Emerging CRISPR/Cas9 applications for T cell gene editing

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Abstract

Gene editing tools are being rapidly developed, accelerating many areas of cell and gene therapy research. Each successive gene editing technology promises increased efficacy, improved specificity, reduced manufacturing cost and design complexity; all of which are currently epitomised by the clustered regularly-interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas9) platform. Since its conceptualisation, CRISPR-based gene editing has been applied to existing methodologies, but also allowed the exploration of novel avenues of research. Implementation of CRISPR/Cas9 has been instrumental to recent progress in the treatment of cancer, primary immunodeficiency, and infectious diseases. To this end, T cell therapies have attempted to harness and redirect antigen recognition function, and through gene editing, broaden T cell targeting capabilities and enhance their potency. The purpose of this review is to provide insights on emerging applications of CRISPR/Cas9 in T cell therapies, to briefly address concerns surrounding CRISPR-mediated indel formation, and to introduce CRISPR/Cas9 base editing technologies that hold vast potential for future research and clinical translation.

Introduction

Targeted gene editing has transformed the scientific landscape allowing for site-specific disruption, activation or repression of genes, targeted integration of exogenous or corrective DNA sequence and more recently in situ base conversion, all producing cells with heritable DNA modifications. While first established with Zinc Finger Nucleases (ZFNs) [1], a number of tools namely meganucleases (MNs) [2, 3], transcription-activator-like effector nucleases (TALENs) [4, 5], megaTALs [6, 7] and lastly clustered-regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas9) [8-10], have since made their appearance in quick succession boasting increased activity, improved specificity, and reduced manufacturing cost and complexity. The immunology field has benefited immensely over the last decade of gene editing opening previously unfathomable avenues.

T cells, represent an essential component of the adaptive immune system, crucial in pathogen clearance and tumour surveillance through the recognition of antigens presented by major histocompatibility complex (MHC) molecules. Antigen specificity is governed by the T cell receptor (TCR)-CD3 complex, acquired during T cell development in the thymus [11]. T cell engineering strategies have been applied to a range of diseases including cancer, acquired immunodeficiency, and inherited disorders.

This review aims to summarise advancements made in the application of emerging CRISPR/Cas9 gene editing technologies in the context of T cell therapies.

CRISPR/Cas9 delivery to T cells

T cells tend to be amenable to genomic manipulation, resisting transformation as a result of pro-apoptotic mechanisms preventing clonal outgrowth, making them ideal targets for CRISPR/Cas9-based editing [12, 13]**(Figure 1)**. Whereas, initial studies relied on plasmid based [14] or lentiviral delivery of entire CRISPR/Cas9 cassettes [15-17], concerns surrounding the prolonged and constitutive expression of prokaryote derived Streptococcus pyogenes (spCas9), deemed such strategies unfit for clinical translation [18, 19] **(Figure 1a and b)**. T cells, have instead mostly benefited from delivery of individual CRISPR/Cas9 components through co-electroporation of single-guide RNA (sgRNA) with Cas9 supplied as mRNA or protein [20, 21] **(Figure 1c and d)**. Initial reports using enzymatically in vitro transcribed (IVT) sgRNAs were riddled by editing efficiencies of below 3%, attributed to their rapid degradation in vitro [22]. Furthermore, incomplete removal of triphosphates at the 5’ end of IVT sgRNAs have been shown to trigger potent innate immune responses in mammalian cells, akin to those against RNA viruses through upregulation of retinoic

acid-inducible gene I (RIG-1) [23]. Automated solid-phase RNA synthesis of sgRNAs, besides offering unprecedently high levels of editing, can assist in evading innate immune sensing through chemical modifications [22, 24]. In particular, 2'-O-methyl 3'phosphorothioate (MS) modifications, have immensely improved stability and editing efficiencies by more than 30-fold (75.7%-83.3%) over non-modified sgRNAs when transiently co-delivered with Cas9 mRNA or as ribonucleoprotein (RNP) complexes in primary T cells and are now being implemented in the vast majority of studies [22, 25, 26].

Double stranded DNA (dsDNA) breaks caused by CRISPR/Cas9 will in the absence of exogenous template be repaired by the error prone non-homologous end joining (NHEJ) pathway, in most cases resulting in loss of function mutations. While desirable for the purpose of genomic disruption, targeted correction requires DNA template containing the desired sequence for initiation of the homologous-directed repair (HDR) pathway. Integration deficient lentiviral (IDLV) or adeno-associated viral (AAV) vectors used to transport large templates or entire genes into edited cells were met with modest efficiency with HDR ranging between 40%-60% [7, 27, 28]. These have predominantly focused on earlier generation editing tools with few studies having tested their efficiency in CRSPR/Cas9 edited T cells [29-31].

Alternative HDR approaches in CRISPR edited T cells have used single-stranded synthetic oligodeoxynucleotide (ssODN) spanning approximately 200bp, 30-60bp of which comprise the 5’ and 3’ homology arms and therefore mostly applicable in site-specific mutation correction or replacement of small sections of genomic DNA [25, 32-34].

A recent study, exploring a non-viral approach delivering dsDNA for correction of inherited autoimmune-associate mutations in T cells, found contrary to expectations [35, 36], that dsDNA templates >1kb, co-delivered with CRISPR/Cas9 RNPs, could integrate into the break site with modest efficiency and little impact on cell viability [34]. However the authors reported non-HDR insertions at both the induced (~1%) and endogenous (~0.01%) dsDNA break site when using these dsDNA templates. Incorrect HDR events were reduced to almost undetectable levels when using long single stranded DNA templates. Technical progressions such as these can broaden applicability and importantly, have a significant clinical impact by dramatically reducing costs and manufacturing hurdles associated with viral approaches.

T cell immunotherapy:

T cell immunotherapies redirect the immune system to specifically target and lyse pathogenic organisms directing these response towards desired antigens while minimising detrimental ‘off-target’ effects on healthy tissue. This initially relied on non-specific stimulation of the host immune

system in an attempt to increase reactivity to tumour antigens [37]. To this end, O'Connell et al, administered methanol extraction residue of Bacillus Calmette-Guerin (MER) to advanced cancer patients. Despite antineoplastic effects seen in pre-clinical models, results from this clinical trial indicated no improvement in medium survival (13 weeks with high dose MER versus 16.5 weeks in the placebo group). Due to such setbacks, approaches relying on non-specific immune activation were widely abandoned in favour of adoptive T cell transfer (ACT) [37, 38]. This involved the *ex vivo* isolation, activation and expansion of autologous antigen specific T cells before being re-administered to the patient [39, 40]. Pioneering experiments such as these, highlighted the ability of the immune system to promote anti-tumour responses through autologous lymphokine-activated killer cells [37] and tumour infiltrating lymphocytes (TILs) [41]. Of note Rosenberg et al, demonstrated the ability to extract and expand TILs from resected melanomas for autologous reinfusion in combination with IL-2 resulting in objective regression in 9 of 15 patients [42]. However TIL based therapy is hampered by the reliance on the pre-existence of TILs, as well as the laborious procedure of isolating and expanding these cells within a suitable time course for the patients [12].

Exploration of allogeneic donor leukocyte infusion (DLI) protocols were next attempted with caution to balance beneficial graft versus leukaemia (GVL) effects against potentially fatal graft versus host disease (GVHD). Despite modest levels of efficacy, early investigations called for refinement of T cell products [43, 44]. This need for refinement is exemplified by Kolb and associates, reporting 2 of 3 patients showing clinically significant GVHD in a HLA matched setting after DLI, which was managed with immunosuppressive therapy [43]. Both autologous and allogeneic cell therapies have greatly improved through advances in T cell engineering allowing *ex vivo* gene modification of peripheral blood T cells to express receptors of desired specificity. There are currently two widely applied methods of redirecting T cells to a specific antigen, outlined below.

Transgenic TCR therapies:

Redirection of T cells towards a specific antigen can be achieved through expression of full-length TCRα and β chains. Complexes formed between transgenic TCR chains and the endogenous CD3 complex (CD3ε, CD3δ, CD3γ, and CD3ζ chains) allows T cells to engage specific short linear peptide epitopes, such as tumour associated antigens in the context of an MHC molecule **(Figure 2a)**. Results from their use in a variety of tumour types including Hepatocellular Carcinoma [45], B-cell malignancies [46], WT1 expressing tumours [47, 48], sarcoma and melanoma [49] have been encouraging.

Although transgenic TCRs for ACT have proven highly effective, competition between endogenously expressed TCR chains for components of the CD3 complex often lower cell surface expression of

transgenic TCRs [47, 50, 51]. In order to mitigate this concern, gene editing tools have been used to knockout endogenous TCR chains using ZFNs [50, 51], TALENs [50, 52], megaTAL [53], and CRISPR/Cas9 [34, 50, 53, 54]. Roth and associates, presented proof of principle that CRISPR/Cas9 gene editing for targeted insertion of a transgenic TCR against NY-ESO-1 into the endogenous T cell receptor alpha constant (TRAC) locus was viable, achieving expression of ~12%. This strategy not only alleviates competition for CD3, but also allows regulated expression of the transgenic TCR from the endogenous promoter [34].

Additionally, mispairing between endogenous and transgenic TCR chains could form TCR-CD3 complexes with unknown specificity, potentially evoking severe autoimmune responses in healthy tissue [47, 52, 55]. Pairing of TCR chains is restricted to αβ and γδ. Legut et al. expressed a γδ transgenic TCR with CRISPR-based knockout of the endogenous β chain, which lessens concerns surrounding TCR mispairing, and negated editing of both endogenous TCRα and β chains [54].

Successful reports of CRISPR/Cas9 gene editing in conjunction with transgenic TCR expression has fuelled a phase 1 clinical trial in multiple myeloma patients (NCT03399448) where patients will receive autologous T cells transduced with an NY-ESO-1 specific TCR, edited for removal of PD-1 and TCRα and β chains.

CAR-T cell therapies

Unlike transgenic TCRs, chimeric antigen receptors (CARs) fuse the highly specific antigen-binding domain, single-chain variable fragment (scFv), of a monoclonal antibody to the activation and costimulatory domains of a TCR, endowing them with MHC-independent target recognition [56, 57] **(Figure 2b)**. Since their conception, a variety of iterations improving upon their efficacy have been developed and used to generate autologous anti-CD19 CAR T cells for the treatment of adult and paediatric B cell acute lymphoblastic leukaemia (B-ALL) [58-61]. While focus has predominantly been on ubiquitously expressed CD19 in B cell malignancies, targeting range to alternative antigens and cell types has been greatly extended. Despite the highly encouraging results, logistical roadblocks in collection of autologous material and considerable variability in manufacture have greatly limited access to CAR T cell therapies **(Figure 3a)**. Similarly, identification of suitable allogeneic matched donors can be labour-intensive and financially burdensome due to the bespoke nature of each manufacture. Gene editing has drastically transformed the field facilitating removal of alloreactive surface antigens in an effort to overcome HLA barriers.

A seminal study led by Qasim et al. first illustrated the clinical outcome of 'off-the-shelf' universal CAR T cell (UCART19) therapies. TALEN-edited $TCR^{-}CD52^{-}$ allogeneic anti-CD19 CAR T cells devoid of

endogenous alloreactive TCR and resistant to CD52-targeting chemotherapeutic antibody Alemtuzumab. The UCART19 cells exhibited highly potent anti-leukaemic effects in two paediatric lymphoblastic leukaemic relapse patients, entering molecular remission by day 28 prior allogeneic stem cell transplantation [62].

Further refining of 'off-the-shelf' allogeneic CAR T cells was aided by CRISPR/Cas9, which owing to its reduced complexity and size, allowed incorporation of TRAC targeting sgRNA within the 3' long terminal repeat (LTR) of a CAR19-expressing lentiviral vector with transient delivery of Cas9 mRNA [63]. This enabled coupling of CRISPR edits with transgene expression yielding highly pure and homogenous $TCR^{-}CAR^{+}$ therapeutic T cells exhibiting strong anti-leukaemic effects in a xenograft mouse model. Scalability of the manufacture confirmed its clinical potential with early phase safety studies imminent **(Figure 3b)**.

An alternative strategy exploring knocking out through knock-in, used AAV carrying DNA template coding for a promoterless anti-CD19 CAR flanked by TRAC homology arms in CRISPR/Cas9 edited allogeneic T cells [30]. HDR-mediated site-specific integration of CAR19 into the TRAC locus resulted in CAR19 expression off the endogenous TRAC promoter offering a level of regulatory control and as a result, reduced T cell exhaustion effects.

Aside from B-ALL, CAR T cell therapies have been considered for T cell acute lymphoblastic leukaemia (T-ALL) accounting for approximately 15% and 25% of paediatric and adult ALL cases, respectively. Despite a long-term-survival rate of over 75% in paediatric and ~50% in adult patients, levels of morbidity and mortality are extremely high in relapse cases [64, 65]. While CAR-T cells have been highly effective in lysing B cells, targeting of T cells has proven significantly more challenging due to fratricidal T-on-T effects hampering manufacture [66, 67]. CD5 T cell antigen, is highly expressed by T-ALL blasts; Mamonkin et al. showed that targeting of CD5 surface antigen resulted in potent anti T-ALL effects in vivo [68]. In an effort to reduce fratricide, however, Raikar et al. designed CRISPR/Cas9 CD5 knockout anti-CD5 CAR T cells, which were able to overcome barriers of self-activation while preserving their potency [67]. Definitive T cell marker, CD3, presents an alternative targeting option for elimination of $CD3^{+}$ lymphomas. High levels of CD3 expression on CAR T cells, necessitates the removal of the TCRαβ/CD3 complex to prevent fratricide [69]. Critically scheduled TALEN-mediated TRAC disruption in anti-CD3-specific CAR T cells allowed for propagation of $3CAR^{+}TCR^{-}$ cultures with potent anti-leukaemic effects against primary T-ALL targets, however, variability of CD3 expression in T-ALL stipulates combinational targeting of surface antigens such as CD7, an option facilitated by CRISPR/Cas9.

An example of this was demonstrated by Cooper et al. who generated CRISPR-edited universal 'off-the-shelf' allogeneic anti-CD7 CAR T cells (UCART7) devoid of endogenous CD7 and TCR preventing both fratricide and minimising GvHD, while maintaining robust in vivo CD7-directed killing of primary T-ALL cells [66].

Although expression of CD7 is naturally restricted to T cells, NK cells and their precursors, it has also been reported to be aberrantly expressed by therapy-resistant leukaemic clones in approximately 30% of acute myeloid leukaemia (AML) cases. To this end, Gomes-Silva et al. demonstrated the feasibility of this hypothesis using CRISPR/Cas9 edited $CD7^-$ anti-CD7 CAR T cells and reported effective clearance of AML lines and primary AML blasts with no impact on normal myeloid progenitor and mature cells [70, 71].

Checkpoint inhibitor blockade

T cell exhaustion as a result of perpetual stimulation can often lead to tumour re-emergence. Programmed cell death protein-1 (PD-1), transiently up-regulated on T cells during early activation, has also been identified as key regulator of T cell fate and marker of exhaustion [72, 73]. The clinical significance of targeting PD-1 becomes apparent in the tumour microenvironment setting where inhibitory ligand PD-L1 up-regulation impairs cytotoxic T cell function [74]. Benefits of antibody-mediated PD-1 blockade on anti-Her2 CAR T cells have previously been reported [75], however antibody durability and half-life as well as its non-targeted delivery can restrict its use. This has been addressed through permanent CRISPR/Cas9 mediated ablation of the PDCD1 gene in primary cytotoxic T lymphocytes (CTLs) with reports of increased cytokine production and enhanced killing against multiple myeloma cells [76] and Epstein Barr virus (EBV) positive gastric cancer cells [77]. In addition, ex-vivo delivery of PD-1 targeting sgRNA as a Cas9-sgRNA RNP complex in lentivirally transduced CAR19 cells has now been reported to enhance anti-tumour efficacy [78]. This study however, exposed two important considerations; firstly, expression of endogenous TCR would undoubtedly result in severe auto-reactive or allo-reactive effects, and importantly, enhancing CAR activity through PDCD1 knockout may be undesirable resulting in unmanageable cytokine release syndrome (CRS) [78]. These have partially been addressed by multiplexing CRISPR knockouts through co-electroporation of three distinct sgRNAs against TCRα and β chains, beta-2 microglobulin and PD-1 alongside Cas9 mRNA in lentivirally transduced anti-CD19 or anti-PSCA CAR T cells [79]. Separately, the authors investigated the incorporation of TCR and HLA class I CRISPR cassettes within the CAR19-encoding vector achieving >70% double knockout [80]. As a safeguard, pro-apoptotic Fas receptor

(FasR) CD95, the interaction of which to tumour necrosis factor (TNF) Fas ligand (FasL) has direct links to cancer progression and attenuation of anti-tumour potency by CAR T cells [81], was also targeted in a triple TCR/HLA-1/FasR sgRNA knockout experiment. Effector $CAR19^{+}TCR^{-}HLA\text{-}1^{-}FasR^{-}$ cells appeared less prone to apoptosis in vitro and in vivo with elevated levels of degranulation and enhanced killing [80]. Checkpoint inhibitor cytotoxic T lymphocyte-associated protein 4 (CTLA-4) is also an attractive target as showcased by the significant improvement of anti-tumour activity of CTLs following its disruption [82]. To a similar effect, CRISPR/Cas9 deletion of lymphocyte activation gene-3 (LAG-3), a negative regulator of T cell activity, was assessed on CD19 CAR T cells. While there was no significant detriment to their immune phenotype, there was no reported advantage to the LAG-3 knockout [83]. The synergetic mode of operation of LAG-3 with PD-1 may imply that dual knockout is essential for a superior functional outcome [84].

PD-1 blockade may be more profound in the more challenging solid tumour setting where tumour microenvironment can lead to suppression of CAR T cells from PD-L1 expressing cancer cells. This was demonstrated in human triple-negative breast cancer where CAR T cells against overexpressed tumour differentiation antigen mesothelin, treated with CRISPR/Cas9 RNPs for PD-1 disruption exhibited enhanced tumour clearance and relapse prevention [85]. Similar observations were made in hepatocellular carcinoma targeting GPC3-CAR T cells devoid of PD-1, presenting with improved anti-tumour efficacy, cytokine production, infiltration and survival of the $PD\text{-}1^{-}$CAR T cells [86].

CRISPR/Cas9 has already made its debut in the clinic at the Sichuan University in Chengdu for the targeted disruption of PD-1 for treatment of metastatic non-small cell lung cancer (NCT02793856). Autologous T cells underwent CRISPR-mediated ablation of PD-1 preceding administration to 8 patients, 2/4 of which in the higher dose cohort presented with stable disease [87]. The same approach has been implemented in clinical trials currently underway for prostate cancer (NCT02867345), bladder cancer (NCT02863913) and renal cell carcinoma (NCT02867332).

While the benefits of PD-1 knockout have clearly been demarcated across these studies, genome-wide screening identifying PDCD1 as a master gene involved in suppression of oncogenic T cell signalling, with mutations leading to T cell lymphomas, should not be discounted and treated with extreme caution [88].

Targeting of primary immunodeficiencies

Gene editing of T cells however, extends beyond the realms of immunotherapies. Attempts for the correction of primary immunodeficiency (PID) X-linked hyper-immunoglobulin M (hyper-IgM)

syndrome (XHIM), affecting immunoglobulin class switching due to mutations in CD40 ligand (CD40L), have been made through conventional gene addition/replacement strategies of the affected gene. Expression of CD40L from a constitutive promoter however, lead to dysregulation of gene expression and abnormal lympoproliferation [89]. TALEN-mediated editing first showed the ability to deliver wild-type sequence by AAV to the target locus, restoring physiologic expression of the gene [90]. Kuo and colleagues using both TALEN and CRISPR/Cas9 reagents achieved high levels of genome modification (30.6%-33.7%) and ultimately site-specific integration (16.2%-20.8%) of corrective sequence bypassing all known disease causing mutations in patient-derived T cells. Importantly, similar results were also achieved in long-term repopulating haematopoietic stem cells (HSCs), which could provide permanent immune reconstitution [91].

HIV targeting therapies

Human immunodeficiency virus (HIV) entry relies on interaction with the CD4 receptor and subsequent binding to either chemokine receptor 5 (CCR5), or CXC chemokine receptor (CXCR4) [92, 93]. Infection eventually leads to progressive loss of CD4+ T cells, resulting in a highly immunocompromised state. With >30 million HIV patients, the need for treatment is imperative [94]. Highly active antiretroviral therapy (HAART) can supress HIV replication to almost undetectable levels [95], however, some advanced immunodeficiency patients fail to completely reconstitute CD4+ T cell counts >500 cells/mm^3 [96]. Furthermore, discontinuation of HAART treatment results in reappearance of replicating HIV from persistent viral reservoirs, indicating the requirement for life-long drug administration [97].

CRISPR/Cas9 Gene Editing Promotes HIV Resistance

Individuals homozygous for the CCR5Δ32 gene mutation were reported to be naturally resistant to HIV-1 infection [98, 99]. This was clinically demonstrated in 2007 when an HIV-1 positive patient received HLA matched allogeneic CD34+ stem cells homozygous for CCR5Δ32, after which infection became undetectable [93]. Due to limited CCR5Δ32 donors, this treatment is not possible for the majority of patients. This barrier, has been addressed through knockout of the CCR5 locus to generate primary CD4+ T cells resistant to CCR5 tropic HIV-1 infection using ZFNs [100, 101], TALENs [102-104], and megaTALs [6, 7, 94].

Robust CCR5 disruption has also been demonstrated using CRISPR/Cas9 in human CD4+ T cell lines utilising lentiviral vectors expressing both spCas9 and sgRNA [16, 17, 105]. The chosen delivery

method, did not result in editing of primary T cells. CCR5 and CXCR4 knockout in primary T cells, appeared more successful when utilising DNA plasmid, or RNP complex electroporation. Importantly, this translated to marked protection and selective advantage of $CCR5^{-}CXCR4^{-}$ edited cells when challenged with either CCR5 or CXCR4 tropic HIV-1 [17, 105].

Alternative strategies have included expression of anti-HIV restriction factors such as Rev M10, APOBEC3G D128K, and hrhTRIM5α [106] or C46 HIV fusion inhibitor [7] by targeted knock-in into the CCR5 locus establishing multiple parallel blocks to HIV-1 infection. A similar concept from the University of Washington aimed to combat persistent viral reservoirs by HDR-mediated integration of an anti-HIV-CAR into the CCR5 locus [7, 94]. Although at the time of writing, such methods have yet to employ CRISPR/Cas9, it is expected that further application will involve this highly versatile tool.

Editing of the Latent viral reservoir:

Elimination of proviral DNA from latently infected viral reservoirs has potentially curative implications. Gene editing using ZFNs [107, 108] and TALENs [109, 110] have already been used to recognise HIV proviral DNA sequences. Similarly, CRISPR/Cas9 is widely being adopted for this purpose, delivered either transiently to inactivate existing HIV provirus, or stably expressed, providing defence towards invading HIV-1.

Ebina et al. aimed to validate CRISPR/Cas9 gene editing efficiency in models of latently infected cells by targeting the highly conserved TAR sequence, present within the repeat region of both proviral LTRs [111]. Transfection of CRISPR reagents into a T cell line transduced with an inducible GFP expressing lentiviral vector, resulted in >60% reduction of reporter expression [111]. As well as demonstrating efficient viral inactivation through CRISPR/Cas9 indel formation within the HIV-1 LTR, full proviral excision was observed in approximately 30% of the population. Similar results were demonstrated by Hu et al. providing evidence that stable expression of sgRNA and spCas9 can grant healthy cells protection from HIV-1 infection [112]. Further work screening protospacer sequences across the HIV-1 genome, concluded that targeting the HIV-1 Rev gene resulted in highest levels of disruption [113].

Despite initial encouraging results, two groups independently reported HIV-1 re-emergence in CD4+ T cells expressing proviral-specific sgRNAs [114, 115]. Viral escape was attributed to non-deleterious indel formation following NHEJ averting proviral excision and stimulating viral production. These CRISPR-induced indels, destroyed protospacer recognition sequences, allowing HIV-1 to rapidly

mutate out of this treatment strategy. To avoid CRISPR-mutation escape, Ophinni et al. tested 3 sgRNAs targeting conserved regions of two genes critical for viral transcription, Rev and Tat indicating that combinational disruption provides enhanced repression of HIV-1 infection [116]. Alternatively, combination of CRISPR and RNA interference allows assault on both genetic elements of HIV-1, DNA and RNA, resulting in greater inhibition of viral escape compared to when delivered individually [117]. Significant barriers to this mode of therapy still exist. However, the potential of eliminating latent viral reservoir has inspired much interest. Recent investigations have focused on a Tat inducible nuclease to reduce possible immunogenicity and 'off-target' cleavage events [107], as well as novel CRISPR/ Cas9 gesicle base delivery tools [118].

CRISPR uncut: Base Editing

CRISPR/Cas9 gene editing specificity has seen improvements by using truncated protospacers [119], paired D10A nickase Cas9 [120], or high-fidelity Cas9 variants [121]. There still however remains potential for off-target indel formation, large deletions extending over kilobases, and chromosomal translocations [122-124]. In silico prediction software attempts to rank sgRNAs based on their on and off target potential, however, this method is not without limitations [125]. To this end novel in vitro techniques that rely on high throughput sequencing of genomic DNA treated with RNP complexes have been developed [126-130]. Nevertheless, there has yet to be an industry standard for detection of off-target events.

In 2016, the Liu group described the first CRISPR/Cas9 founded base editing tool capable of generating precise base conversions without DNA DSBs, or exogenous template DNA [131]. First generation base editors (BE1) fused a rat APOBEC1 cytidine deaminase to a catalytically dead spCas9 endonuclease (dCas9) allowing for C>T conversions. Further refinements saw the addition of a uracil DNA glycosylase inhibitor (UGI) and replacement of dCas9 with D10A Cas9 (nCas9) nickase forming a third generation base editor (BE3) [131, 132]. Later work described Adenosine deaminase base editors (ABE), able to generate adenosine (A) to guanine (G) modifications by replacing rAPOBEC1 with a modified E.coli TadA enzyme [133].

Since their conceptualisation, base editors have been rapidly optimised [134-136] and developed using alternative deaminase [137-139]. Moreover, changes to the deaminase activity window have increased both precision and utility [140, 141]. High-fidelity base editors, as well as broadened

targeting range Cas9 nucleases are also now in development [142, 143]. Expansion of the number of targetable bases has also been achieved by replacing the spCas9 nickase with other endonuclease such as saCas9 [141, 144] or dCas12a [135].

Applications have included crop improvement [137, 145, 146], generation of animal models [147-149], development of gene disruption tools [141, 150, 151], exon skipping strategies [152], and correction of pathogenic single nucleotide polymorphisms (SNPs) [131, 153]. The Liu group offer an elegant review of these advances [154].

Despite reports of base editing in a number of mammalian cells types, including fibroblasts [134, 136], post mitotic sensory cells [155], cancer cell lines [136], blastocysts and embryonic cells [147, 148, 153, 156, 157], its application to date in primary T cells has been modest. Only recently has a report showcased its potential for the generation of 'off-the-shelf' CAR-T cells [158].

Conclusion

Gene editing technologies have generated significant breakthroughs across broad scientific disciplines through their inherent ability to create precise genomic modifications, facilitating modelling, prevention and correction of disease. Most noteworthy, however, has been their successful implementation in the oncology field accelerating clinical treatment development. Rapid advancements in gene editing technologies have witnessed the emergence of novel tools, namely CRISPR/Cas9, augmenting targeting capabilities and maximising accessibility. Innovative refinements aim to increase their safety profile and endow them with clinically relevant features. The versatility and omnipresence of CRISPR/Cas9 has revolutionised T cell based therapies, unleashing the power of the immune system and propelling the transition from bench to bedside.

Summary points

1. CRISPR/Cas9 has been widely incorporated by several research groups, primarily due to its simplistic design requiring minimal specialist knowledge, enhanced targeting efficiency and affordability.
2. CRISPR/Cas9 based immunotherapies have stimulated advancements in the manufacture of transgenic TCR products, and 'universal-off-the-shelf' T cell therapies.
3. CRISPR/Cas9 is under development for inducing T cell resistance to HIV-1 infection as well as disruption of the latent viral reservoir with potentially curative implications.

4. CRISPR-mediated indels have potentially unknown long-term effects; advancements in base editing aim to mitigate such concerns and hold vast potential for T cell engineering.

Figure legends

Figure 1. Delivery strategies of CRISPR/Cas9 reagents into T cells. a. Integrating viral vectors encoding single or multiple CRISPR sgRNA cassettes can be delivered to T cells by viral transduction. Cas9 sequence can be coupled in the sgRNA vector, delivered as a separate vector or supplied transiently as mRNA or protein. Viral entry proceeds with uncoating, and stable integration of CRISPR/Cas9 sequences into cell genome. Transcription of CRISPR/Cas9 sequence and translation of Cas9 mRNA into protein is followed by ribonucleoprotein (RNP) complex formation and trafficking into nucleus for targeted genomic cleavage. **b.** Plasmid DNA encoding CRISPR sgRNA cassettes either coupled to Cas9 or as separate plasmids can be delivered to T cells by transfection or electroporation. Episomally expressed DNA will undergo transcription of sgRNA and Cas9 and following its translation will form RNPs that will enter the nucleus and cleave the target genome. **c.** Enzymatically or chemically synthesised sgRNAs can be transiently transported along with Cas9 mRNA into T cells by electroporation. Following mRNA translation and RNP formation complexes will enter the nucleus and edit the target sequence. **d.** Cas9 protein can be pre-complexed with enzymatically or chemically synthesized sgRNA to form an RNP before electroporating into T cells. RNPs will traffic to the nucleus and cleave target sequence.

Figure 2. Tumour target recognition by transgenic TCR or CAR expressing T cells. a. The transgenic T cell receptor (TCR) comprises an α and β chain which are closely associated with the γ, δ and ε chains and the signal activating ζ chain to form the CD3 complex. Tumour peptide recognition is carried out in an MHC class I dependent manner. Engagement of the transgenic TCR results in its activation for the degranulation of the target cell. **b.** The chimeric antigen receptor (CAR) comprises a single chain variable fragment (scFv) of a monoclonal antibody, linked to a transmembrane stalk, a 41BB or CD28 co-stimulatory domain and a CD3ζ signalling domain. Tumour antigen recognition occurs in an MHC class I independent fashion. Engagement of scFv with tumour antigen activates the CAR T cell to lyse the target cell.

Figure 3. Autologous versus allogeneic CAR T cell therapies. a. Autologous CAR T cell therapies are bespoke treatments using the patient's own cells for the development of a personalize therapeutic. The procedure involves harvesting of peripheral blood T lymphocytes from the patient that are modified ex vivo with a viral vector delivering a CAR construct to the cells. Following expansion, the autologous CAR T cells are re-infused into the patient and monitored for tumour clearance. **b.** Allogeneic gene edited CAR T cell therapies use healthy donor material for the treatment of

unmatched recipients. Healthy peripheral blood T lymphocytes are harvested and gene modified ex vivo to express a CAR construct. CRISPR/Cas9 is employed for the knockout of alloreactive or chemotherapeutic antibody targets. Gene edited CAR T cells are expanded and administered to multiple patients without the requirement for donor matching.

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Declaration of interest

The authors declare that they have no conflict of interest.

Author contributions

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