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REVIEW ARTICLE

CAR T-cell Therapy: A New Era in Cancer Immunotherapy

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Abstract: ***Background***: Cancer is one of the leading causes of death worldwide. Over the years, a number of conventional cytotoxic approaches for neoplastic diseases has been developed. However, due to their limited effectiveness in accordance with the heterogeneity of cancer cells, there is a constant search for therapeutic approaches with improved outcome, such as immunotherapy that utilizes and enhances the normal capacity of the patient's immune system.

Methods: Chimeric Antigen Receptor (CAR) T-cell therapy involves genetic modification of patient's autologous T-cells to express a CAR specific for a tumor antigen, following by *ex vivo* cell expansion and re-infusion back to the patient. CARs are fusion proteins of a selected single-chain fragment variable from a specific monoclonal antibody and one or more T-cell receptor intracellular signaling domains. This T-cell genetic modification may occur either *via* viral-based gene transfer methods or non-viral methods, such as DNA-based transposons, CRISPR/Cas9 technology or direct transfer of *in vitro* transcribed-mRNA by electroporation.

Results: Clinical trials have shown very promising results in end-stage patients with a full recovery of up to 92% in Acute Lymphocytic Leukemia. Despite such results in hematological cancers, the effective translation of CAR T-cell therapy to solid tumors and the corresponding clinical experience is limited due to therapeutic barriers, like CAR T-cell expansion, persistence, trafficking, and fate within tumors.

Conclusion: In this review, the basic design of CARs, the main genetic modification strategies, the safety matters as well as the initial clinical experience with CAR T-cells are described.

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1. INTRODUCTION - CANCER IMMUNOTHERAPY

Conventional cytotoxic approaches for neoplastic diseases have only modest efficacy in treating cancer of advanced stage. Treatment responses vary considerably among patients and a high relapse rate with poor prognosis continues to be a major challenge. It is thus interesting that immunotherapy has emerged as a challenging approach, altering the face of cancer treatment during the last decades. Immunotherapy utilizes and enhances the normal capacity of the immune system and is considered one of the most promising approaches for the treatment of various serious diseases (including cancer, autoimmune diseases, allergic - hypersensitivity reactions) [1].

The idea of redirecting the immune response to fight cancer is based on the knowledge that cancer progresses *via* close interaction between tumor cells and the immune system. Every known immune mechanism is involved in the recognition and elimination of tumor cells in physiological conditions. This can be explained by the cancer immunoediting theory of the three Es stages: Elimination, Equilibrium, Escape. Cancer immunoediting is an extrinsic tumor suppressor mechanism that engages only after the cellular transformation has occurred and intrinsic tumor suppressor mechanisms have failed. The stage of neoplastic cell "Elimination" refers to mechanisms of both innate and adaptive immunity, combined to destroy developing tumors long before they become clinically visible. The first phase of the "Elimination" stage is the identification of cancer cells by the mechanisms of natural immunity. When the tumors grow more than 2-3 mm, the nutritional requirements induce angiogenic mechanisms and layer remodeling, which in turn cause secretion of cytokines and attract NK cells, γδT-cells, macrophages and dendritic cells to the tumor. If these phases go to completion, then the host remains free of cancer and thus "Elimination" stage represents the full extent of the im-

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munoediting process. If, however, a rare cancer cell variant survives in the "Elimination" stage, it may then enter the "Equilibrium" stage, in which its outgrowth is prevented by immunologic mechanisms. Cancer cells have a vast evolutionary potential, owing to their rapid proliferation and lack of proper DNA damage control, and eventually develop effective immune evasion strategies. Any tumor cell that survived "Elimination" stage as well as the host immune system enter the dynamic "Equilibrium" stage during which the immune system continues to exert a selection pressure on tumor cells but not enough to fully extinguish them. As a result, a tumor bed (containing many genetically unstable and mutating tumor cells) is developed and exerts reduced immunogenicity. "Equilibrium" is the longest of the three stages and may occur up to 20 years, between initial transforming event to the clinical detection of the tumor. In the "Escape" phase, the genetic and epigenetic changes in the tumor cell confer resistance to immune detection and/or elimination, allowing the tumors to expand and become clinically detectable. The breach of the host's immune system can result from alterations that affect tumor recognition [downregulation or loss of expression of classical Major Histocompatibility Complex (MHC) class I: HLA-A, HLA-B and HLA-C] or development of escaping mechanisms for immune destruction (production of cytokines, causing apoptosis of activated T-cells) [2]. Cancer immunoediting and the "Three Es theory" describe the relationship between the tumor cells and the immune system, which is the key for understanding all the processes that lead to immunologic tumor rejection and therefore for identifying which immune system's compartments need to be enhanced to facilitate natural protection against tumors [2]. Immunotherapy has the potential to make cancer cells "visible" to the immune system again, triggering immunity-mediated control of cancer, either passively or actively, thus offering direct cytolysis of cancer cells [3].

Adoptive T-cell therapy (ACT) was developed in order to treat advanced cancer with a patient's own T-cells and it has been established over many years through the *ex vivo* manipulation, expansion and reinfusion of T-cells. The first step forward came in 1980, when Rosenberg's team described a novel method for generating large numbers of autologous lymphoid cells capable of lysing fresh, non-cultured, primary and metastatic cancer cells [4]. Lymphokine-Activated Killer Cells, also known as LAK cells, are lymphocytes that in the presence of Interleukin-2 (IL-2) are stimulated to kill tumor cells. The systemic administration of both autologous LAK cells and recombinant IL-2 mediated the regression of established pulmonary and hepatic metastases from patients with metastatic cancer, melanoma, colon cancer or renal-cell cancer and in one patient with a primary lung adenocarcinoma [4]. Shortly after, Rosenberg and colleagues showed that a subpopulation of antigen-specific T-cells, which infiltrate tumors, can cause regression of a variety of advanced metastatic tumors in mouse models [5]. Such rare populations of tumor-antigen-specific T-cells, isolated at the site of the tumor, are known as tumor infiltrating lymphocytes (TILs) [6]. TILs can be isolated from patient's tumor tissue, cultivated, activated and expanded *in vitro* in the presence of IL-2, and then reinfused back to the patient, showing promising efficacy in the treatment of melanoma in the clinic [7]. This therapy was mainly developed to treat viral infections (such as cytomegalovirus or Epstein Barr virus infections). TIL therapy had remarkable results in patients with renal cancer, too, in the presence of pre-conditioning chemotherapy, but had lower efficacy in large clinical trials (~7% complete response) [8]. In the meantime, a cell-based adoptive immunotherapy was developed using a heterogeneous cell population generated from lymphocytes, co-cultured with an anti-CD3 antibody and many other cytokines *in vitro*, showing antitumor cytotoxicity against multifarious tumor cells *in vitro* and *in vivo*. These cells, known as cytokine-induced killer (CIK) cells, were first discovered in the 1990s and their effective action relied on a perforin-based mechanism and Fas/Fas ligand interactions [9, 10].

The effectiveness of cancer immunotherapy approaches is based on antigen specificity of T-cells. This specificity can be enhanced by the genetic modification and redirection of T-cells to target antigens that are overexpressed in tumors. Patient's own T-cells can be engineered to express modified TCRs (so-called TCR therapies) or Chimeric Antigen Receptors (CARs) that will enhance antigen specificity. These approaches overcome the fundamental limitations associated with central and peripheral tolerance, generating T-cells more efficient at targeting tumors without the requirement for the *de novo* T-cell activation in the patient [11].

This review emphasizes on CAR T-cell therapy, a promising approach to immunotherapy by engineering patients' own lymphocytes to express CARs, in order to treat advanced cancers, thus yielding promising results in clinical trials.

2. CAR T-CELL THERAPY DESIGN

CAR T-cell therapy depends on efficient, stable and safe gene transfer platforms. Autologous T-cells, isolated through leukapheresis, are harvested and genetically modified *ex vivo*, using viral and non-viral transfection methods. Modified T-cells are then expanded *in culture*. When the CAR T-cell product is prepared and passed all the quality control testing, the patient in most cases receives lymphodepleting chemotherapy, following by CAR T-cell infusion. The first chimeric receptor was designed in 1989 by Eshhar's group at the Weizmann Institute of Science in Israel [12].

The extracellular domain of the CAR consists of the antigen binding moiety and a spacer. These antigen binding moieties could be: a) a scFv (single-chain fragment variable), derived from antibodies; b) a human Fab fragment, selected from phage display libraries; or c) nature ligands that engage their cognate receptor [13]. More specific, the scFv is a variable monoclonal antibody fragment, derived from mouse monoclonal antibodies (mAbs), humanized Abs or fully human Abs and it is responsible for recognizing and binding to tumor-associated antigens (TAAs), expressed on the tumor cell surface.

CARs recognize unprocessed antigens, as well as carbohydrate and glycolipid structures, typically expressed on the cell surface of a tumor cell, without the requirement of antigen presentation through the MHC [9], in contrast to normal TCRs. By bypassing MHC class I and class II restriction, CAR T-cells of both CD8+ and CD4+ subsets can be recruited for redirected recognition of the target cell. The

mechanism of CAR-mediated tumor elimination by redirected CD4+ and CD8+ T-cells, predominantly use at least two pathways in executing cytolysis, *e.g*. perforin and granzyme exocytosis and to some extent death receptor signaling *via* Fas/Fas-ligand (Fas-L) or TNF/TNF-receptor (TNF-R) [14].

The simplest form of spacer is the hinge region of IgG1 and is sufficient for most scFv-based constructs [15]. A spacer is the connection between the antigen binding domain and the transmembrane domain (TM). This TM domain is connected with an intracellular signaling moiety. The most stable receptor is the CD28 TM. The most common component of the intracellular domain is CD3ζ shown to deliver the fisrt signal for T-cell activation and function. Concomitant co-stimulatory signals (CD28 or 4-1BB) are needed as the second signal, critical for increased secretion of cytokines (IL-2) and the *in vivo* expansion and persistence of T-cell [15, 16]. The intracellular signaling domain has been extensively evaluated both preclinically and clinically and can greatly affect the functional activity of CARs [17]. Since the initial development of CARs in 1989, CAR T-cells can be divided into four generations according to the structure of the intracellular domain [15].

First generation CARs comprised of the ζ chain of complex TCR/CD3 (CD3ζ). Second generation CARs are characterized by the dual signal for T-cell activation: one triggered by the antigen recognition and another produced by a co-stimulatory molecule, such as CD28/B7, which promotes the IL-2 synthesis to complete the activation of T-cells and avoid apoptosis [15]. Third generation CARs achieved enhanced responses by combining sequences of co-stimulatory signals, such as OX40 (CD134), CD28, 4-1BB (CD137), CD27, DAP10 or other molecules, in combination with CD3ζ [18, 19]. The combination of multiple co-stimulatory signals may enhance CAR T-cell function *via* increased cytokine production, T-cell proliferation and killing in the setting of recursive exposure to antigen [17]. However, these treatments have not improved the patients' outcomes relative to those with second generation CARs (small number of cases studied). More studies are needed to explore the safety and efficacy of third-generation CARs [13].

Many reports suggest the further optimized design of CARs, such as CAR T-cells redirected for universal cytokine killing (TRUCK). TRUCK cells produce and then release a transgenic product, such as IL-12 or IFN-γ [20]. IL-12 can activate innate immune responses against tumor cells, invisible to CAR T-cells, while IFN-γ can contribute to the antigen-independent destruction of tumor cells through IFN-γR, which is expressed in the tumor stroma [20, 21] (Fig. **1**).

The design of a biphasic CAR (tandem CAR - TanCAR), a single transgenic receptor which recognizes two distinct antigens, offers synergistic killing and enhanced function. The recognition domains for the two different antigens are in tandem and separated by a flexible hinge. This strategy enables bypassing antigen loss and tumor escaping; if one target antigen is downregulated or mutated, TanCAR is still functional and preserves the cytolytic ability of T-cells [22]. In order to achieve improved tumor specificity, Wilkie, *et al*. and Kloss, *et al*. proposed dual specific CARs: the co-expression of two different CARs in the same T-cell population, each recognizing a different tumor antigen and providing complementary signals. This strategy could be used as "tumor barcoding"; only double-antigen positive tumors are killed. These CAR T-cells include a CAR that provides suboptimal CD3ζ-mediated activation upon binding of one antigen and a chimeric co-stimulatory receptor, containing only CD28 and 4-1BB, that recognizes a second antigen. This provides CAR T-cell specificity and prevents off-target effects, ensuring complete T-cell activation as soon as it meets both CARs' targets [23, 24]. In addition to antigen-specific approaches, two "universal" CAR systems have been reported. These systems include CARs with scFv for avidin [25] or antifluorescein isothiocyanate (FITC) [26], which ensure the identification of tumors associated with, biotinylated or bound by FITC, mAbs.

T-cells that recognize antigen on both tumor and off-target tissues can be restricted to tumor only by using an antigen-specific inhibitory CAR (iCAR), introduced into the T-cells to protect the off-target tissues. iCARs provide a dynamic, self-regulating safety switch, to prevent rather than treat, the consequences of inadequate T-cell specificity. These T-cells, beside the tumor-antigen targeting CAR, have a second CAR, named iCAR, targeting a different, off-target, tissue antigen combined with an intracellular strong acute inhibitory signaling domain, based on PD-1 or CTLA-4 molecules. These cells can selectively limit cytokine secretion, cytotoxicity and proliferation, following their interaction with the off-target tissue antigen [27-29].

The combinational strategies with CAR T-cell therapy and checkpoint inhibitor blockade, using antagonistic antibodies against the negative regulators CTLA-4 and PD-1/PD1-L, have a great potential. It has been demonstrated that the specific blockade of the PD-1 immunosuppressive pathway significantly enhanced the function of anti-HER2 CAR T-cells, leading to tumor eradication in immune competent HER2 transgenic mice [30].

3. GENETIC ENGINEERING OF T-CELLS.

Genetic engineering methods have been upgraded, since 1970, from simple physical-chemical laboratory methods to viral and non-viral transfection methods, trying to achieve high transgene expression with less toxic or oncogenic adverse effects. This review describes the basic design of CAR-engineered cells with multiple gene transfer methods applied in clinical practice, including viral transduction, transposons and mRNA transfection methods as well as nanoparticles, liposomes, electroporation or using CRISPR/Cas9 technology. Advantages and drawbacks of these methods are described, emphasizing to long-last transgene expression with fewer safety concerns.

3.1. Viral Transduction

Viral transductions are currently the preferred procedures to equip T-cells with CARs, including retroviruses (lentivirus and γ-retrovirus), adenovirus and adeno-associated virus. Viral vectors of the family "*Retroviridae*" are now the most commonly used vectors for gene therapy applications. Major advantages of viral gene transfer vectors are the relative ease of manufacturing and production as well as their capacity to stably integrate the genetic material into the host genome. In

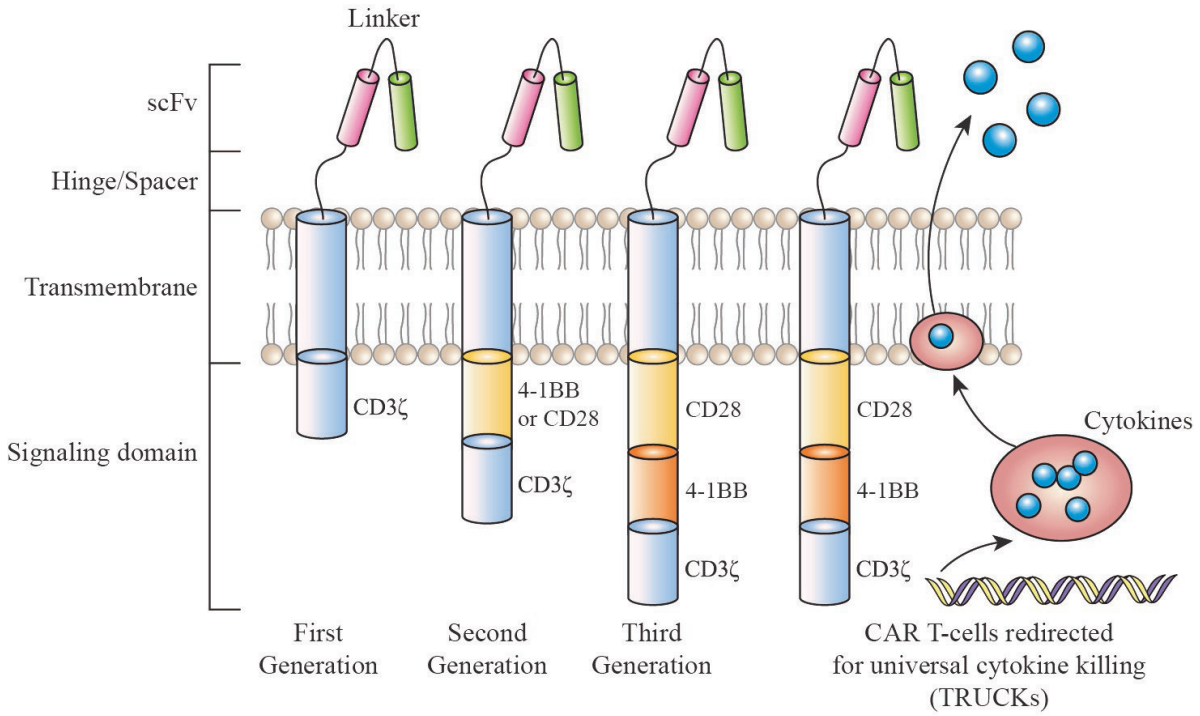

Fig. (1). Schematic representation of chimeric antigen receptor (CAR) structure. CARs' extracellular domain consists of the scFv from a monoclonal antibody, which recognizes a tumor-associated antigen (TAA). Various hinges and TM domains are used to link the recognition domain with the intracellular signaling molecules. While first-generation CARs signaled through the CD3ζ chain only, second-generation CARs further include a signaling domain from a co-stimulatory molecule, for example, CD28 or 4-1BB (illustrated). Third generation CARs incorporate two co-stimulatory signaling domains in tandem with the CD3ζ chain. TRUCK cells are engineered to secrete proinflammatory cytokines, such as IL-12, which can activate an innate immune response against the tumor (Art Design by authors).

order to comply with clinical safety standards, viral vector platforms must demonstrate replication incompetence, low genotoxicity and low immunogenicity. Two defining characteristics of retroviruses make them particularly suited to act as vectors for gene transfer: (i) most of the viral genome can be replaced with a transgene or transgenes of interest; (ii) upon transduction, the viral genome is permanently integrated into the host cell genome. For these reasons, simple γ-retroviruses, such as the Moloney murine leukemia virus (Mo-MLV), were the first to be successfully engineered to serve as advanced packaging systems for gene transfer. The most commonly used lentiviral vectors are based on the human immunodeficiency virus (HIV). To generate a CAR-vector, the essential genes *gag, pol* and *env* (plus *rev* for lentivirus) are removed from the viral backbone and they are provided *in trans*, in helper plasmids, for viral production. In place of these viral genes, CAR transgene is introduced. A packaging cell line is transfected with the CAR transgene vector plus the helper plasmids (with *gag, pol* and *env* genes), in order to generate a stable virus-producing cell line for large-scale production. Stimulated T-cells, with OKT3/CD28 beads, are incubated with retroviral particles for genomic integration. Upon the fusion of viral and host membrane, the virion core is released into the cytosol and transported along the microtubules to reach the nucleus. This method permits the generation of T-cells, expressing high levels of CAR. Transduction efficiency of the CAR transgene through viral vectors reach up to 68% for retroviruses, depending on the multiplicity of infection [31, 32].

The long terminal repeats (LTRs) are the viral control center for gene expression, acting as enhancer, promoter, transcription initiation (capping), transcription terminator and polyadenylation signal. Although, 3 ΄LTR and 5 ΄LTR have the same sequence, 3 ΄LTR usually acts in transcription termination and polyadenylation, but not as a promoter. The basis for one form of retroviral oncogenesis relies on the disruption in 5 ΄LTR and the conversion of 3 ΄LTR to a promoter [33]. The security level of these vectors is high nowadays, resulting from the partial deletion of the U3 region of the 3 ΄LTR and the use of the cytomegalovirus (CMV) promoter to replace the U3 region at the 5 ΄LTR to start the transcription. This strategy drastically reduces the transcriptional activity from the LTR of the virus. However, there is still a risk of insertional oncogenesis at random sites within the genome and possible immune-mediated toxicity, caused by long-term persistence and activity of engineered T-cells. There are also restrictions on the size and number of genes that can be packed in these vectors and suboptimal efficacy. In addition, heterogeneous copy numbers can result in T-cell populations with highly variable cytotoxic capabilities, due to different levels of expression on the cell surface.

There are additional manufacturing issues related to viral carriers, which have high production costs. While the scale of viral production was sufficient for Phase I/II clinical trials, cost-effective implementation of the virus-mediated CAR treatment for rapid and broad clinical translation, would be a major barrier.

3.2. Transposons

Transposons are dual mobile genetic elements composed of: (a) one plasmid carrying the CAR (transposon) and (b) another plasmid carrying the transposase. These bi-component vector systems, such as Sleeping Beauty [34] and piggyback [35], can lead to the stable integration of a transgene. The main mechanism of these systems includes the transposase, which acts on the inverted terminal repeats (ITRs) flanking the CAR sequence, thus leading to excision and subsequent integration at a TA dinucleotide sequence in the target cell genome. DNA plasmids carrying the CAR (as the transposon) as well as the transposase are electroporated into T-cells. Following transposition and stable genomic integration, the CAR protein is expressed on the surface of T-cell. Transposon-mediated CAR therapy is substantially more effective, less toxic, with reduced cost of manufacturing and more rapid preparation compared with the conventional plasmids, when transfected into mammalian cells.

Monjezi's group engineered CD19 CAR T-cells through non-viral Sleeping Beauty stable transposition of CAR genes from supercoiled, minimal DNA vectors, called minicircles (MCs). MC-derived CAR transposon's integrants were observed into genomic safe harbor loci, minimalizing the potential for insertional mutagenesis and genotoxicity, in comparison with LV-derived CAR, integrating highly expressed and cancer-related genes [36]. Compared to Sleeping Beauty, piggyback system seems to have a higher efficiency of gene transfer, without integration near proto-oncogenes, and generates functional CAR T-cells, although not tested in the clinic [37].

3.3. CRISPR/Cas9

At the beginning of 2000, the scientific community's interest turned to genetic "editing" methods. Zinc finger (ZFNs) and nucleoside transcription activator-like effector nucleases (TALENs) were developed. ZFNs and TALENs are chimeric, tailored restriction enzymes that are engineered to target specific genetic sites, even validated as safe-harbor sites. So far, in CAR therapy, this technology has been used to knock out the endogenous TCR receptor in allogenic T-cells, which could prevent unwanted graft-versus-host disease (GvHD), although the CAR transgene was virally transfected (Cellectis - UCART19) [38, 39]. Genome-editing strategies could also be used to prevent or delay the rejection of CAR T-cells by the recipient's immune system *via* eliminating or decreasing the expression of histocompatibility antigens on the donor T-cells.

The breakthrough in the genetic "editing" was the CRISPR/Cas9 technology. The type II CRISPR protein Cas9 is directed to target almost any region in the genome by a short RNA guide (gRNA), where it functions as an endonuclease. The endonuclease can be transferred *via* liposome-mediated transfection, electroporation, chemical transduction or as part of a viral genome [40] in the form of Cas9 protein/gRNA ribonucleoprotein (RNP) [41], or in the form of a plasmid, driven by either U6 or H1 promoters for transcription after transfection of mammalian cells. Next, a donor template, typically in a plasmid form, is used to integrate the desired transgene by homology-directed repair (HDR). Furthermore, an alternative non-viral approach is applied through nanomaterials. One of these approaches is based on the biotin-streptavidin conjugate and the transport and binding of the templates from the donor to the Cas9 modified human cells, increasing the rates of gene transfer up to 5 times more than the conventional methods [31]. Finally, the co-injection of Cas9 [delivered as *in vitro* transcribed mRNA (IVT-mRNA, see below)] with a single species of gRNA increased in the rate of genomic cleavage in some cells [42]. CRISPR technology has been used to produce CAR T-cells with a high degree of homogeneity and promising survival results in mouse models. More specifically, the introduction of the CAR sequence into the "alpha constant"-TRAC endogenous T-cell receptor locus improved the cytotoxicity of the CAR T-cell [43]. However, the efficacy of gene editing for CAR knocking remains low, with success rates up to 20% and there is still the problem of the off-target mutagenesis [44].

PD-1 and the endogenous TCR have been knocked out by CRISPR/Cas9 in T-cells of patients with lung cancer during the first clinical trial of CRISPR/Cas9. However, CAR or TCR wasn't introduced into T-cells in this trial [45]. Similar trials with PD1-knockout autologous T-cells for prostate (NCT02867345), bladder cancer (NCT02863913) and renal cell carcinoma (NCT02867332) are also being initiated. The main goal is the elimination of random integration of viral delivery systems as well as the control of CAR's integration. It is though unclear if the removal of some inhibitory signals from the T-cells leads to the uncontrolled proliferation of cells or to severe autoimmunity [44].

3.4. Non-viral Transfer Methods

Other approaches concerning the genetic engineering of T-cells use non-viral transfer of plasmid DNA or IVT-mRNA, because of its low immunogenicity and low risk of mutagenesis. The use of mRNA for gene therapy applications was first described by Malone *et al.*, in 1989 [21], using liposome-mediated transfection. Transfer of TCR genes, *via* electroporation of mRNA, into primary T-cells has been described by Zhao *et al.*, in 2006 [20]. The development of a therapeutic approach using mRNA dealt with several concerns and seemed to be a major challenge, because of its characteristics, such as sensitivity and susceptibility to degradation, instability, negative charge, the insufficient translation in the host's cells and immunostimulatory effects. These challenges have been circumvented to some extent by an improved understanding of the relationship between structure and stability of mRNA as well as the development of a wide range of chemical modification methods. The various structural modifications of the mRNA are the addition of anti-reverse cap analogs (ARCAs) and polyadenylate tail. These modifications increase the efficiency of translation and the stability of the mRNA. The poly (A) tail is preferred to be greater than 100 residues. Another modification is the replacement of adenylate-uridylate rice (AREs) elements with more stable 5'UTR (untranslated region) and 3'UTR of the β-globin gene. The widely studied AREs are important signals of mRNA degradation in the 3'UTRs of most eukaryotic mRNAs. mRNAs containing AREs indicate decreased stability, perhaps due to the removal of the poly (A) tail. However, the stability is increased when AREs are replaced with the 3'UTR of a more stable mRNA, such as the β-globin

mRNA. These modifications increase mRNA stability and allow its expression for longer periods.

The mRNA transfer represents a cytoplasmic expression system; it does not need to enter the nucleus to mediate its function. IVT- mRNA can be synthesized with the structural modifications that increase its stability. Further improving the delivery modalities of mRNA is, therefore, necessary for its development as a therapeutic tool. IVT-mRNA delivery can be mediated by either disruption of the cell membrane (electroporation, gene gun) or by endocytosis using several nanoparticles [46], such as viromers, protamine-mRNA complexes, lipid nanoparticles, polymeric nanoparticles, lipid-polymer hybrid nanoparticles and gold nanoparticles [47]. Lipofectamine is commonly used as a cationic carrier for introducing IVT-mRNA into cells. Lipofectamine is composed of cationic lipids that form liposomes with positively charged surfaces and facilitates the entry of mRNA, through endocytosis, into the eukaryotic cell as follows: positively charged liposomes crosslink with the phosphate groups of the nucleic acid backbone and form a complex that reacts with the negative charged cytoplasmic membrane, allowing the complex to fuse therewith. The complex accumulates intracellularly, escapes from the endosome and the genetic material enters the cytoplasm to be expressed [48]. Electroporation is one of the most promising strategies for introducing the CAR IVT-mRNA construct into T-cells. It has been reported that, under certain circumstances, IVT-mRNA transfection *via* electroporation was efficient enough, with low electroporation-related apoptosis [49]. Many studies reported the successful tumor toxicity of IVT-mRNA CAR electroporated T-cells and NK cells in pre-clinical models [50]. The mRNA mediated transfection systems allow more rapid changes in CAR design and are safer compared to long-term, integrating, viral expression systems. The use of IVT-mRNA transfection technology gives CAR therapy extra safety and, thus, the required clinical advantage, despite the short lifetime and transiency of their expression. In fact, IVT-mRNA degradation over time ensures complete removal of the CAR from the patient without the need for suicide genes [51]. Thus, IVT-mRNA mediated transfection systems are easier to move into a good manufacturing practice (GMP)-compliant system with potentially lower cost and less complex release testing [52]. In fact, there is the need for only a few repetitive infusions (3-9 infusions) of CAR T-cells in patients to raise a long-lasting response [53]. CARs transfected into T-cells using mRNA are currently being investigated in early clinical trials at the University of Pennsylvania (Philadelphia, PA; NCT02624258, NCT01837602, NCT02277522, NCT02623582). Furthermore, an attempt was made to cope with solid tumors with CAR T-cells modified by electroporated IVT-mRNA [54]. However, electroporation sometimes leads to cell death, especially when electrical fields cause permanent membrane permeation and the consequent loss of cell homeostasis, in a process known as irreversible electroporation. When the electroporation field is applied to the skin, using surface plate electrodes, the main "potential" drop develops along the skin rather than along the target subcutaneous tissues. Skin swelling is a common consequence of *in vivo* electroporation. Most electroporation protocols aim to penetrate only the plasma membranes. Electroporation of the nucleus requires an additional step, using higher voltage and less pulse length (nucleoporation). In addition, although the principle of electroporation is applicable to all cell types, its effectiveness depends on the electrical properties of the cells. Smaller, in size, cells require a higher field to penetrate. Cells with less conductive contents (such as fat cells) are less sensitive. Thresholds for different cells in a heterogeneous tissue will, therefore, vary [55].

Due to the elimination of intentional integration into the host genome, the use of IVT-mRNA rather than MC- or plasmid-encoded transposase and CAR gene is preferable. However, the main drawbacks of these systems are the long *ex vivo* culture time to generate therapeutic doses of gene-modified T-cells and the severe cell damage, which may follow the electroporation of plasmid DNA.

4. CAR T-CELL THERAPY TOWARDS BLOOD MALIGNANCIES

Currently, anti-CD19 CAR T-cells were demonstrated to be dramatically effective in pediatric and adult patients, for the treatment of R/R (relapsed or refractory) B-cell malignancies, such as B-cell non-Hodgkin lymphoma (NHL), Acute Lymphoblastic Leukemia (ALL) and Chronic Lymphocytic leukemia (CLL), with percentage of complete remissions ranging from 70 to 94% in different trials [15]. Since the initial successful reports of CD19 CAR therapy, follow-up trials have been conducted in higher numbers of patients with follicular lymphoma (FL), CLL, and ALL as well as for patients with other B-cell malignancies [56]. CAR T-cell therapy has demonstrated promising outcomes by targeting CD19 [13, 57-64], CD20 [65], or CD30 [66], expressing either a CD28 or a 4-1BB co-stimulatory domain, although the most captivating success has been achieved in CD19 CAR-T cells for B-ALL [67, 68]. Furthermore, successful treatment with CD19 CAR T-cells was observed in cases of multiple myeloma (MM) [69, 70].

Despite the remarkable high response rate of CAR T-cells targeting CD19 in lymphocytic leukemias, antigen escape (the loss of detectable CD19 on the surface of tumor cells) has been also observed in ~10 to 20% of pediatric B-ALL patients, treated with CD19-directed immunotherapy [71]. Thus, there is the need for discovery of more novel targeting hematologic markers. More clinical trials are undergoing concerning: a) MM, targeting CD138 or B-cell maturation antigen (BCMA) [72, 73] as well as b) acute myelogenous leukemia (AML), targeting CD33 and CD123 [74].

FDA, EMA and various regulatory agencies have recognized that the use of CAR T-cells is a revolutionary therapeutic approach. In fact, "tisagenlecleucel-T" (Kymriah, Novartis) is the first therapy using CAR technology to enter the marketplace, indicated for use in pediatric and young adult patients (age 3 to 25 years) with R/R ALL. The product got the green light from the FDA, on August 30, 2017, and its cost reaches $475,000. Furthermore, Kymriah is under regulatory review by the FDA for adults with R/R diffuse large B-cell lymphoma (DLBCL), an aggressive subtype of NHL, and in Europe for R/R B-cell ALL and DLBCL. Kymriah is also being assessed in FL, second-line DLBCL, CLL and MM [75]. FDA approved the second T-cell therapy, on Oc-

tober 18, 2017, called "axicabtagene ciloleucel" (Yescarta, Kite Phama), for the treatment of patients with R/R aggressive B-cell NHL, who are ineligible for autologous stem cell transplant (after at least two lines of systemic therapy) and its price is up to $373,000. Yescarta is under review in Europe and it is also being assessed for mantle cell lymphoma (MCL) and indolent NHL subtypes, including FL [75]. Both FDA-approved CAR therapies use retroviral vector-based gene therapy products, including the potential for generation of replication-competent retroviruses (RCR) and vector-induced genotoxicity. In addition, there are some concerns about delayed adverse events, related to insertional mutagenesis. They also include the risk of severe side effects of cytokine release syndrome (CRS) and neurotoxicity; thus, FDA also requires post-marketing studies to assess the long-term safety and risk of secondary malignancies [75].

Unfortunately, at this moment, one single treatment with these therapies comes with a high price tag. However, as the production scale increases and companies (like Cellectis) are pioneering in the development of either allogeneic or off-the-shelf CAR T-cell therapy (that do not require an individualized manufacturing process), it is estimated that the cost would significantly be reduced over the next few years. Furthermore, considering that a single treatment can lead to durable results in comparison with other therapies, *e.g*. antibodies that require prolonged treatment at considerable cost, the pricing of CAR T-cell therapies would not be that prohibitive. With the first therapies already in the market, the way is open for more and better, faster and cheaper alternatives to arrive in the near future. In 2026, the market for CAR T-cell therapies for hematological malignancies - R/R NHL (DLBCL, FL, MCL and CLL) and MM - is estimated to $1.1 billion [75].

5. CAR T-CELL THERAPY TOWARDS SOLID TUMORS

Targeting solid tumors is more difficult than targeting hematological malignancies; CAR T-cells deal with a huge range of challenges. The genetic instability of tumor cells means they can stop expressing antigens targeted by T-cells or lack the mechanisms that present them. Furthermore, the adoptive CAR T-cell therapy for solid tumors has shown limited success so far, due to the tumor histopathological characteristics, the inadequate "trafficking" of CAR T-cells to tumor sites, as well as local strong immunosuppressive microenvironment, tumor heterogeneity and shortage of specific antigens [76]. The dense micro-tumor environment is characterized by hypoxia, low pH, lacking arginine or tryptophan, inhibitory effects of tumor-derived cytokines and by inhibitory pathways against activated T-cells, including up-regulation of inhibitory receptor's effector functions after T-cell activation, leading to rapid loss of functional activity and therapeutic efficacy of CAR T-cells [77-79]. Moreover, rapid death caused by the "on-target off-tumor" cross-reaction of CAR T-cells has been reported [80], administered anti-ErbB2 CAR T-cells localized to the lung, immediately following the infusion, were triggered to release cytokines by the recognition of low levels of ErbB2 on lung epithelial cells, highlighting the important priority of enhancing CAR T-cell therapy's safety. To overcome these barriers, several ingenious strategies have been deployed, including: (a) the design of iCARs [28], (b) the design of logic-gated CARs [81], (c) the introduction of chemokine receptor genes, that match the chemokines produced either by tumor or tumor-associated cells (*e.g*. CCR2b, which binds to CCL2-secreting neuroblastoma cells [82]) or (d) endowing CAR T-cells with basement membrane-degrading enzyme (*e.g*. heparanase [83]). T-cell therapies combined with immunomodulatory agents [such as checkpoint inhibitors and cytokines, and/or small-molecular antagonists that block biochemical pathways, crucial for tumor growth (*i.e*. adenosine)] constitute exciting opportunities, that may have synergistic effects in augmenting antitumor responses [27].

Although clinical trials for CAR T-cell therapy against solid tumors have a promising outcome, these responses do not approach those of CD19 CAR T-cells [84]. These approaches target the epidermal growth factor receptor (EGFR) [85], the variant III of the epidermal growth factor receptor (EGFRvIII) [86], the human epidermal growth factor receptor 2 (HER2) [80, 87], the carcinoembryonic antigen (CEA) [88], disialoganglioside 2 (GD2) for neuroblastoma [89], mesothelin (MSLN) [53, 90], the prostate-specific membrane antigen (PSMA) [91] and interleukin-13Ra2 (IL13Ra2) for glioblastoma [54], which are commonly expressed on solid tumors and play a critical role in tumorigenesis [20]. Also, there is T4 immunotherapy using ErbB-targeted CAR (T1E28z) and an IL-4-responsive chimeric cytokine receptor (4ab) for head and neck squamous cell carcinoma [92] as well as few non-tumor targets that are considered as essential for tumor growth and survival *in vivo*, such as VEGFR [93] or FAP [94]. Moreover, CAR T-cell therapy can alternatively target not only the typical mutated or over-expressed tumor antigens, but a new class of tumor targets, resulting from post-translational modifications, such as the cancer-associated Tn-glycoform of the membrane mucin (MUC1), which was uniform across several types of cancer but undetected on normal tissues [95]. Juno Therapeutics announced for its pipeline an armored MUC16-targeted JCAR020 in combination with IL-12 in order to boost the immune response in the tumor microenvironment of ovarian cancer [75]. Aurora BioPharma is developing AU105, an adoptive bispecific CAR T-cell therapy, targeting HER2 and CMV for breast cancer glioblastoma (completed phase I/II). At present, more and more scientists are devoted to searching for potential targets [96].

6. SAFETY CONSIDERATIONS FOR CAR THERAPY

CAR T-cell application has produced impressive antitumor responses, but it is still associated with several safety concerns about the side-effects it may cause. Various toxicities are observed, immediately or weeks following CAR T-cell infusion, which can pose significant risks (Table **1**) [97, 98].

The most common short-term adverse effect of CAR T-cell therapy is the CRS, normally in concomitance with neurotoxicity. CRS after CD19 CAR-T cell therapy reported to occur in 54-91% of patients, including severe CRS in 8.3-43% [99]. The patients, who received "tisangenlecleucel" during the phase II trial for R/R B-ALL, were reported with severe CRS (47%) and with neurotoxicity (15%) [100]. The patients, who received "axicabtagene ciloleucel" during the

Table 1. Toxicities, caused during CAR T-cell therapy, for the treatment of blood or solid tumors [97, 98].

Type of Toxicity	Caused by:	Observed During:
"On-target on-tumor"	• Rapid destruction of a large tumor mass • Massive release of tumor cell components into the circulation	• Leukemia treatment • Treatment of solid cancer (low toxicity)
"On-target off-tumor"	• Engagement of cognate antigen on healthy tissues	• Treatment of solid tumors • Treatment of leukemia/lymphoma (low toxicity)
"Off-target off-tumor"	• Inflammatory reaction beyond the targeted tumor tissue	• Independently of the CAR specificity and the malignant disease
Cytokine Release Syndrome	• Release of supra-physiological serum levels of proinflammatory cytokines by CAR T-cells (IFN-γ, IL-6, TNF-α)	• Treatment of hematologic malignancies
Neurotoxicity	• Diffuse encephalopathy, mainly due to systemic cytokines' trafficking to the CSF	• Independently of the CAR specificity and the malignant disease

pivotal trial for aggressive B-NHL, reported with severe CRS and neurotoxicity in percentages 13% and 28%, respectively [101]. CRS is characterized by severe dyspnea, often in combination with bronchospasm, hypoxia, fever, shiver, hives, coagulopathy and capillary leak, appearing 1 or 2 hours right after the first infusion. Following recognition of CD19+ tumor or normal B-cells, activation of CAR T-cells result to their proliferation, lysis of target cells, and proinflammatory cytokine secretion (TNF-α, IL-6 and IFN-γ) that can be associated with the clinical evidence of CRS and neurotoxicity. CRS may be also associated with some manifestations of tumor lysis syndrome, such as hyperuricemia, hyperkalemia, hypocalcemia, hyperphosphatemia, acute renal failure, elevated LDH, and acute respiratory failure, even death. Acute respiratory failure may be accompanied by interstitial pulmonary infiltration or swelling, an evident in chest X-rays. However, these symptoms seem to be transient and reversible without long-term deficits, yet with unknown mechanisms [102].

Neurotoxicity is typically presented as a wide range of neurological and psychiatric manifestations, including seizures, delirium, aphasia, and hallucinations and it is caused by systemic cytokines, which cross the blood brain barrier (BBB) [103]. Gust, *et al.,* reported neurotoxicity (incidence of ~40%) in 133 lymphodepleted patients with R/R B-cell malignancies (62 patients with B-NHL, 47 patients with B-ALL and 24 patients with CLL) after CD19 CAR T-cell infusion. Severe neurotoxicity was characterized by endothelial activation, including increased BBB permeability, vascular leak and disseminated intravascular coagulation. It has been also demonstrated that neurotoxicity is associated with high concentrations of serum cytokines, including those that activate endothelial cells, such as IL-6, IFN-γ, and TNF-α [104]. On the other hand, CAR T-cells have been found in cerebrospinal fluid (CSF) of patients, suggesting the enhancement of CAR T-cell infiltration in the CSF, caused by hyperthermia and IL-6, released during CRS [103]. On rare occasions, CRS can evolve into CAR T-cell related encephalopathy syndrome (CRES) or into fulminant hemophagocytic lymphohistiocytosis (HLH), also known as the macrophage activation syndrome (MAS). HLH/MAS is characterized by severe immune activation, lymphohistiocytic tissue infiltration and immune-mediated multiorgan failure [105]. The physiology of CRS and HLH/MAS syndromes may have some overlap [106].

Beside the above toxicities, the concerns about RV/LV virus-derived CAR T-cells include genotoxicity, due to the potential for generation of RCR and insertional mutagenesis *in vivo*, leading to overexpression of adjacent genes or disruption of genes at the site of integration [107]. Another potential adverse with integrating vectors is the insertion in hotspots, resulting in oncogene deregulation with malignant transformation (*e.g*., secondary leukemias). The insertional mutagenesis based on "clonal" sovereignty and tumorigenesis has been observed in RV-derived hematopoietic stem cells in gene therapy clinical trials for severe x-linked immune deficiency [108], chronic granulomatous disease and Wiskott-Aldrich syndrome [109]. Depending on the vector type, viruses always retain inherent weaknesses, including potential immunogenicity, tumorigenicity, limited load carrying capacity, complex production processes, *etc*. Viral transfection methods are also expensive and require highly trained personnel, with high security requirements.

According to Neelapu and his team, the acute toxicities associated with CAR T-cell therapy can be managed in a three-step approach. Firstly, it is important to determine the nature of the CAR T-cell related toxicity, according to the patient's clinical and biological symptoms, and reach to the correct diagnosis: CRS, CRES, and HLH/MAS [105]. Secondly, it is necessary to determine the severity of the detected syndrome, according to some criteria for grading. More specific, the symptoms or signs of CRS reflect to a scale from Grade 1 to Grade 4. The CRS grade should be determined at least twice a day, and whenever a change in vital signs or organ toxicities in the patient's status is observed [105]. The third step in the management of CAR-related toxicities approach is the selection of the appropriate treatment, based on the toxicity grade, according to specific management algorithms [105, 106].

CRS-related toxicities are effectively managed in clinical trials with tocilizumab, an IL-6 receptor antagonist. Tocili-

zumab is widely used to treat rheumatologic disorders; however, it is considered as an "off-label" treatment for reducing or abrogating CRS, following CAR T-cell infusions. Furthermore, systemic corticosteroids are used effectively to abrogate CRS-related toxicities, by inhibiting CAR T-cell persistence and antimalignant efficacy. Neurotoxicity may difficulty be managed due to the BBB; in fact, tocilizumab is a monoclonal antibody and its size makes unlikely the efficient BBB penetration [104]. The treatment with dexamethasone is often chosen in this context because of efficient BBB penetration [98].

In principle, the treatment of B-cell malignancies with CAR-T cells leads to almost entire B-cells' repertoire depletion (B-cell aplasia), an on-target off-tumor toxicity. CD19 is expressed on most B-cell malignancies, however, it is also expressed on normal B-cells. Thus, CAR T-cell response would also deplete normal B-cells [110]. In early studies, B-cell aplasia has been reported in patients for a year or longer after CAR T-cell infusion. This toxicity requires pooled γ-globulin administration and/or antibiotics, till the recovery of B-cells. B-cell aplasia may not occur, due to the preserved humoral immunity based on the persistence of B-cells that do not express CD19 and are able to secrete antibodies [111].

Nowadays, there is no clinical or regulatory standard to guide the toxicity management, while prophylaxis, medicine and supportive care were used in the practice [102]. In Kite's clinical trial concerning aggressive B-NHL, on October 2016, about one-third of the patients developed serious neurological side effects, and 18% developed CRS, leading to the death of two of the 62 patients, as a result of the treatment [112]. The deaths in clinical trials (Kite and Juno) brought safety at the forefront of the regulatory committee's considerations [75].

CONCLUSION

As of the end of December 2016, there were about 113 CAR T-cell trials registered at clinicaltrials.gov, with an enrollment of more than 8,000 patients worldwide. Most trials (85%) have been held in the USA and China and 65% of the studies are directed against hematological malignancies [113]. Today, autologous CAR T-cell therapy's cost is high enough as the cost of a bone marrow transplantation, but this may change in the case of an "off-the-shelf" solution, such as allogenic CAR T-cells sourced from a healthy donor and ready to go when the patient needs it, as a cryopreserved product [114]. Cellectis' UCART123 recently received FDA approval to start clinical trials, making it the first study for allogenic CAR T-cells in humans [115]. Cellectis is also developing allogenic CAR T-cell therapies targeting CD19, CD22, CD38 and CS1 [75].

The solution for safer and more efficient approaches is through optimizing the design of CARs and the discovery of new, more specific antigen-targets. A promising approach is the evaluation of therapies, which combine CAR T-cell therapy with: (a) the increasing arsenal of immunomodulatory agents, targeting T-cell inhibitory molecules (CTLA-4, PD-1) [30, 116]; (b) safety switches; (c) the improvement of suicide genes and deletion methods (inducible caspases and antibody deletion targets); and (d) the use of alternative transfection strategies. Future T-cell products may have the potential of facing the GvHD by removing/suppressing endogenous TCR through genome engineering. These approaches not only increase CAR T-cell general efficacy, but they may solve many problems concerning immunotoxicity and/or autoimmunity [117].

In conclusion, CAR therapy shows the way for a potential paradigm shift in the treatment of refractory or relapsed cancers. Unlike conventional approaches used to manage cancer disease, CAR T-cell therapy is a patient-specific, "living" and self-replicating drug [118]. Although CAR therapy has many successes in hematological cancers, this is only the beginning of exploring the powerful potential of CAR redirected immune system in the elimination of resistant, metastatic or recurrent non-hematological cancers.

LIST OF ABBREVIATIONS

1G, *etc*. CARs	=	First Generation, *etc*. CARs
ALL	=	Acute Lymphocytic Leukemia
AREs	=	Adenylate-uridylate Rice Elements
BBB	=	Blood Brain Barrier
CAR	=	Chimeric Antigen Receptor
CLL	=	Chronic Lymphocytic Leukemia
CMV	=	Cytomegalovirus
CRES	=	CAR T-cell Related Encephalopathy Syndrome
CRS	=	Cytokine Release Syndrome
CSF	=	Cerebrospinal Fluid
CTLA-4	=	Cytotoxic T-lymphocyte-associated Protein 4
DLBCL	=	Diffuse Large B-cell Lymphoma
EGFR	=	Epidermal Growth Factor Receptor
FITC	=	Fluorescein Isothiocyanate
FL	=	Follicular Lymphoma
GvHD	=	Graft-versus-host Disease
HER2	=	Human Epidermal Growth Factor Receptor 2
HLA	=	Human Leukocyte Antigen
HLH	=	Hemophagocytic Lymphohistiocytosis
iCAR	=	Inhibitory CAR
IFN-γ	=	Interferon Gamma
IL	=	Interleukin
LAK	=	Lymphokine-Activated Killer Cells
LTR	=	Long Terminal Repeat
LV	=	Lentivirus
mAb	=	Monoclonal Antibody
MAS	=	Macrophage-activation Syndrome

MCs = Minicircles

MHC = Major Histocompatibility Complex

MM = Multiple Myeloma

NHL = Non-Hodgkin Lymphoma

NK cells = Natural Killer Cells

PD-1 = Programmed Cell Death Protein 1

RCR = Replication-competent Retrovirus

RNP = Ribonucleoprotein

R/R = Relapsed or Refractory

RV = Retrovirus

scFv = Single-chain Fragment Variable

TAA = Tumor-associated Antigens

TALEN = Transcription Activator-like Effector Nuclease

TanCAR = Tandem CAR

TCR = T-cell Receptor

TIL = Tumor Infiltrating Lymphocytes

TM domain = Transmembrane Domain

TNF = Tumor Necrosis Factor

TRUCKs = T-cells Redirected for Universal Cytokine Killing

UTR = Untranslated Region

ZFN = Zinc Finger

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

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

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