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Genome-editing technologies in adoptive T cell immunotherapy for cancer

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Abstract

Purpose of Review—In this review, we discuss the most recent developments in gene-editing technology and discuss their application to adoptive T cell immunotherapy.

Recent Findings—Engineered T cell therapies targeting cancer antigens have demonstrated significant efficacy in specific patient populations. Most impressively, CD19-directed chimeric antigen receptor T cells (CART19) have led to impressive responses in patients with B-cell leukemia and lymphoma. CTL019, or KYMRIAH™ (tisagenlecleucel), a CD19 CAR T cell product developed by Novartis and the University of Pennsylvania, was recently approved for clinical use by the Food and Drug Administration, representing a landmark in the application of adoptive T cell therapies. As CART19 enters routine clinical use, improving the efficacy of this exciting platform is the next step in broader application.

Summary—Novel gene-editing technologies like CRISPR-Cas9 allow facile editing of specific genes within the genome, generating a powerful platform to further optimize the activity of engineered T cells.

Keywords

CRISPR-Cas9; TALEN; zinc-finger nucleases; adoptive cell therapy; chimeric antigen receptor T cells (CART); immunotherapy; PD-1; gene-editing

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Conflict of Interest

Nathan Singh and Junwei Shi each declare no potential conflicts of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Introduction

Re-direction of the immune system to target and eliminate cancer cells has been a goal in cancer biology for over 100 years. The specificity and potency of the immune response make this strategy highly attractive, and decades of research have led to significant refinements of this platform. The ability of T cells to mediate anti-cancer effects was first demonstrated in a series of studies both *in vitro* and *in vivo* (1–3). In a seminal study published in 1979 it was observed that stem cell transplantation from syngeneic donors was less effective than sibling donors in preventing relapse of leukemia, (4) suggesting a "graft-versus-leukemia" (GVL) effect contributed to the efficacy of allogeneic transplantation. Subsequently, it was demonstrated that relapse rates are higher when T cells were removed from the allograft, (5) and that relapse could successfully be treated by infusion of donor lymphocytes (6) or withdrawal of T cell-directed immunosuppression, (7) all pointing to the importance of T cells in the anti-cancer response after allogeneic transplant. Since then, unleashing the T-cell compartment has been the focus of much of cancer immunotherapy. Allogeneic hematopoietic cell transplantation (allo-HCT) is, as such, the first and most fundamental form of adoptive cell therapy (ACT), i.e. the transfer of immune cells to obtain an anti-tumor effect. In allo-HCT, matched donor allografts including CD34+ hemopoietic stem cells and mature lymphocytes, are infused into recipients with the intention of reconstituting the immune system, as well as invoking a GVL effect. The next step in ACT took the form of donor-leukocyte infusion (DLI), in which the original donor's leukocytes are re-infused into the recipient at time of relapse in the hopes of re-initiating a GVL effect (8). While DLI has demonstrated efficacy in the treatment of some forms of leukemia and lymphoma, it's efficacy has been limited in other diseases, (9) such as acute leukemia and myelodysplastic syndrome. Additionally, both allo-HCT, and much more significantly DLI, are associated with toxicity in the form of graft-versus-host disease (GVHD), a significant cause of morbidity and mortality. As such, more targeted approaches to T-cell immunotherapy could balance the power of T lymphocytes while tempering their toxicity. The identification of tumor-infiltrating lymphocytes (TILs) within surgically-resected melanoma samples opened the field to the use of antigen-targeted T cells for tumor immunotherapy. While early clinical trials demonstrated efficacy in the treatment of metastatic melanoma, (10) the technical barriers to culture and manufacture of TILs has limited their broad-scale application. Additionally, early studies showed that tumor-infiltrating lymphocytes can be tumor-specific, (11) but often do not exert significant anti-tumor effects due to T-cell exhaustion. (12) However, novel strategies to select potent tumor-reactive TILs have been developed (13) and have demonstrated clinical activity in selected patients. (14)

The broad application of genetic engineering technology, such as retro- and lenti-viral transduction, has permitted the introduction of artificial transgenes into primary T cells, resulting in a redirected antigen-specificity against tumor-associated antigens. (15) The two genetically-engineered T cell therapies that have been most heavily investigated are transgenic T cell receptor (tTCR) therapies and chimeric antigen receptor (CAR) therapies. (15) tTCRs are synthetic receptors that maintain the structure of the native TCR but are designed for antigen-selectivity and high affinity. Several TCR specificities are being tested

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clinically, such as NYESO-1-directed tTCR for multiple myeloma. (16) Alternatively, CARs are synthetic hybrid molecules expressing the antigen recognition domain of the B-cell receptor, and the co-stimulatory domains of the T-cell receptor. CAR therapy targeting the B-cell surface molecule CD19 have demonstrated remarkable success in the treatment of relapsed or refractory B-cell acute lymphoblastic leukemia (B-ALL), with complete remission observed in >90% of patients. (17, 18) Significant clinical activity has also been observed in patients with highly-refractory chronic lymphocytic leukemia (CLL), with overall response rates of >50%. (19) The recent FDA approval of the first engineered cellular therapy (KYMRIAH™, tisagenlecleucel) has been a watershed moment for the application of cellular therapy in cancer, and demonstrates the power of CARs in mediating effective anti-tumor responses.

Most clinical adoptive T cell trials to date used autologous T cells collected from patients at time of enrollment. Unfortunately, for some patients, cell manufacturing is not feasible or fails due to poor lymphocyte counts, poor T cell quality and/or high tumor burden. (20) Furthermore, even those patients who do achieve successful collection must wait weeks to months for cell manufacturing and quality control – a delay that can be untenable in patients with aggressive malignancies. For these reasons, many groups have been invested in developing “off-the-shelf” CAR T cells, derived from allogeneic donor T cells, to solve the problem of poor T cell collections and long manufacture delays. The greatest barrier to implementation of this therapy is prevention of GVHD, and thus innovative gene-editing technologies are being investigated to help balance on-target activity with off-target toxicity. Another relevant issue in ACT is that, while CAR/tTCR therapy allows for both highly-targeted and highly-active anti-tumor activity, these cells are subject to tumor-driven immunosuppression. Gene-editing serves as a potential tool in overcoming this barrier.

Significant efforts have been made to use gene-editing technologies to develop next-generation adoptive T cell therapies. Indeed, the application of gene editing to immunotherapy can potentially allow for finer tuning of the immune response, permitting more specific activity, enhanced immune cell function and the potential for “off-the-shelf” use. Several gene-editing technologies have recently been applied to immunotherapy, the most-novel and powerful of which is the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated 9 (Cas9) platform. (21–23) In this review, we describe the latest gene-editing technologies and discuss how they are being applied to adoptive T cell immunotherapy.

Gene-editing strategies

In contrast to genetic engineering, which allows for the introduction and expression of a novel (or native) protein, gene editing is most often used to silence or modify endogenous protein expression at the genome-level. The gene-editing platforms that have been most commonly utilized are zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and CRISPR-Cas9 technologies.

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Zinc finger nucleases (ZFN)

The first gene editing tool to be broadly applied was ZFN technology. Zinc fingers are large multimeric proteins, wherein each individual finger targets a three to four base pair sequence within genomic DNA. Linking several zinc fingers together can thus create highly-specific recognition sites.(24, 25) These multimeric zinc finger proteins are linked to the *FokI* endonuclease to create a ZFN, capable of site-specific double-stranded DNA cleavage resulting in homologous recombination (HR) or non-homologous end joining (NHEJ). (26) This technology is certainly effective and specific, however it relies on protein engineering for appropriate target selection, which typically requires time-consuming optimization.

Transcription activator-like effector nucleases (TALEN)

Similar to ZFNs, TALENs are hybrid molecules of DNA-recognition proteins, in this case transcription factors, linked to an endonuclease. Originally identified from bacteria of the *Xanthomonas* genus, TALs are units of 33–35 amino acids that are able to recognize a single base pair of genomic DNA. (27) Linkage of several TALs together with an endonuclease generates a site-specific TALEN. (28) The primary advantage of TALEN technology over ZFN is cost, however its optimization for a specific site appears longer than CRISPR-Cas9. (29)

Clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9

CRISPR-associated 9 (Cas9) endonuclease technology is the most recently developed gene-editing platform, and has generated a great deal of excitement due to its efficiency and simplicity of use. Originally identified in *Streptococcus pyogenes*, the CRISPR/Cas9 complex serves as a bacterial immune system to cleave foreign viral DNA. (30) CRISPR sequences are normally present within the host bacterial genome, and upon viral entry into the host cell, viral DNA is inserted between CRISPR sequences. These CRISPR complexes undergo transcription and processing into CRISPR RNAs (crRNAs), composed of bacterial CRISPR sequences, but also short segments of viral sequences (protospacers), and intervening sequences known as protospacer adjacent motifs (PAMs). These crRNAs form a complex with the cas endonuclease, and upon recognition of the homologous protospacer and PAM sequence, the cas endonuclease enacts a double stranded break, invoking DNA repair. (31) This system has been applied to gene editing by introduction of short-guide RNA (sgRNA) to serve as the crRNA. (23, 32) Thus, by introduction of cas9 and a target sgRNA, gene editing can be accomplished efficiently, specifically and simply. Several methods exist to deliver the Cas9/sgRNA components to cells, including viral vector-based delivery and RNA/protein electroporation.

These technologies have been, to varying degrees, applied to many aspects of molecular biology and translational therapy in the last 15 years. ZFN and TALEN have previously been clinically applied to adoptive T cell therapy, while CRISPR-Cas9 is now being evaluated clinically and is poised to be evaluated in multiple settings (see Table 1).

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Gene-editing to improve ACT

Universal off-the-shelf T cells

The use of allogeneic T cells in CAR therapy is limited by their potential for initiating GVHD, which is primarily mediated through T cell receptor (TCR) activation. The availability of off-the-shelf T cell products have the potential of significantly increasing the number of patients with access to these therapies. As such, the application of gene-editing technologies to T cell therapy could generate a universal T cell product that could be infused to any unmatched recipient. For example, a group at the MD Anderson Cancer Center generated a ZFN able to specifically knock out the endogenous TCR, blocking their ability to recognize recipient peptides resulting in GVHD. (33) Disruption of the T cell receptor alpha chain (TRAC) gene has also been achieved using TALEN technology in CD19 CAR T cells (UCART19) (34), which have recently been evaluated clinically. (35) Two pediatric patients with relapsed B-ALL were treated with UCART19, and while both patients experienced CAR-mediated disease control, they both also developed GVHD, likely as a result of the remaining TCR+ cells in the infusion product, highlighting the importance of gene editing efficiency. Several exciting pre-clinical studies have been reported using CRISPR technology to both broaden the application of CAR T cells, as well as enhance CAR T cell activity. In a recent report from our group, multiplex genome editing was achieved using the CRISPR/Cas9 system to simultaneously disrupt TCR alpha and beta chains (TRAC and TRBC) in NY-ESO1 TCR engineered T cells. Optimization of sgRNA delivery resulted in >95% disruption of TCR expression, and this disruption significantly impaired allogeneic activity *in vitro*. (36) In a seminal report from Memorial Sloan Kettering Cancer Center (37), CRISPR technology was used to target the TRAC locus but, as opposed to previous reports which have only disrupted the TRAC gene to prevent TCR expression, here investigators delivered a CD19 CAR construct that was engineered with flanking homology domains that resulted in integration of the CAR gene within the TRAC locus. The primary advantage of this technology is site-specific integration of the CAR gene, which eliminates the possibility of an integration event that leads to disruption of an essential gene, as well as endogenous promoter-driven expression. Moreover, integration of the CAR19 gene into the TRAC locus allowed for a more physiological regulation (TCR-like) of CAR function, leading to better anti-tumor activity as compared to standard CAR engineering.

An essential requirement for the development of universal T cells is prevention of donor T cell rejection. TCR-negative universal T cells could potentially be rejected by recipient T cells through recognition of non-self HLA. Elimination of HLA molecules from T cells using gene-editing technologies like ZFN or CRISPR-Cas9 has been proposed as a mechanism to bypass this rejection. (38, 39) Meganucleases can also be used to knock out beta-2-microglobulin (together with the TCR) to obtain HLA class I negative T cells and therefore avoid T cell mediated rejection ((40), *abstract #200).*

Exhaustion-resistant T cells

In normal T-cell physiology, naturally-occurring “off signals” exist to ensure appropriate control of the robust and cascading T-cell response. These inhibitory signals terminate T-cell activation and inhibit further effector functions, avoiding possible side effects as

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autoimmunity or uncontrolled inflammation. Several studies have demonstrated that increased expression of these inhibitory molecules such as PD-1, CTLA-4, TIM-3 and LAG-3) in T cells permits immune evasion by tumor cells, (12) and thus therapies have been developed that specifically block this inhibitory signal in an effort to prevent T-cell immunosuppression. Referred to as “checkpoint blockade”, this therapy has demonstrated impressive success in both solid (41–46) and hematologic malignancies (47). However, although able to potently re-activate anti-tumor T cells, checkpoint inhibitors can also lead to autoimmunity by dis-inhibiting self-reactive T cells. (48) CAR and tTCR T cells used in ACT are still subject to immunosuppression by tumors and the tumor micro-environment. Therefore gene-editing technologies have been recently applied to ACT to interrupt pathways controlling T-cell exhaustion. As part of the report discussed above with TCR and HLA class I knock out in CAR T cells (49) the authors also disrupted PD-1 in CD19 CAR T cells. These cells demonstrated enhanced anti-leukemia activity in a xenograft models of B-ALL, suggesting that genetic disruption of checkpoint molecules may enhance CAR T cell activity. Further development of this technology allows the integration of several sgRNAs into the CAR lentiviral gene vector to achieve high-efficiency multiplex gene editing using a single electroporation. This system demonstrated high fidelity in the disruption of up to four genes (TCR, HLA, PD-1 and CTLA-4) to generate universal CAR T cells resistant to two inhibitory pathways. (49)

Several clinical trials have opened evaluating the safety of using CRISPR/Cas9 technology to knockout exhaustion markers (see Table 1), however the trial data have not been published yet. Our group at the University of Pennsylvania was recently granted the first FDA approval to investigate the use of CRISPR/Cas9-edited cells in the United States (doi:10.1038/nature.2016.20137). Using CRISPR gene editing, autologous T cells will be modified to lose expression of PD-1 and the native TCR, and then engineered using lentiviral vectors to express a transgenic T-cell receptor targeted to tumor antigens. This strategy will remove two barriers to the success of engineered cell therapies, while maintaining potent and highly-effective antigen targeting.

Other applications

While not directly attempting to improve the activity of engineered T cell therapies, T-cell gene-editing has been applied to several other platforms. The most relevant clinical studies applying ZFN technology as an immune-editing tool have been in HIV. The chemokine receptor CCR5, primarily present on the surface of CD4 T cells, serves as the primary co-receptor for HIV entry. ZFNs targeting the T-cell surface molecule CCR5 were developed to cause double-stranded breaks in the proximal coding region of *CCR5*, and resulted in efficient disruption of CCR5 expression. (50) Given the results of this pre-clinical study, twelve patients were treated in a phase I trial of these gene-edited T cells. There was a notable survival advantage in of the *CCR5*-modified cells *in vivo* that was observed when patients were taken off of anti-retroviral therapy, suggesting efficacy. Interestingly, analysis in this small cohort suggested that biallelic disruption of the *CCR5* gene correlated with cell survival. Only one adverse event occurred, which consisted of flu-like symptoms and did not require intervention. This study confirmed the safety and feasibility of this therapy, and highlighted that homozygous modification was likely necessary for most effective results.

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(51) Lastly, a group in China is performing a trial (NCT03164135, see Table 1) for patients with hematologic malignancies and HIV, using a stem-cell transplant approach with CD34+ stem cells engineered to be CCR5-deficient using CRISPR/Cas9 technology, with the goal of reconstituting a T cell compartment that is HIV-resistant.

CRISPR technology has been used to allow for the development of CAR technologies that would otherwise not be feasible. The development of myeloid-directed immune therapies has been hindered by the lack of target antigens that are unique to malignant myeloid clones. In contrast to B-cells, the immunodeficiency that results from the depletion of normal myeloid cells cannot be overcome with pharmacologic supplementation and would leave patients vulnerable to life-threatening infections. Our group has developed a strategy to circumvent this issue. Using CRISPR/Cas9 guide RNAs targeting CD33, hematopoietic stem cells (HSCs) were engineered to be devoid of CD33. These HSCs demonstrated normal myeloid compartment development and normal function of mature myeloid cells. T cells were then engineered to express CARs targeting CD33, and demonstrated activity against un-edited myeloid cells but not CD33-deficient cells. (52) This proof-of-concept demonstrates a novel method in the development of myeloid-directed immune therapy.

Along a similar path, the group from Baylor has designed a method to engineer CAR T cells to target T-cell antigens. A major hurdle in the design of T-cell immunotherapies targeting T cells is that these engineered cells are fratricidal, in that their cytotoxicity is activated by antigens they themselves express. In a recent report, the authors used CRISPR/Cas9 editing to knockout CD7, an antigen unique to T cells, and then engineered CD7-deplete cells to express a CAR targeting CD7.(53) These CD7 CAR T cells demonstrated efficacy *in vitro*, confirming the feasibility of this platform. The immunodeficiency resulting from pan-T cell depletion, however, would remain as a significant barrier to the clinical translation of this strategy.

Conclusions and future directions

CART19 (Kymriah, Novartis) is now an FDA-approved drug and it is likely that other T cell therapies will follow in the coming few years. As such, there is great interest in building upon this technology to further expand access to these treatments and improve responses. The development of technologies that allow surgical gene-editing has opened new avenues for engineering of T cells. CRISPR-Cas9, TALEN and other machineries allow efficient and relatively simple gene knock-out and are being applied to the generation of universal T cells, exhaustion-resistant T cells and to reducing the on-target toxicity of redirected T cells. The preclinical results and data from early clinical trials are promising but more clinical experience with these technologies, in particular CRISPR-Cas9, is required to confirm safety and efficacy. Several trials are ongoing and early results should be available in the next few years. The rapid progression of ACT has generated great promise, and the application of gene-editing technology generates an opportunity to successfully treat many more patients using engineered T-cell products.

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Table 1

T cell gene-editing trials in immunotherapy for cancer (as of 9/16/2017)

	Product	Target	Tumor	Start date	Academia	Industry	Phase	Construct (scFv-CD-SD)	Gene-editing	Active clinical trials	Reports	Status
1	CCR5KO CD34+ HSC	NA	HIV+hematologic malignancies	May-17	Affiliated Hospital to Academy of Military Medical Sciences, China	NA	1	NA	CCR5 CRISPR KO	NCT03164135	NA	R
2	PD1 KO T cells	NA	Advanced Esophageal Cancer	Mar-17	Hangzhou Cancer Hospital, China	Anhui Kedgene Biotechnology Co.,Ltd	2	NA	PD-1 CRISPR KO	NCT03081715	NA	R
3	PD1 KO T cells	NA	Muscle-invasive Bladder Cancer	Sep-16	Peking University, China	Cell Biotech Co., Ltd.	1	NA	PD-1 CRISPR KO	NCT02863913	NA	NYR
4	PD1 KO T cells	NA	Castration Resistant Prostate Cancer	Nov-16	Peking University, China	Cell Biotech Co., Ltd.	1	NA	PD-1 CRISPR KO	NCT02867345	NA	NYR
5	PD1 KO T cells	NA	Metastatic Renal Cell Carcinoma	Nov-16	Peking University, China	Cell Biotech Co., Ltd.	1	NA	PD-1 CRISPR KO	NCT02867332	NA	NYR
6	PD1 KO T cells	NA	Metastatic Nonsmall Cell Lung Cancer	Aug-16	Sichuan University, China	Chengdu MedGenCell, Co., Ltd.	1	NA	PD-1 CRISPR KO	NCT0279385	NA	R
7	UCART19	CD19	B-cell neoplasms	Nov-16 Aug-16 Jun-16	KCL/UCL, London, UK	Servier/Cellectis	1	4g7-CD8-41 BB-CD3z	TCR and CD52TALEN KO	NCT02735083 NCT02746952 NCT02808442	(35)	R R R
8	UCART19	CD19	B-cell leukemia/ lymphoma	Jun-17	Chinese PLA General Hospital, China	NA	1	4-1 BB-CD3z	TCR, B2M CRISPR KO	NCT03166878	NA	R
9	UCART123	CD123	BPDCN/AML	Jun-17 Jun-17	Weill Cornell Medical College, US	Cellectis S.A	1	NA-CD8-41BB-CD3z	TCR and CD52TALEN KO	NCT03203369 NCT03190278	NA	S S
10	EBV-CTL	EBV antigens	Advanced Stage Epstein-Barr Virus (EBV) Associated Malignancies	Apr-17	The Affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School	NA	1/2	NA	PD-1 CRISPR KO	NCT03044743	NA	R

HIV=human immunodeficiency virus; BPDCN= blastoid plasmocitoid dendritic cell neoplasm; AML=acute myeloid leukemia; R= recruiting; NYR= not yet recruiting