Human Immunology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

# CRISPR/Cas9 and CAR-T cell, collaboration of two revolutionary technologies in cancer immunotherapy, an instruction for successful cancer treatment

Hasan Mollanoori[a], Hojat Shahraki[b,c], Yazdan Rahmati[a], Shahram Teimourian[a,*]

[a] Department of Medical Genetics, Iran University of Medical Sciences (IUMS), Tehran, Iran
[b] Cellular and Molecular Research Center, Zahedan University of Medical Sciences, Zahedan, Iran
[c] Department of Laboratory Sciences, School of Allied Medicine, Zahedan University of Medical Sciences, Zahedan, Iran

ARTICLE INFO

*Keywords:*
CAR T cell
Cancer immunotherapy
CRISPR/Cas9 technology
Biotechnology companies

ABSTRACT

Clustered regularly interspaced short palindromic repeats/CRISPR associated nuclease9 (CRISPR/Cas9) technology, an acquired immune system in bacteria and archaea, has provided a new tool for accurately genome editing. Using only a single nuclease protein in complex with 2 short RNA as a site-specific endonuclease made it a simple and flexible genome editing tool to target nearly any genomic locus. Due to recent developments in therapeutic engineered T cell and effective responses of CD19-directed chimeric antigen receptor T cells (CART19) in patients with B-cell leukemia and lymphoma, adoptive T cell immunotherapy, particularly CAR-T cell therapy became a rapidly growing field in cancer therapy and recently Kymriah and Yescarta (CD19-directed CAR-T cells) were approved by FDA. Therefore, the combination of CRISPR/Cas9 technology as a genome engineering tool and CAR-T cell therapy (engineered T cells that express chimeric antigen receptors) may lead to further improvement in efficiency and safety of CAR-T cells. This article reviews mechanism and therapeutic application of CRISPR/Cas9 technology, accuracy of this technology, cancer immunotherapy by CAR T cells, the application of CRISPR technology for the production of universal CAR T cells, improving their antitumor efficacy, and biotech companies that invested in CRISPR technology for CAR-T cell therapy.

## 1. Introduction

The increasing burden of cancer in the human population is the major concern in today's societies, therefore finding safe and effective alternative therapies had become one of the main goals of researchers throughout the world. One of these alternative approaches is harnessing the power of the body's own immune system to battle against cancers which is termed immunotherapy. Considering the use of the body's own immune system, immunotherapy promises a more effective and durable treatment than conventional treatments, and chimeric antigen receptor-T cell therapy (CAR-T), a type of immunotherapy, can transform the future of cancer treatment. CAR-T cells are genetically engineered T cells that express artificial proteins known as chimeric antigen receptors, which navigate this CAR-expressing immune cells to surface tumor antigens. Recently significant advances were reported in therapeutic engineered T cells and effective responses were shown by the use of CD19-directed chimeric antigen receptor T cells (CART19) in patients with B-cell leukemia and lymphoma. Kymriah and Yescarta are two CD19-directed genetically modified autologous T cells were recently approved by FDA as CAR-T cells that used in patients who had acute lymphoblastic leukemia (ALL) and certain types of non-Hodgkin lymphoma (NHL), respectively [1,2]. Considering the overlap between genome editing approaches and CAR-T cell therapy in the context of genetic engineering enables us to use both of them together in combination. Making targeted manipulation in the genome for therapeutic goals requires highly efficient systems to modify existing DNA patterns with great accuracy. Programmable nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR-Cas9 provide powerful tools for editing and engineering the genome that can be used to study the function of genes and therapeutic goals. Among aforementioned nucleases, the use of CRISPR/Cas9 has surpassed others for ease, pliability and the possibility of multiplex gene editing [3,4]. CRISPR-Cas9 is taken from type II acquired immune system in bacteria and archaea that used to shield

* Corresponding author at: Department of Medical Genetics, Iran University of Medical Sciences (IUMS), Crossroads of Shahid Hemmat & Shahid Chamran Highways, P.O. Box: 15875-6171, Tehran 1449614535, Iran.
*E-mail address:* teimourian.sh@iums.ac.ir (S. Teimourian).

https://doi.org/10.1016/j.humimm.2018.09.007
Received 17 June 2018; Received in revised form 23 September 2018; Accepted 23 September 2018

**Table 1**
Comparison of the different types of programmable nucleases in genome editing.

| Features | CRISPR/Cas9 | TALEN | ZFN |
|---|---|---|---|
| DNA binding component | RNA | Protein | Protein |
| Cost | Low | Moderate | High |
| Nuclease | Cas9 | FokI | FokI |
| Toxicity | Low | variable – high | Low |
| System design | Simple | Complex | Complex |
| Efficiency | High | High | High |
| Multiplex editing capability | High | Low | Low |
| Disadvantages | Off targets | expensive and time consuming<br>Pairs of ZFNs are required to target<br>any specific locus<br>Complexity of protein domains<br>Off targets | time consuming<br>Pairs of TALENs are required to target<br>any specific locus<br>Complexity of protein domains<br>Large size of TALE molecules difficult to deliver to the cells |
| Limitations | Narrowed target sequences due to necessity of presence of PAM sequences | Binding efficiency depends on the presence of thymidine nucleotide before the 5′ end of a sequence | recognition 3–6 nucleotide sequences |

themselves from bacteriophage and plasmid of cellular invaders [5,6]. Among the types of CRISPR/Cas systems identified so far type II (CRISPR/Cas9) system has been studied the most and has been modified and adjusted for use in genome editing in eukaryotic systems [7,8]. Therefore, CRISPR/Cas9 technology, as a strong genome engineering platform is able to significantly expand the kind of cancers and patients that can be treated with CAR-T cells. Also, further improves the efficiency and safety of these therapies. This article explains how CRISPR/Cas9 technology works in the targeted genome editing and its therapeutic application in man, issues and problems related to the accuracy of this technology and the approaches used to solve these problems. Then, CAR-T Cells-based therapeutic technology and the use of CRISPR/Cas9 technology to produce universal CAR-T cells and increasing the potency of these therapeutic approaches are discussed. At the end, we introduce biotech companies that invested in CRISPR technology for CAR-T cell therapy (see Table 1).

## 2. Mechanism and application of CRISPR-Cas9 technology in the treatment of human diseases

This immune system in bacteria and archaea contains a short sequence of invaded bacteriophage or plasmid DNA that cleaved from them and stored in the CRISPR locus of these organisms as a novel spacer sequence. This DNA transcribed and processed into RNA for recognition of subsequent invade of the same virus or plasmid to eliminate them [9]. The Protospacer Adjacent Motif (PAM) sequence, a sequence contains a few nucleotides, is the recognition site in the invaders DNA and recognized by Cas nuclease but not integrated into the host genome with Protospacer sequence. Subsequent invades result in transcription of an RNA from the spacers in CRISPR locus which is called pre-crispr RNA (pre-crRNA). In type II of CRISPR system (CRISPR/Cas9) a second RNA, termed trans-activating CRISPR RNA (tracrRNA), is transcribed and results in the maturation of pre-crRNA by binding to the repeated sequences of pre-crRNA and forming a RNA duplex structure that is cleaved by RNase III. This structure also works as a scaffold among crRNA and Cas9 [10,11]. Finally crRNA/tracrRNA duplex recruits Cas9 nuclease to form a ribonucleoprotein complex that fulfills Cas9 function and navigates it to align with target DNA by base pairing with complementary crRNA spacer [5,6]. To further simplify this genome editing system for laboratory application, CRISPR/Cas9 diminished into 2 parts: Cas9 protein alongside a sole sgRNA, an artificial RNA containing crRNA and tracrRNA. Cas9 nuclease activity requires the presence of PAM sequence immediately after the 3′ end of the sgRNA complementary DNA sequence (5′-NGG-3′ for SpCas9), so in the presence of PAM sequence in target DNA Cas9 nuclease induces a double-strand break (DSB) at three nucleotides upstream of the PAM [7,12,13]. Generally, DSBs that initiates the process of two intrinsic mechanisms for DSB repair can be categorized into two different repair approaches: homologous directed repair (HDR) approach that is used to knock-in desire DNA and either leading to precise repair of the mutation or creating disease-specific mutations, and Non-homologous end joining (NHEJ) approach for knock outing the desire DNA or deletion of the target site (by inducing two cuts at two borders of desired locus) (Fig. 1) [14]. Therefore, by generating DSBs at the desired sites in the genome and using two aforementioned repair approaches, the genome can be edited for different purposes, recently the use of this system has become popular for therapeutic purposes (see Fig. 2).

CRISPR-Cas9 system as an adaptive immune defense system has shown therapeutic potential in manipulating and disabling viral genome to obstruct virus infection, such as HIV, human papillomavirus, Hepatitis B virus, Herpes viruses, JC Virus and hepatitis C virus [15]. Gene therapy applications have also been examined for monogenic disorders, including disruption of the PCSK9 gene for treatment of cardiovascular disease, deletion of an intronic region in the CEP290 gene that comprises a mutation which disrupts the gene coding sequence by generating an aberrant splice site for Leber Congenital Amaurosis type 10 (LCA10), correction of the sickle cell mutation and induction of fetal hemoglobin (HbF) expression for sickle cell disease (SCD), correction of point mutation in fumarylacetoacetate hydrolase (FAH) for Hereditary Tyrosinemia type I and other diseases such as cystic fibrosis, Duchenne muscular dystrophy, Epidermolysis bullosa, X-linked chronic granulomatous disease [16].

CRISPR-Cas9 technology for treating cancer has so far been used in three areas: cancer immunotherapy, manipulation of cancer genome and epigenome, and elimination or inactivation of carcinogenic viral infections.

## 3. Obstacles and limitations of using CRISPR/Cas9 for therapeutic applications

Due to the simplicity, high efficiency, high pliability, low cost, ability to multiplex genome editing, and wide applicability (in a wide range of cell cultures and animal models) for targeted genome editing the attention of many researchers have been attracted to the CRISPR/Cas9 technology. This rapidly expanding technology has begun a new vista in the treatment of a variety of human diseases, although some technical challenges and barriers related to the accuracy, efficiency, and delivery of this technology remain ahead and delay its therapeutic potential. Accuracy of the CRISPR–Cas9 technology is defined by the ability of editing desired target locus in the genome, but we understand the Cas9-sgRNA binding to the target DNA can tolerate sequence mismatches and besides target DNA sequences, also identical, or highly

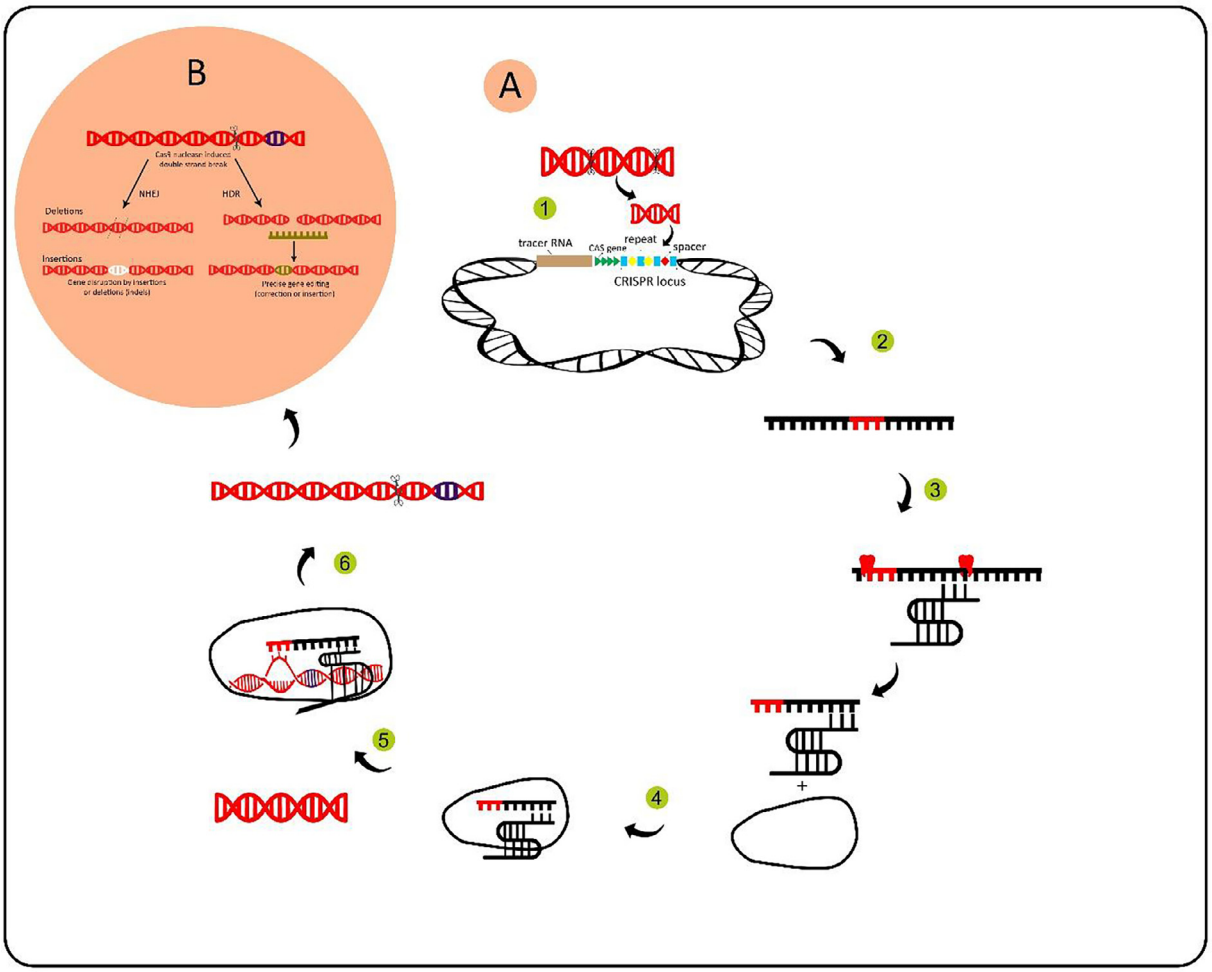


**Fig. 1.** Natural CRISPR/Cas9 pathway and its usage in gene editing approaches. A: 1. A short sequence of invaded bacteriophage or plasmid DNA incorporated into the CRISPR locus as a novel spacer sequence. 2. This sequence transcribed and processed into pre-crRNA after subsequent invade. 3. A second RNA, tracrRNA, is transcribed and results in maturation of pre-crRNA by binding to the repeated sequences of pre-crRNA and forming a RNA duplex structure that is cleaved by RNase III and produces the guide RNA 4. Inactive Cas9 nuclease recruited by guide RNA and becomes activated. 5. The activated guide RNA/cas9 complex binds to the target DNA. 6. The Cas9 nuclease cleaves the invaded DNA and inactivates it. B: In genome editing approaches DSBs initiates two intrinsic mechanisms for DSB repair: homologous directed repair (HDR) approach that used to knock-in desire DNA and either leads to precise repair of mutation or creates disease-specific mutations, and Non-homologous end joining (NHEJ) approach for knock outing desire DNA or deletion of the target site.

homologous DNA sequences can be cleaved. This will lead to mutations in undesired genomic loci, called off-target effects. Off target effects are the major barrier to the potential use of CRISPR/Cas9 technology in therapeutics approaches that lead to mutations or chromosomal re-arrangements because of re-ligation between cuts on different chromosomes. These off target effects are just as deleterious as genomic defects, so in order to have a safe therapeutic application of CRISPR/Cas9 system, reduce off-target events derived from the endonuclease remain a priority and should be diminished, as much as possible.

Various approaches have been explored to minimize Cas9-mediated off-target events that are briefly mentioned below. The precise selection of sgRNAs is the first and most effective approach for reducing potential off-target effects. Several online tools based on predictive algorithms are available that calculate the interaction between synthetic editing systems and DNA for designing sgRNAs with maximum specificity for a desired genomic locus [17]. Use of a truncated sgRNAs, 17 bp, showed similar efficiency as full-length gRNA while showed a reduced off-target effect and more sensitivity to sgRNA/DNA mismatches. The 5′-end nucleotides in sgRNAs are not essential for full sgRNA activity and may lead to mismatches at other positions along the sgRNA-target DNA interface, so the use of shorter sgRNA improves the specificity of CRISPR/Cas9 by increasing the sensitivity to mismatches. The reduction of extra binding energy of the Cas9/sgRNA complex to target DNA may lower nonspecific mismatches of the sgRNA/DNA target-locus jointing, thereby leads to ameliorated specificity of targeting [18]. Modified Cas9 proteins will produce novel Cas9 variants with altered PAM preference that have improved the range of available PAMs and present more sgRNA options for providing a better chance to recognize those with less off-target activity. CRISPR/Cas9 recognition of the target site by sgRNA will navigate Cas9 to a complementary sequence in the target site, but Cas9 requires recognition of a short neighboring PAM for its nuclease activity. This restriction leads to a challenge in the implementation of genome editing applications that require precision in a small area of the genome, such as homology-directed repair (HDR), the introduction of indel mutations into genetic elements with small size such as microRNAs, splice sites, etc., and allele-specific editing. Engineered Cas9 variants with novel PAM specificities can circumvent targeting range limitations, also by providing novel PAM sequence we can improve the precise selection of more specific sgRNAs [19]. High-fidelity Cas9 endonucleases arise from the structure-guided design of CRISPR/Cas9 system based on the detailed crystal structure of Cas9–gRNA complex bound to its target DNA. Kleinstiver et al. 2016 created multiple substitution mutations in the amino acids that bind to the phosphate backbone of the target DNA strand and found one variant

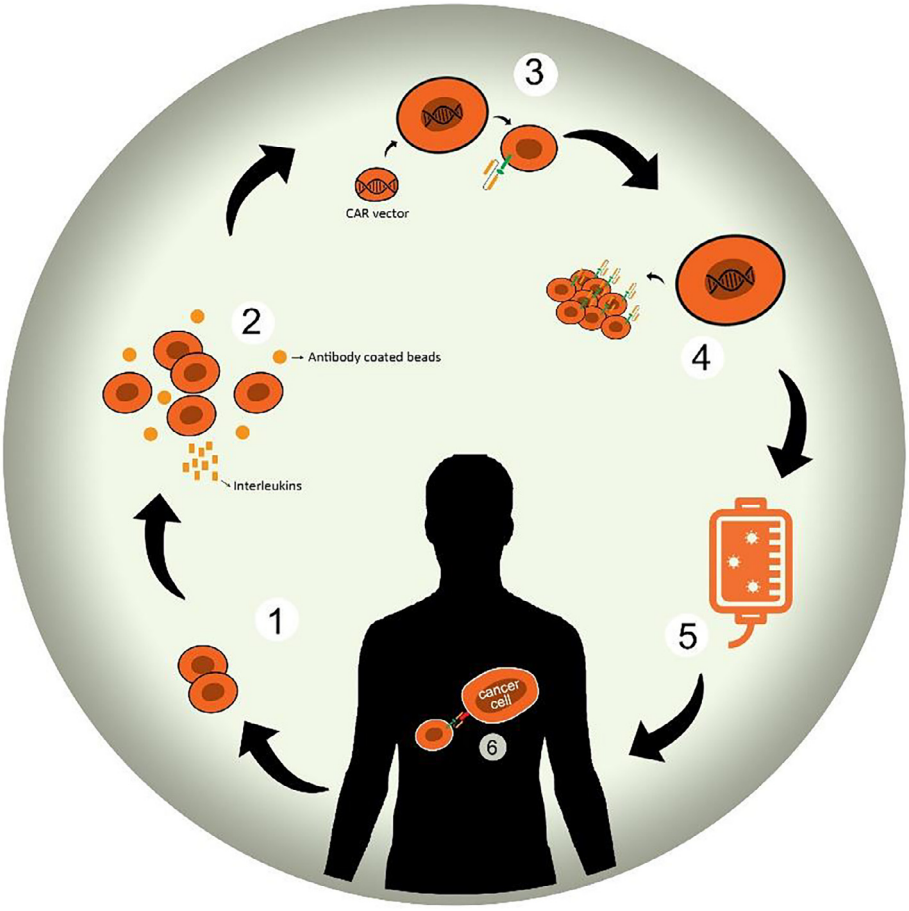


**Fig. 2.** CAR T cell therapy: 1- The first step in CAR T therapy is collection of T cells from patients by leukapheresis. 2- Collected T cells are activated and expanded by antibody coated beads and interleukins. 3- T cells transduced with CAR gene by using lentiviral vectors, retroviral vectors, or non-viral methods like electroporation and sleeping beauty system to express CAR receptor on their surface are called "CAR T cells". 4- CAR T cells are expanding. 5- After quality control CAR T cells infused into the patient. 6- In the patient's body CAR T cell track down and kill tumor cells.

with four substitutions functioned as a high fidelity Cas9 (Cas9HF) with more on-target activity and minimal off-target activity. Cas9 nuclease has some non-specific interactions with its target DNA site, such as four direct hydrogen bonds to the phosphate backbone of the target DNA strand that made by N497, R661, Q695, Q926 residues. The disruption of these non-specific contacts reduces the extra energy of the Cas9/sgRNA complex, enables it not to bind to mismatched off-target sites in the presence of a desired target DNA site [20]. With a similar approach, positively charged amino acids involved in the interaction with the complementary DNA strand mutated and demonstrated enhanced accuracy.

Nuclease function is activated through strand separation by helicase activity of Cas9. The crystal structure of Cas9/sgRNA/target DNA complex reveals a positively-charged groove, positioned between the HNH, RuvC, and PAM-interacting domains in Cas9 that is likely to be involved in stabilizing the non-target strand of the target DNA, so neutralization of these positive charges can significantly reduce off-target cutting whilst maintaining on-target activity, by that means more stringent Watson-Crick base pairing between the guide RNA and the target DNA strand produced [21]. One of the molecular modifications of Cas9 to reduce off-target effects involves inducing mutations in one of the two nuclease domains, HNH or RuvC, to produce a modified Cas9 that nicks one strand of the target DNA (termed a Cas9 nickase). In this strategy Cas9nickase has been combined with a pair of sgRNAs complementary to opposite strands of the target locus and in a way similar to dimeric ZFNs and TALENs cleaves the target locus by the synergistic interaction of two independent sgRNA, whilst nicking of both DNA strands by a pair of Cas9 nickases leads to site-specific DSBs, individual nicks are predominantly repaired by the high-fidelity base excision repair pathway (BER), thereby minimizes off-target effects by each individual Cas9n-sgRNA complex [22]. Fusion of a catalytically inactive Cas9 protein (dCas9) to the dimerization-dependent nonspecific FokI cleavage domain generates an RNA-guided nuclease that its nuclease activity depends on the presence of a pair of FokI domains to form a dimer. This approach improves CRISPR accuracy due to the fact that FokI is just active as a dimer, thereby navigated via a pair of sgRNAs to cleave a larger DNA in their target site (about 40 bps). This larger sequence fortunately almost always is unique [23]. The exposure time of genome to Cas9 or high levels of Cas9 has been associated with the off target effect, so control of Cas9 activity results in reducing the off-target effects and improving the specificity. Different strategies used for regulating Cas9 nuclease activity, for example conditional expression of Cas9 by an inducible promoter, fusion of Cas9 with an estrogen receptor domain (ERT2) that sequester Cas9 to the cytoplasm in the absence of 4-HT, control of the Cas9 enzymatic activity via self-splicing properties of inteins, control of Cas9 activity by stimulating with blue light through fusion with light-responsive elements and use of self-restricted CRISPR/Cas9 system [17].

Another barrier to the use of CRISPR/Cas9 technology in therapeutic approach is the lower HDR efficiency than NHEJ, however, so far several approaches for increasing HDR rate have been explored [16]. By solving current challenges and improving safety and delivery methods, the CRISPR-Cas9 system can be applied for clinical applications in patients. Recently this system has been approved by advisory committee at the US National Institute of Health as the first clinical trial to attack cancer cells [24].

## 4. Why CAR-T cell therapy?

Conventional therapies have several restrictions to become reliable and to produce lasting outcome in patients with advanced, relapsed and refractory malignancies, therefore the development of new therapeutic

approaches with suitable outcomes are critical to successful cancer treatment. Immune system, particularly T lymphocytes has a critical role in identification of tumor-specific antigens, therefore harnessing the power of the patient's immune system to eradicate cancerous cells has been regarded in the scope of immunotherapy. Adoptive T cell transfer (ACT), a type of immunotherapy, involving the ex vivo expansion of fragments of resected tumors or genetically engineered patients' derived T cells and sending them back to the patients for eliminating tumor cells, divided into three scopes: tumor-infiltrating lymphocytes (TILs), T cell receptor (TCR) -engineered T cells, and chimeric antigen receptor (CAR) -modified T cells [25]. There are 2 major T cell sources that can be used in the ACT, including tumor-infiltrating lymphocytes (TILs) and peripheral blood T lymphocytes that engineered to express alpha/beta T-cell receptors (TCRs) or chimeric antigen receptors (CARs). In TIL therapy approach antigen-specific tumor-infiltrating lymphocytes (TILs) are derived from tumor tissue and cultured with lymphokines such as interleukin-2 and then these TILs infused into the patient to induce lysis of tumor cells and tumor regression. Adoptive T-cell therapy (ACT) using TILs is a powerful immunotherapeutic approach against metastatic melanoma, but several important limitations exist in TIL therapy: first the requirement to perform an invasive method to gain tumor tissue to grow TILs, second the difficulty in identifying antigen-specific T cells in other cancer types, and third failure to grow TILs in ~10% to 15% of patients with melanoma [26,27]. The ability to modify lymphocyte genes to induce expression of the desired T-cell receptor (TCR) or chimeric antigen receptor can circumvent these limitations. Chimeric antigen receptor/T cell receptor (CAR/TCR) engineered T cells are autologous T-cells that harbor a transgene encoding antigen receptors (CARs) or genetically engineered T cell to express modified T-cell receptors (TCRs) chain that will elevate antigen affinity [28]. Both CAR- and TCR-engineered T cells have unique advantages and disadvantages to each other. Engineered TCRs recognize peptides derived from either cell surface or intracellular proteins in an HLA-dependent manner that provide the option to target intracellular tumor antigen. However, restriction to HLA also has been a limitation. CAR T cell contrary to TCRs operates in an MHC-independent manner. Engineered TCRs recognize MHC restricted peptides taken from cell surface as well as intracellular proteins and transcription factors, so provides access to a greater number of targets than CARs, however CAR-T cells also can be develop against carbohydrate and glycolipid antigens to target malignant cells expressing these antigens via the activation of cytoplasmic co-stimulation [29–31]. The CAR is a single chimeric protein that artificially made by fusing an extracellular antigen-recognition moiety, single-chain variable fragment (scFv) antibody is frequently used for the antigen-recognition domain also human Fab fragments and natural ligands that engage their cognate receptor can be used, to one or several intracellular T-cell signaling and costimulatory domains [32,33]. CAR T-cells divided into four generations, the first generation has used a single chain antibody such as CD3ζ or FcεRIγ that links the immunoreceptor tyrosine-based activation motifs (ITAM) at transmembrane region, second and third generations include costimulatory molecule such as CD28, CD137(4-1BB), CD134(OX40), CD27, DAP10 and CD244 that can ameliorate proliferation, cytotoxicity, sustain response, and prolong the life of CAR-T cells in vivo (the second generation has one costimulatory molecule and the third generation has two costimulatory molecules). The fourth-generation CARs were generated by adding a second factor, including cytokines or ligands such as IL-12, IL-18, CD40L and 4-1BBL etc. to the foundation of the second-generation constructs to ameliorate efficacy and durability [34–36]. CAR-T cell therapy as one of the most novel and successful agents in cancer immunotherapy entered into the clinic and recent significant responses in clinical trial in B-cell malignancies have generated great enthusiasm to the ultimate smart cancer therapeutics which turned on the lights of hope in cancer patients. After showing complete responses in refractory ALL and CLL patients using a CD19-directed CAR T cell product, US Food and Drug Administration (FDA) recognized CAR-T cell therapy as a "breakthrough therapy" and approved it to treat leukemia and lymphoma which opened the path to commercializing CAR-T cell by a number of pharmaceutical and biotechnology companies [37,38].

## 5. Combined use of CRISPR-Cas9 and CAR-T cell in cancer immunotherapy

Conventional CARs in the majority of CAR T-cell clinical trials derived from patient's own autologous T cells, however, the generation of these autologous CAR T-cells have some limitation, including, time-consuming and expensive process, difficulty in collecting T cells with good quality and quantity in patients with crucial diseases that limit the use of these modified T cells to a low number of patients. These limitations have led to the idea of making universal or off-the-shelf T cells, genetically modified allogeneic T cells derived from healthy donors, that have the potential to overcome these limitations, simplify the manufacturing of engineered CAR-T cells and available for many number of patients. Universal CAR-T cell achievement requires passing from two main obstacles, graft-versus-host disease (GVHD), rejection of the infused allogeneic T cells in the recipient, and improved biosafety profile for more powerful disease-targeted activity. The endogenous αβ T-cell receptors (TCRs) on allogeneic T cells that responsible for antigen recognition may recognize recipient alloantigens and result in GVHD. The presence of intrinsic human leukocyte antigen (HLA) molecules on the allogeneic CAR-T cells that recognized as foreign HLA molecules, usually triggers rapid immunologic rejection in recipients and prevents their application in allogeneic setting as the off-the-shelf CAR-T cells. To solve these obstacles, researchers investigated ways to silence or disrupt both TCRs and HLA molecules in allogeneic universal T cells and as mentioned above, CRISPR/Cas9 technology because of the possibility of highly effective multiplex gene editing at a time, simplicity to use and high pliability can be used for this aim. In several studies CRISPR/Cas9 mediated multiplex knock out of TCR beta chain and beta-2-microglobulin (B2M), an essential subunit of the HLA-I molecule, have been used in the production of universal CAR-T cells. Results show these universal cells retain function both in vitro and in vivo without causing GVHD [39–41]. Knocking out B2M and the production of HLA-I negative CAR T-cells, leading to a problem in this CAR T-cells. HLA-I negative CAR T-cells are potential target for Nk cells that would lead to the rejection of theses universal T cells. There are some approaches to circumvent this problem, including applying an engineered T cell that express HLA-E or anti-NK cell depletion antibody [42].

T cell inhibitory receptors or signaling molecules, such as CTLA-4, PD-1, LAG-3, and TIM-3 are naturally occurring "off signals" to ensure proper control of T cell response. The expression of these inhibitory receptors on CAR-T cells leads to T cell exhaustion. Recent studies showed tumor cells use this characteristic for immune evasion, for example, tumor cells upregulated PD-1 ligand that causes reduced immune responses [43]. Therapeutic approaches specifically designed to target and inhibit these inhibitory receptors by immune checkpoint related antibodies, such as anti-PD-1, PD-L1 and CTLA-4, displayed great success in the treatment of solid tumors in addition to hematologic malignancies [44–46]. The reported successes of inhibiting these inhibitory signals by related antibodies have led to the use of CRISPR/Cas9 technology to destroy them. Studies indicate an improvement in the antitumor efficacy and clinical outcome of these modified CAR-T cells [39,41,47–49]. T cell response, can be mediated by Fas receptor that is a member of the tumor necrosis factor α family of death receptors. This receptor and its ligand (FasL) involved in T cell apoptosis that can attenuate CAR T cell activity because of cell Fas/FasL-dependent activation induced cell death (AICD). Therefore, ablating Fas induced cell death by knocking out the Fas receptor by CRISPR/Cas9 can result in the generation of CAR T cells that are resistant to apoptosis and enhances CAR T cell function [41].

In a new manner, recently, CRISPR technology was used to target

TRAC locus. In this approach CRISPR/Cas technology used as a delivery system to knock in a CD19-specific CAR into TRAC locus that placed the CAR expression under control of TCR promoter and caused enhanced potency, uniformed CAR expression, more memory characteristics and less exhausted phenotype in this CAR [50].

In summary, studies that used CRISPR/Cas9 technology to modify CAR T cells indicate that this technology is a highly efficient approach to generate genetically modified CAR T cells with a targeting efficiency about 90% for single gene disruption (the efficiency decreased as the number of targeted genes increased, maybe due to the competition of the gRNAs for Cas9) without impairing effector function a pure population of CAR T cells could be achieved by enriching the genetically modified T cells.

As above mentioned, CRISPR/Cas9 can be easily used for silencing or disrupting any desired genomic locus, so, the combination of this technology with CAR-T cell therapy can be an instruction for successful cancer treatment and as mentioned in the next section several companies invest in the improvement of CAR-T cell therapy by CRISPR/Cas9 technology.

## 6. Big biotech companies in commercializing therapeutic approach

Conventional autologous CAR T-cell therapy has some limitations and high cost, but the idea of universal allogeneic CAR T-cells that sourced from a healthy donor and undergo gene editing changed the condition and several big companies have invested in this field. Indeed, CRISPR technology by realizing the dream of manipulating the genome opened a new vista in the treatment of human disease. Unlike traditional genome-editing technologies that have had limited use due to design complexity, transfection inefficiencies and limitations in ability to multiplex gene editing, the emergence of CRISPR technology results in a significant enhance in genome editing investment in the last years because of its extraordinary potential in genome editing, simplicity and low cost [51]. Editas Medicine (Editas medicine. 2018), Intellia Therapeutics (Intellia Therapeutics. 2018) and CRISPR Therapeutics (CRISPR Therapeutics. 2018) are three big biotech companies that invested in CRISPR-based gene-editing and play a major role in this field. Intellia established in 2014 by Caribou Biosciences to expand the Caribou CRISPR-Cas9 platform and in January 2015, Intellia initiated collaboration with Novartis to explore options for using CRISPR to engineer chimeric antigen receptor (CAR) T cells and hematopoietic stem cells (HSCs). Intellia Therapeutics uses in vivo approaches for targeting liver diseases, including transthyretin amyloidosis, alpha-1 antitrypsin deficiency, hepatitis B virus, and inborn errors of metabolism. Its ex vivo approaches include chimeric antigen receptor T cells and hematopoietic stem cells. Editas Medicine targets diverse range of diseases including LCA10, β-thalassemia, SCD, DMD, AATD, CF and cancer (chimeric antigen receptor/T cell receptor (CAR/TCR) engineered T cells). In May 2015, Editas Medicine entered into collaboration with Juno Therapeutics (a leader in the emerging field of immuno-oncology) to ameliorate the efficacy and safety of Juno's chimeric antigen receptor/T cell receptor (CAR/TCR) engineered T cells by its own CRISPR platform. CRISPR Therapeutics develops CTX101, a donor-derived allogeneic anti-CD19 CAR-T cell for targeting CD19-positive malignancies and also anti-CD70 CAR-T cell for both hematologic malignancies and solid tumors. Anti-B-cell maturation antigen (BCMA) for multiple myeloma. Due to the potential of CRISPR/Cas9 technology to target any desire locus in the genome and abundance of genetic based diseases, including monogenic disease, and common diseases such as cancer, CRISPR market worth's billion dollars globally.

## 7. Conclusion and outlooks

The rapidly growing field of immunotherapy has created many hopes in the treatment of cancer and CAR-T19 has recently achieved FDA approval. So, development of this therapeutic approach has attracted much attention nowadays. Furthermore, CRISPR-Cas9 technology has opened a new vista to genome editing and its utilization in the engineering of T cells provides an extraordinary potential to build CAR-T cell and streamlined the emerging field of immunotherapy. But several technical challenges still need to be solved regarding accuracy, safety and efficiency issues of this technology. After solving these current challenges and improvement of the delivery methods, the CRISPR-Cas9 system can apply for clinical applications in patients.

## Acknowledgement

Authors would like to thank Mr. Mohsen Mollanoori for Figures drawings.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2018.09.007.

## References

[1] D.L. Porter, B.L. Levine, M. Kalos, A. Bagg, C.H. June, Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia, N. Engl. J. Med. 365 (8) (2011) 725–733.
[2] J.N. Kochenderfer, W.H. Wilson, J.E. Janik, M.E. Dudley, M. Stetler-Stevenson, S.A. Feldman, et al., Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19, Blood 116 (20) (2010) 4099–4102.
[3] S. Teimourian, R. Abdollahzadeh, Technology developments in biological tools for targeted genome surgery, Biotechnol. Lett. 37 (1) (2015) 29–39.
[4] P. Mali, K.M. Esvelt, G.M. Church, Cas9 as a versatile tool for engineering biology, Nat. Meth. 10 (10) (2013) 957–963.
[5] P. Horvath, R. Barrangou, CRISPR/Cas, the immune system of bacteria and archaea, Science (New York, NY). 327 (5962) (2010) 167–170.
[6] B. Wiedenheft, S.H. Sternberg, J.A. Doudna, RNA-guided genetic silencing systems in bacteria and archaea, Nature 482 (7385) (2012) 331–338.
[7] P. Mali, L. Yang, K.M. Esvelt, J. Aach, M. Guell, J.E. DiCarlo, et al., RNA-guided human genome engineering via Cas9, Science (New York, NY). 339 (6121) (2013) 823–826.
[8] H. Wang, H. Yang, C.S. Shivalila, M.M. Dawlaty, A.W. Cheng, F. Zhang, et al., One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering, Cell 153 (4) (2013) 910–918.
[9] P.D. Hsu, E.S. Lander, F. Zhang, Development and applications of CRISPR-Cas9 for genome engineering, Cell 157 (6) (2014) 1262–1278.
[10] J.A. Doudna, E. Charpentier, Genome editing. The new frontier of genome engineering with CRISPR-Cas9, Science (New York, NY) 346 (6213) (2014) 1258096.
[11] F. Jiang, J.A. Doudna, CRISPR-Cas9 Structures and Mechanisms, Annu. Rev. Biophys. 46 (2017) 505–529.
[12] T. Koo, J.S. Kim, Therapeutic applications of CRISPR RNA-guided genome editing, Brief. Funct. Genom. 16 (1) (2017) 38–45.
[13] I. Fonfara, A. Le Rhun, K. Chylinski, K.S. Makarova, A.L. Lecrivain, J. Bzdrenga, et al., Phylogeny of Cas9 determines functional exchangeability of dual-RNA and Cas9 among orthologous type II CRISPR-Cas systems, Nucl. Acids Res. 42 (4) (2014) 2577–2590.
[14] F.A. Ran, P.D. Hsu, J. Wright, V. Agarwala, D.A. Scott, F. Zhang, Genome engineering using the CRISPR-Cas9 system, Nat. Protoc. 8 (11) (2013) 2281–2308.
[15] J.A. Soppe, R.J. Lebbink, Antiviral goes viral: harnessing crispr/cas9 to combat viruses in humans, Trends Microbiol. 25 (10) (2017) 833–850.
[16] H. Mollanoori, S. Teimourian, Therapeutic applications of CRISPR/Cas9 system in gene therapy, Biotechnol. Lett. 40 (6) (2018) 907–914.
[17] S. Chira, D. Gulei, A. Hajitou, A.-A. Zimta, P. Cordelier, I. Berindan-Neagoe, CRISPR/Cas9: transcending the reality of genome editing, Mol. Therapy Nucl. Acids 7 (2017) 211–222.
[18] Y. Fu, J.D. Sander, D. Reyon, V.M. Cascio, J.K. Joung, Improving CRISPR-Cas nuclease specificity using truncated guide RNAs, Nat. Biotechnol. 32 (3) (2014) 279–284.
[19] B.P. Kleinstiver, M.S. Prew, S.Q. Tsai, V.V. Topkar, N.T. Nguyen, Z. Zheng, et al., Engineered CRISPR-Cas9 nucleases with altered PAM specificities, Nature 523 (7561) (2015) 481–485.
[20] B.P. Kleinstiver, V. Pattanayak, M.S. Prew, S.Q. Tsai, N. Nguyen, Z. Zheng, et al., High-fidelity CRISPR-Cas9 variants with undetectable genome-wide off-targets, Nature 529 (7587) (2016) 490–495.
[21] I.M. Slaymaker, L. Gao, B. Zetsche, D.A. Scott, W.X. Yan, F. Zhang, Rationally engineered Cas9 nucleases with improved specificity, Science (New York, NY). 351 (6268) (2016) 84–88.
[22] F.A. Ran, P.D. Hsu, C.Y. Lin, J.S. Gootenberg, S. Konermann, A.E. Trevino, et al., Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity, Cell 154 (6) (2013) 1380–1389.

[23] N. Wyvekens, V.V. Topkar, C. Khayter, J.K. Joung, S.Q. Tsai, Dimeric CRISPR RNA-guided FokI-dCas9 nucleases directed by truncated gRNAs for highly specific genome editing, Hum. Gene Ther. 26 (7) (2015) 425–431.
[24] S. Reardon, First CRISPR clinical trial gets green light from US panel, Nature 531 (2016).
[25] C. Yee, Adoptive T cell therapy: points to consider, Curr. Opin. Immunol. 51 (2018) 197–203.
[26] S.L. Goff, F.O. Smith, J.A. Klapper, R. Sherry, J.R. Wunderlich, S.M. Steinberg, et al., Tumor infiltrating lymphocyte therapy for metastatic melanoma: analysis of tumors resected for TIL, J. Immunother. 33 (8) (2010) 840–847 Hagerstown, Md : 1997.
[27] H. Shi, L. Liu, Z. Wang, Improving the efficacy and safety of engineered T cell therapy for cancer, Cancer Lett. 328 (2) (2013) 191–197.
[28] M.B. Geyer, R.J. Brentjens, Review: current clinical applications of chimeric antigen receptor (CAR) modified T cells, Cytotherapy 18 (11) (2016) 1393–1409.
[29] G. Gross, T. Waks, Z. Eshhar, Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity, PNAS 86 (24) (1989) 10024–10028.
[30] A. Morello, M. Sadelain, P.S. Adusumilli, Mesothelin-targeted CARs: driving T cells to solid tumors, Cancer Discov. 6 (2) (2016) 133–146.
[31] D.T. Harris, D.M. Kranz, Adoptive T cell therapies: a comparison of T cell receptors and chimeric antigen receptors, Trends Pharmacol. Sci. 37 (3) (2016) 220–230.
[32] Y. Pang, X. Hou, C. Yang, Y. Liu, G. Jiang, Advances on chimeric antigen receptor-modified T-cell therapy for oncotherapy, Mol. Cancer 17 (1) (2018) 91.
[33] M. Sadelain, R. Brentjens, I. Riviere, The basic principles of chimeric antigen receptor design, Cancer Discov. 3 (4) (2013) 388–398.
[34] C. Zhang, J. Liu, J.F. Zhong, X. Zhang, Engineering CAR-T cells, Biomarker Res. 5 (2017).
[35] N. Muhammad, Q. Mao, H. Xia, CAR T-cells for cancer therapy, Biotechnol. Genet. Eng. Rev. 33 (2) (2017) 190–226.
[36] O.O. Yeku, R.J. Brentjens, Armored CAR T-cells: utilizing cytokines and pro-inflammatory ligands to enhance CAR T-cell anti-tumour efficacy, Biochem. Soc. Trans. 44 (2) (2016) 412–418.
[37] S.S. Neelapu, F.L. Locke, N.L. Bartlett, L.J. Lekakis, D.B. Miklos, C.A. Jacobson, et al., Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma, N. Engl. J. Med. 377 (26) (2017) 2531–2544.
[38] S.L. Maude, N. Frey, P.A. Shaw, R. Aplenc, D.M. Barrett, N.J. Bunin, et al., Chimeric antigen receptor T cells for sustained remissions in leukemia, N. Engl. J. Med. 371 (16) (2014) 1507–1517.
[39] X. Liu, Y. Zhang, C. Cheng, A.W. Cheng, X. Zhang, N. Li, et al., CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells, Cell Res. 27 (1) (2017) 154–157.
[40] J. Ren, X. Liu, C. Fang, S. Jiang, C.H. June, Y. Zhao, Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition, Clin. Cancer Res. 23 (9) (2017) 2255–2266.
[41] J. Ren, X. Zhang, X. Liu, C. Fang, S. Jiang, C.H. June, et al., A versatile system for rapid multiplex genome-edited CAR T cell generation, Oncotarget 8 (10) (2017) 17002–17011.
[42] X. Liu, Y. Zhao, CRISPR/Cas9 genome editing: fueling the revolution in cancer immunotherapy, Curr. Res. Transl. Med. 66 (2) (2018) 39–42.
[43] E.J. Wherry, M. Kurachi, Molecular and cellular insights into T cell exhaustion, Nat. Rev. Immunol. 15 (8) (2015) 486–499.
[44] D.P. Carbone, M. Reck, L. Paz-Ares, B. Creelan, L. Horn, M. Steins, et al., First-line nivolumab in stage IV or recurrent non-small-cell lung cancer, N. Engl. J. Med. 376 (25) (2017) 2415–2426.
[45] A.M.M. Eggermont, V. Chiarion-Sileni, J.-J. Grob, R. Dummer, J.D. Wolchok, H. Schmidt, et al., Prolonged survival in stage III melanoma with ipilimumab adjuvant therapy, N. Engl. J. Med. 375 (19) (2016) 1845–1855.
[46] S.M. Ansell, A.M. Lesokhin, I. Borrello, A. Halwani, E.C. Scott, M. Gutierrez, et al., PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma, N. Engl. J. Med. 372 (4) (2015) 311–319.
[47] L.J. Rupp, K. Schumann, K.T. Roybal, R.E. Gate, C.J. Ye, W.A. Lim, et al., CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells, Sci. Rep. 7 (1) (2017) 737.
[48] Y. Liao, L. Chen, Y. Feng, J. Shen, Y. Gao, G. Cote, et al., Targeting programmed cell death ligand 1 by CRISPR/Cas9 in osteosarcoma cells, Oncotarget 8 (18) (2017) 30276–30287.
[49] Y. Zhang, X. Zhang, C. Cheng, W. Mu, X. Liu, N. Li, et al., CRISPR-Cas9 mediated LAG-3 disruption in CAR-T cells, Front. Med. 11 (4) (2017) 554–562.
[50] J. Eyquem, J. Mansilla-Soto, T. Giavridis, S.J.C. van der Stegen, M. Hamieh, K.M. Cunanan, et al., Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection, Nature 543 (7643) (2017) 113–117.
[51] K. Brinegar, K.A. Yetisen, S. Choi, E. Vallillo, G.U. Ruiz-Esparza, A.M. Prabhakar, et al., The commercialization of genome-editing technologies, Crit. Rev. Biotechnol. 37 (7) (2017) 924–932.