transmembrane domain, is another important domain, and changing its length and composition significantly affect CAR-T-cell activity. However, results from different studies have shown controversial results about the use of various kind of spacer domains in CARs structure (21, 22). For example, Guest et al. (23) added CH2CH3 spacer to four CARs with no spacer, this addition specified them for some antigens such as carcinoembryonic antigen (CEA), neural small adhesion molecule (NCAM), the oncofetal antigen 5T4 and B-cell with CD19. While 5T4- and CD19-specific CAR-T cells with CH2CH3 spacer had some enhanced effector function, but superior anti-tumor activity of CEA- and NCAM-specific CAR-T cells was without spacer. As well as, Hudecek et al. (19) used spacers with different size such as CH2-CH3 spacer (229 amino acids (AA)), CH3 spacer (119 AA), and short spacer (12 AA), on ROR1-specific CARs and showed that CAR-T cells with short spacer have better anti-tumor activity. These studies illustrated that spacer region have a potential role in CAR-T cells activity and it is necessary to determine an optimum form of this region for all CARs.

The transmembrane domain of CARs connects extra and intracellular domains and so has a critical role in CAR stability. There are some different transmembrane domains such as CD3-ζ, as an earliest transmembrane domain, and CD4, CD8, and CD28 molecules that can be used in CARs structure (24). Currently, CD28 is the most utilized domain in CAR-T cells and provide a significant stability for CARs (25, 26). Ultimately, transmembrane domain

links to an intracellular signaling domain that affords the downstream signaling, such as phosphorylation of immunoreceptor tyrosine based activation motifs (ITAMs) that are present in the cytoplasmic domain, and lead to the activation of CAR-T cells (27). According to the structure of intracellular signaling domain, CARs have been classified into four different generations.

Different generations of CARs

The first generation of CAR-T cells were developed in 1989 by Gross et al. (28, 29). These kind of modified T cells contain a CD3ζ or FcεRIγ as an intracellular signal transmitter, which is sufficient to induce T cell activation through the phosphorylation of ITAMs (11, 12). This activation triggers cytotoxic responses against tumor cells besides secretion of cytokines, especially IL-2 and IFN-γ (30). Some *in vivo* preclinical studies were able to illustrate better survival for tumor-bearing mice using the first generation of CAR-T cells (30, 31). However, this activation signal usually is insufficient to completely activate CAR-T cells and results in T cell anergy, poor cytotoxic activity, poor T cell survival, weak proliferation, and poor persistence of CAR-T cells (32-34).

Therefore, the second generation of CAR-T cells was developed to improve the activation signaling and also enhance the proliferation, persistence and cytokine secretion of CAR-T cells. These were achieved by adding a co-stimulatory molecule (second signaling domain or signal 2) such as CD28 into the intracellular domain of CARs between the transmembrane domain and CD3-ζ domain, which causes complete T cell activation, and prevents apoptosis by promoting the expression of anti-apoptotic proteins such as Bcl-xL (13, 14). CD28 along with 4-1BB, as a part of the tumor necrosis factor (TNF) receptor family are the most broadly applied co-stimulatory molecules in CARs structure (35). CD28-containing CARs have a great potency to activate the T cells. Additionaly, CAR-T cells with CD28 domain are generally able to secrete high amounts of cytokines, such as IL-2, IL-10, TNF-α, and IFN-γ in comparison with other CAR-T cells (25, 36). Moreover, inducible co-stimulation (ICOS) which is a member of CD28 super family and OX40 (part of TNFR family) are two other co-stimulatory molecules that are used as a second signal domain in CARs construction (37). Currently, the second generation of CAR-T cells is major CARs used in clinical trial experiments (table 1).

Hitherto, most experiments have been performed on different type of B-cell malignancy, and CD19 (an extracellular glycoprotein) is the most common targeted antigen on these cells. For example, Kochenderfer et al. used second generation anti-CD19-CAR-T cells containing CD28 co-stimulator for treatment of advanced follicular lymphoma. In this trial, infusion of anti-CD19 CAR-T cells provided partial remission for up to 32 weeks with no remarkable toxicity, and the persistence of CAR-T cells was being monitored using qPCR (38). In another phase I trial study with four patients, Ritchie et al. used second generation autologous anti-LeY CAR T-cells for treatment of acute myeloid leukemia (AML) and tried to estimate the persistence and safety of these adoptive T cells. They delivered totally 1.3×10^{9} T cells to the patients, and observed a significant persistence of infused CAR-T cells in the patients for up to 10 months (39). Recently, Kebriaei et al. expressed the second-generation anti-CD19 CAR-T cells in a non-viral system, through the use of Sleeping Beauty (SB) transposon/transposase system, and applied them as an adjuvant therapy in phase I trial for treatment of 26 patients with advanced non-Hodgkin lymphoma (NHL) and acute lymphoblastic leukemia (ALL). The rate of CAR-T cell persistence in these patients was in average 201 days for the autologous recipient and 51 days for allogeneic recipients. Furthermore, these results emphasized safety of SB system for application in immunotherapy of human diseases (40).

Enhancing the persistence of CAR-T cells after infusion is the main issue to improve the CAR-T cell function. Accordingly, Rossig et al. conducted a

multi-center phase I/II trial study and used Epstein-Barr-virus (EBV)-specific cytotoxic T-cells (EBV CTL) redirected with a CD19-CAR-T cells for treatment of patients with relapsed pediatric ALL. Additionally, they used EBV-directed vaccination which was able to show a significant increase in the persistence of infused CAR-T cells in comparison with T-cells without vaccination (41).

Different co-stimulatory receptors provide various functional properties for CAR-T cells. For example, the second-generation CD28-specific CARs immediately proliferate and are activated against tumor, whereas 4-1BB-specific CARs have the long-term persistence potency in tumor environment (42). Therefore, discrete features of co-stimulatory domains provide an opportunity to be able to further develop CAR-based T cell therapies, and improve their properties for a variety of cancers.

Following features can be provided by third generation of CAR-T cells. This generation, in addition to CD3ζ, contain two co-stimulatory domains with different properties which are able to enhance the activation status of CAR-T cells with powerful cytokine production (for example: CD3ζ-CD28-OX40 or CD3ζ-CD28-41BB) (15). Different *in vitro* (43) and *in vivo* (44) preclinical studies have illustrated that third generation CAR-T cells have a superior potential in activation, proliferation and persistence but there is no sufficient clinical study capable to demonstrate the safety and efficacy of third generation CAR-T cells. Additionally, enormous phenotypic heterogeneity of solid tumors makes limitation on activity of these CAR-T cells. Due to heterogeneity, there are significantly high rate of antigen-negative tumor cells which are not recognized by CAR-T cells which enhance the probability of tumor relapse. This may be resolved through the activation of native T-cells in tumor environment mediated by infused CAR-T cells.

Fourth generation of CAR-T cells with dual anti-cancer cell activities through the inducible IL-12 have been developed (45). Theae are second generation of CAR-T cells additionally engineered with an inducible expression cassette encoding transgenic IL-12 and known as T cell redirected for universal cytokine-mediated killing (TRUCKs) (45). Thereby, inducible recombinant IL-12 (iIL-12) (composed of the p40 and p35 chain) is under control of the NFAT/IL-2 minimal promoter and releasing of iIL-12 is promoted by CAR signaling pathway (46). Releasing of IL-12 by TRUCKs increases T cells activation in tumor environment, and attracts innate immune system which causes the eradication of antigen-negative tumor cells (45). However, similar to third generation CAR-T cells, it is needed to direct more and more experiments to be able to illustrate the efficacy and usability of fourth generation CAR-T cells.

Genome editing technologies enable the generation of allogenic universal CAR-T cells

Despite recent advances in the development of autologous adoptive T-cell immunotherapy (T-cells that are currently applied in the generation of CAR-T cells are mainly achieved from the patients themselves (autologous T cells)), some limitations reduce the efficacy of CAR-T cells including the insufficient quantity of autologous T cells that affect the efficiency and quality of CAR-T cells, and also the generation of autologous T cell production is not cost-effective either in term of time or expense. Therefore, a large number of cancer patients cannot be treated through this approach.

Accordingly, the use of genetically engineered allogenic T cells which are received from healthy donors and known as universal T cells can be a promising opportunity for the development of a highly efficient approach in cancer therapy. Moreover, universal CAR-T cells can be used for treatment of various cancer patients without raising graft-versus-host disease (GVHD). Additionally, allogenic CAR-T cells can be improved regarding their persistence and efficient elimination of the tumor cells. GVHD disease occurs by the recognition of recipient antigens via TCRs related to infused T-cells. Conversely, recognition of foreign

HLAs of infused allogenic T-cell causes rapid rejection. Thereby, it is necessary to be able to silence both TCRs and HLAs in universal allogenic CAR-T cells (47).

Currently, the development of genome editing technologies provided an opportunity to precisely manipulate the genome. Hence, it is possible to use gene editing strategies for silencing of infused T-cell-related HLAs and TCRs. These approaches may provide an opportunity to generate reliable allogenic universal CAR-T cells that can be used for the treatment of different cancer patients without any concern about GVHD or immune response against foreign T-cells and early rejection. Up to now, three different genome editing approaches have been developed. Here we briefly describe the safety concerns regarding their specificity in allogenic CAR-T cells generation.

Zinc finger nuclease (ZFN)

Zinc finger nuclease (ZFNs) are artificial hybrid proteins, and were primarily developed as one of genome-editing tools for specific modifyication of genome. ZFNs are mainly composed of two different domains: a sequence-specific repeated zinc finger protein that recognizes and binds to a predetermined region of the genome, and a non-specific FokI restriction endonuclease domain (48). Each zinc finger protein can recognize three or four base pairs. As a result of this, ZFNs are typically comprised from three or four zinc finger protein which are joined together in tandem repeat to be able to target 9-18 base pairs of the genome (49). Moreover, dimerization of FokI endonucleases is necessary to create a site specific double strand breakage in the genome. Thereby, two ZFN molecules are needed to bind complementarily to adjacent regions of the genome. This site-specific binding and dimerization of two FokI endonucleases provides a highly efficient cleavage of DNA. Consequently, DNA repair systems which include two different strategies: error-prone non-homologues end joining repair (NHEJ) in the absence of a template, and homology-directed repair (HDR) in the presence of a DNA template, are vactivated (50). NHEJ causes disruption in a targeted gene but HDR system provides a template-mediated repair of the targeted gene (51, 53). Torikali et al. used ZFN for genome editing of allogenic CD19-specific CAR-T cells, and they could permanently eliminate the expression of endogenous TCRs (53). However, the difficulty of engineering ZFN monomers with high affinity for a specific site of the DNA and low efficacy with high off-target due to heterodimerization of FokI enzymes, limited their capability for further utilization in the genome editing strategies (54).

Transcription activator-like effector nucleases (TALEN)

In 2009, two independent experiments described details of transcription activator-like effector (TALE) DNA-binding proteins that are secreted by plant-specific pathogens such as *Xanthomonas* and *Ralstonia sp* (55, 56). DNA-binding domain of TALE proteins consist of tandem repeats with highly conserved 34-35 amino acids. Moreover, hypervariable amino acids, located at positions 12 and 13 within each tandem repeats referred to as the repeat variable di-residue (RVD) show a high affinity to specific nucleotide targets. Thereby, each of this tandem repeats are able to recognize a single nucleotide target site. Thereby, selective combination of these specific DNA-binding tandem repeats allows to target a unique sequence in the genome (55). Similar to ZFNs, FokI endonuclease fuses to a reprogrammed TALE domain, and provides a TALE nuclease (TALEN) monomer. Binding of two engineered TALEN monomers in an adjacent region enables FokI enzyme dimerization, and creates a double strand break in targeted sequence (57). Like ZFNs, TALENs based cleavage induces NHEJ or HDR system of targeted cells (58). Developing of TALENs allows to target any specific site of genome. Moreover, the ease of reprogram-ming of TALE domains make TALENs more

interesting than ZFNs for targeted genome-editing (54).

In a pre-clinical study TALEN have been used for multiplex genome-editing of anti-CD19 CAR-T cells (59). In this study, both TCR and CD52 (encodes a protein targeted by alemtuzumab) genes, were knocked out. CD52 elimination, increases the resistance of third-party T cells to lympho depleting/ immunosuppressive effects of alemtuzumab, and significantly reduces their rejection chance by the recipient patient. Ultimately, these results illustrated the highly efficiency of TALEN system for the generation of allogenic adoptive T-cells from third-party healthy donors without raising GVHD (59). Recently, Qasim et al. directed the first clinical trial of TALEN-mediated genome-engineered universal CD19-specific CAR-T cells, and have shown remarkably remission of leukemia in an 11-month-old patient (60). However, TALENs based genome editing is associated with some limitations. For example, the large size of TALEN monomers that requires 34-35 amino acids to specify a single base pair, reduces the delivery performance of both TALEN monomers in a single viral vector. Moreover, unstable nature of TALE tandem repeats makes difficult their packaging in viral vectors (54).

CRISPR/Cas9

An alternative strategy to protein-mediated genome-editing technologies, the clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated protein 9 (Cas9) system has been developed as a novel RNA-guided genome-editing tool. Because of the high efficiency and ease of use, CRISPR/Cas9 system is the widely applied strategy for precisely modifying of the genome (61). Originally, CRISPR/Cas9 system from *Streptococcus pyogenes*, was recognized as a complex system that acts as a bacterial and archaeal immune defense system against bacteriophage DNA or other foreign DNAs like plasmids (62). Briefly, when a foreign DNA was detected by type II CRISPR immune system, some part of its sequence was incorporated into the CRISPR repeat sequences (63). The core components of engineered CRISPR/Cas9 system are Cas9 nuclease and single guide RNA (sgRNA). To date, more than 20 Cas9 homologs have been derived from different species of bacteria and each of them have a specific protospacer adjacent motif (PAM) (64). *Streptococcus pyogenes* Cas9 (spCas9) is the most commonly utilized Cas9 for modifying the human genome (65).

Commonly, Cas9 enzyme is composed from two catalytically active domains consisting of RuvC and HNH. As well as, chimeric sgRNAs are generated by fusion of a programmable CRISPR RNA(crRNA) and a trans-activating crRNA (tracrRNA) (66) in which crRNA is synthesized against a specific target and tracrRNA acts as a scaffold for crRNA and facilitates the interaction between crRNA and Cas9 (66). Accordingly, complementary base paring of sgRNA is necessary for precisely directing Cas9 to the desired genomic region. Cas9-mediated cleavage necessarily requires a PAM which should be located precisely downstream of the sgRNA-targeted sequence. PAM acts as a binding signal for Cas9, and typically has 3-5 bp in length. Binding of sgRNA to the targeted sequence along with the presence of PAM provide an opportunity to Cas9 to create a double strand cleavage between the 3rd and 4th base upstream of the PAM (67, 68).

Because of the use of sgRNA instead of protein constructions, CRISPR/Cas9 system is much easier in comparison with other genome editing approaches. This system was developed as a great therapeutic approach to the generation of allogenic CAR-T cells. As well as, efficient multiplex genome editing by CRISPR/Cas9 system enables to simultaneously knock out different types of gene such as programmed cell death protein 1 (*PD1*), cytotoxic T-lymphocyte associated protein 4 (*CTLA4*), TCR beta chain, and beta-2-microglobulin (*B2M*), which reduce the expression of HLA class I antigen on the CAR-T cells surface.

Up to now, several pre-clinical studies demonstrated a significant efficacy of CRISPR/Cas9 system-based allogenic CAR-T cells (69). Most recently, Ren et al. used one shot CRISPR/Cas9 protocol which was enabled to integrate multiple sgRNAs into a CAR-lentiviral vector (70). Consequently, they could generate allogenic CAR-T cells that were simultaneously disrupted in four gene loci including *TCR*, *HLAI*, *PD1*, and *CTLA-4* genes (71). Thereby, double knocking out of *TCR* and *HLAI* eliminates the risk of both GVHD and early rejection. Moreover, disruption of PD1 and CTLA4 which are considered as inhibitory signals, provided inhibitory resistant universal CAR-T cells (70). Currently, the main focus of researches is the production of highly homogenous population of allogenic CAR-T cells. Therefore, in another recently performed pre-clinical study, scientists developed a self-inactivating lentiviral based vector in which a specific sgRNA was incorporated in 3' long terminal repeat (LTR). In the mentioned investigation, they could generate highly homogenous allogenic engineered-T cells that were 96% CAR positive and 99% TCR negative (72).

Even though variety of studies has been directed to treat advanced refractory B cell malignancy, there are significant challenges about the use of this therapeutic strategy for treatment of solid tumors (73). Zhang et al. demonstrated that the disruption of lymphocyte activation gene 3 (*LAG3*), as a negatively regulating factor of T cells, by CRISPR/Cas9 system, remarkably increased the efficiency of CAR-T cells against solid tumors (74). According to these and some other successful pre-clinical studies, generation of highly efficient allogenic CAR-T cells by CRISPR/Cas9 system were carried out in clinical trial stages, and now some different clinical studies are ongoing in phase 1 and 2 (Table 1).

However, one of the crucial challenges relatedto CRISPR/Cas9 system as a safe genome editing approach, is undesirable cleavage of the genome (off-targets) that should be reliably reduced (75). Consequently, there are some suggestions that could be performed to improve the efficacy of this system. For example, designing proper sgRNAs, using programmable systems such as *tet-on* for controlling the expression of Cas9 or using Cas9 mRNA and protein instead of plasmid containing Cas9 gene, because mRNA is very sensitive and is rapidly degraded by RNases after on-target cleavage, can be performed (76). Moreover, the nickase form of Cas9, in which Cas9 is able to cut just a single strand of DNA, can be used. Therefore, creating a double strand breakage in DNA requires two sgRNAs and two Cas9 which significantly reduces the off-target rate (67). As well as, it is possible to use dead Cas9-FokI as a fused protein to create an on-target double strand cleavage. In this strategy, dCas9 is unable to cut DNA but will direct the FokI enzyme to a specific region of the genome that sgRNA have paired. Thereby, double strand cleavage is needed to pair FokI domains adequately close to create a dimer (two target sites should not be more than 25 bp apart, approximately 13-25 bp) (77).

Another utility of the CRISPR/Cas9 system is its application as a gene knock-in platform to insert a DNA sequence in a desired region of genome. Viral delivery vectors such as lentivirus and retrovirus are the traditional approaches that are used to integrate CAR fragments into the T-cells genome. Nevertheless, this kind of uncontrolled integration has the great risk of causing insertional mutagenesis (78).

CRISPR/Cas9 mediates effective gene knock-in in various kinds of cells, but integration of large fragments such as CARs into T-cells genome through the knock-in approach is a great challenge. However, most recently Sather et al. showed that knock-in of CAR into T-cells is possible through the megaTAL and an adeno-associated virus HDR template (79).

Table 1. Overview of CAR-T cell clinical trials

Publication date	2015 (84)	2014 (17)	2014 (83)	2012 (82)	2015 (15)	2011 (81)	2015 (80)
Country	United States, Maryland	United States, Pennsylvania	United States, Texas	United States, Maryland	United States, New York	United States, Pennsylvania	Sweden
Location	National Institutes of Health Clinical Center, 9000 Rockville Pike	http://www.chop.edu/service/oncology/pediatric-cancer-research/cart-19-trial.html	-Houston Methodist Hospital -Texas Children's Hospital	National Institutes of Health Clinical Center, 9000 Rockville Pike	Memorial Sloan Kettering Cancer Center	Abramson Cancer Center of the University of Pennsylvania	Uppsala University Hospital, Dept of Oncology
NCT Number	NCT01593696	NCT01626495	NCT00586391	NCT00924326	NCT01044069	NCT01029366	NCT02132624
Generation	Second	Second	Second	Second	Second	Second	Third
Condition or diseases	ALL B Cell Lymphoma Leukemia Large Cell Lymphoma Non-Hodgkin	B Cell Leukemia B Cell Lymphoma	B Cell Lymphoma Chronic Lymphocytic Leukemia Acute Lymphocytic Leukemia	Primary Mediastinal B-cell Lymphoma Diffuse, Large B- cell Lymphoma Transformed From Follicular LymphomaMantle Cell	-Leukemia -Acute Lymphoblastic Leukemia	chronic Lymphocytic Leukemia (CLL) Acute Lymphoblastic Leukemia (ALL)	B Cell Lymphoma B Cell Leukemia
Phase	Phase 1	Phase 1	Phase 1	Phase 1 Phase 2	Phase 1 Phase 1	Phase 1 Phase 1	Phase 1 Phase 2
Interventions	Biological: Anti-CD19- CAR	Biological: CART-19	Genetic: CD19CAR-28-zeta T cells Drug: Ipilimumab	Drug: Fludarabine Drug: Cyclophosphamide Biological: Anti-CD19-CAR PBL	Biological: gene-modified T cells targeted	Biological: CART-19	Biological: Autologous 3rd generation CD19-targeting CAR T cell
Costimulatory domain	CD28	CD28, 4-1BB	CD28	CD28	CD28	CD137	CD28 and 4-1BB
Number of participants	21	30	14	8	5	23	15
clinical benefits	OS:51.6% ORR:66.7%	OS[1]: 78% CR:90% PFS[1]:67%	-	CR[1]:12% PR[1]:62%	ORR:80%	ORR[1]: CLL %4.29 ALL 8.%33	OCRR[1]:40%
Adverse events	Yes	Yes	Yes	Yes	Yes	Yes	Yes

1 Overall complete response rate
2 Overall Response Rate
3 Complete remission
4 Partial remission
5 Overall survival
6 Progression-free survival

Conclusion

Immunotherapy of cancer based on CAR-T cells therapy has been found to be a novel and hopeful strategy that is approved for commercial application. For example, Kymriah is the first CAR-T cell therapy approved in Canada which is used for the treatment of adult patients with relapsed and refractory (r/r) diffuse large B-cell lymphoma (DLBCL). Because of highly number of clinical trials which are registered in clinicaltrial.gov, it is possible that other CAR-T cell-based therapies will follow the next few years. However, in order to produce CAR-T cells with minimum toxicity and high number of complete responders, achieving more knowledge about various elements impacting the clinical outcome, as well as creating novel CAR constructions with highly stability is required. In addition, autologous CAR T-cell therapy is not cost-effective, because of the high cost of bone marrow transplantation. Thereby, emergence of allogenic CAR-T cells as "off-the-shelf" approach is considered as a solution to this challenge. Moreover, genome editing by CRISPR/Cas9 provided a great therapeutic opportunity to enhance the capability of allogenic CAR-T-cell-based immunotherapy, but there are some challenges that restrict the use of CAR-T cell therapies based on genome editing strategies. Some pre-clinical and early stage clinical trials attempted to demonstrate the safety of CRISPR/Cas9 based genome editing strategy through minimizing off-target rate. However, more clinical trials are required to achieve a high degree of accuracy that confirm the efficiency and safety of this technology.

Naturally occurring immune responses against cancer require two signals that are provided by two different types of receptors; the first signal which is an antigen-specific and known as a recognition signal, is provided by T-cell receptors (TCRs), and the second signal is mediated by co-stimulatory receptors (7, 8). Therefore, co-stimulatory receptors are positive signals and their binding is required for full T cells activation. In contrast, there are alternative receptors on T cells with negative effect and known as co-inhibitory receptors, because they are able to act as modulator or through feedback mechanisms and prevent T cells activation (7, 8). For instance, programmed cell death protein 1 (PD1) and cytotoxic T lymphocyte-associated antigen (CTLA4) are the most important co-inhibitory receptors and so many tumors upregulate related ligands (PD-L1 and PD-L2 act as inhibitory ligands for PD-1 receptors and CD80 and CD86 are inhibitory ligands for CTLA-4) to inhibit T cells function (9). Tumors may also downregulate major histocompatibility complex (MHC) to avoid binding of TCRs to their peptide-MHC ligands and evade an efficient immune reaction (10).

Conflict of interest

The authors declared no conflict of interest.

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