Immunotherapy to get on point with base editing

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Engineered immune cell therapy is revolutionising the field of cancer therapeutics. US Food and Drug Administration (FDA) approval of two chimeric antigen receptor (CAR)-T cell products for the treatment of haematological malignancies paved the way for individualised cancer treatment. However, multiple genetic edits will be required to improve the efficacy of CAR-T cell therapies if they are to treat refractory malignancies successfully, particularly solid tumours. Off-target effects of CRISPR–Cas9-mediated multiplex editing are likely to hinder its safety and application in the clinic. Novel base editing technologies offer a promising and safer alternative for simultaneous editing that could enhance allogeneic engineered immunotherapies for targeting solid tumours and other complex human diseases.

Keywords: Cell therapy; Base editing; CRISPR; Gene editing; CAR-T; Immunotherapy

Introduction

The capacity to alter *ex vivo* the way that immune cells behave and then to re-infuse these modified cells into a patient as a therapeutic would have seemed far-fetched only a few years ago. However, the ability to manipulate the genome rapidly and relatively precisely with gene-editing machinery, coupled with the capacity to expand and maintain sterility and activity of freshly isolated T cells *in vitro*, has made this a reality. In 2017, the US Food and Drug Administration (FDA) approved two genetically engineered T cell therapeutics, axicabtagene ciloleucel (Yescarta®) and tisagenlecleucel (Kymriah®), for the treatment of patients with B cell malignancies. Cell-based therapeutics are now seen as a potential new mode of treatment for many human diseases, with the caveat that most will require genetic engineering to modify or direct cell behaviour. Gene editing is a fast-moving field, with new developments being published almost every week, which push the development of cell therapeutics forward. However, caution is needed when editing or modifying multiple genes, as is required for the treatment of solid tumours with engineered T cells.

Autologous cell therapies

The autologous transplantation of T cells that are extracted from a patient, genetically modified *in vitro*, and subsequently re-infused into the same patient describes the process approved for Yescarta® and Kymriah®. These treatments are examples of adoptive cell therapies, which harness the immune system to target malignant cells through the introduction of a recombinant T cell receptor (TCR) or chimeric antigen receptor (CAR) that targets antigen expressed on the surface of the cancer cell. Yescarta® and Kymriah® were designed with a CAR directed against CD19, a protein expressed on the surface of large B cell non-Hodgkin lymphoma and B cell acute lymphoblastic leukaemia (B-ALL), respectively [1–3]. Brexucabtagene autoleucel (Tecartus®), which shares the same engineering approach and CAR as Yescarta®, and lisocabtagene maraleucel (Breyanzi®), another CD19-directed CAR-T therapy, were more recently approved for the treatment of relapsed or refractory mantle cell lymphoma [4] and relapsed or refractory large B cell lymphoma [5], respectively.

Although the initial response of patients to CAR-T cells is high, durable disease remission is not always evident. Of the

patients who showed an initial response to Yescarta® or Kymriah®, only half maintained disease remission during the first year after infusion. This highlights that further improvements are required to ensure continued efficacy of the cell product and ultimately prevent disease relapse [1,2]. Such improvements could be brought by additional gene engineering, but other limitations of autologous CAR-T cells, in particular their complex manufacture, question whether the paradigm of autologous CAR-T therapy is sustainable.

To be successful, autologous cell therapy requires highly coordinated and reliable manufacturing and shipping processes, which must be delivered in a safe and timely manner. Although some studies reported the successful manufacture of Yescarta® for 99% of patients enrolled in a clinical trial with 91% of those patients eligible to receive their edited T cells after manufacture [2], other clinical trials have shown higher attrition rates, with almost 30% of patients being unable to receive their modified cells [6,7]. The process from blood collection through to re-infusion of the engineered T cells takes at least 3 weeks [3]; therefore, patients with aggressive disease or rapid disease progression, such as in acute leukaemia, might not withstand this delay and will no longer be eligible for safe infusion, or will have died by the time the autologous cell product is available [1–3,6,7]. In addition to logistical limitations, the patient's own T cells might be too few or too fragile as a result of prior chemotherapy and exposure to an immune-suppressive tumour microenvironment (TME), thereby precluding the feasibility of autologous cell therapy [3,7,9]. The high cost of treatment, in the range of US $350 000–500 000 (excluding additional healthcare need or potential price increases), further limits the commercialisation and equitable deployment of autologous CD19-directed CAR-T therapies.

Allogeneic cell therapies

Manufacturing CAR-T cells from healthy donors circumvents the problems of insufficient, variable, or poor-quality source material from the patient and overcomes time constraints that exist when working with cells that need to be re-infused into the patient from whom they were extracted. This also offers opportunities for more complex genetic fine-tuning to increase the persistence and efficacy of infused cells. Thus, the generation of premanufactured, off-the-shelf engineered T cells presents an attractive strategy to overcome the limitations of autologous therapies for the treatment of cancer. However, unlike autologous cells, genetic alteration of allogeneic cell therapies is required to circumvent donor-derived alloreactivity or host-mediated rejection.

Early gene-editing tools, such as zinc finger nucleases (ZFN) and transcription-activator-like effector nucleases (TALEN), have been applied in clinical trials to engineer and improve the efficacy of CAR-T cell therapies. The complex and demanding protein re-engineering required for each gene and the lengthy manufacture of ZFN and TALEN, coupled with low *in vivo* editing efficiency and low product yield, has hampered their therapeutic application [10–12]. By contrast, the versatile, programmable, and cost-effective attributes of CRISPR–Cas9 genome engineering, as well as its ease of use and scalability, have fast-tracked its role in the design of CAR-T cells (Table 1).

Removal of αβ-TCR on allogeneic T cells prevents TCR-mediated recognition and targeting of specific antigen expressed by the transfused cells from the patient, thereby preventing the toxic effects of graft-versus-host disease (GvHD). Qasim *et al.* showed that TCR-depleted CD19 CAR-T cells did not result in GvHD in two paediatric patients and that the transfused CAR-T cells were active and able to target CD19-expressing B cells [11]. Additional removal of major histocompatibility complex (MHC) class I/II molecules is required to minimise immunogenicity and prevent rapid rejection of the allogeneic CAR-T cells by the immune cells of the patient receiving the transfusion. Knockout of beta-2-microglobulin (B2M), the nonpolymorphic subunit of human leukocyte antigen (HLA)-I complex, prevented the infused allogeneic T cells presenting antigen in the context of MHC class I molecules, thereby mitigating a host-versus-graft (HvG) response.

Additionally, lymphodepleting agents, such as alemtuzumab, which targets CD52 expressed on the surface of mature lymphocytes, have been used to deplete patient T and B cells to decrease the likelihood of GvHD or HvG response. To enable such agents to be used in patients already infused with CAR-T cells that also express CD52, Poirot *et al.* disrupted the gene encoding CD52 and rendered CD19-directed CAR-T cells resistant to the lytic properties of alemtuzumab [10]. Cellectis also used this strategy when treating two paediatric patients with B-ALL. TALENs were used to knock out both the TCR alpha chain (TRAC) and CD52 in these allogeneic CAR-T cells, known as UCART19, and both patients entered a molecular remission [11]. These encouraging results led to two additional clinical trials of UCART19 in patients with ALL, specifically the CALM trial in adults (NCT02746952) and the PALL trial in paediatric patients (NCT02808442) (Table 1) [13]. CRISPR–Cas9 has also been used for dual knockout of TRAC and CD52 in allogeneic CAR-T cells targeting both CD19 and CD22 (CTA101), and initial clinical trial data (NCT04227015) showed safety and efficacy for the treatment of B-ALL. It is with much anticipation that clinicians and researchers await long-term follow-up results [14]. Aside from enabling the development and use of allogeneic CAR-T cells, genetic engineering can overcome some of the current limitations of autologous and allogeneic CAR-T cells that are apparent in patients treated with these therapeutics.

Genetic fine-tuning

Lack of CAR-T cell persistence is a common impediment to effective treatment. Constant exposure to antigen can result in exhaustion and functional deterioration of effector CAR-T cells, characterised by decreased expansion and proliferation, decreased cytokine production and killing capacity, altered cell metabolism, and increased expression of multiple inhibitory receptors, including cytotoxic T lymphocyte antigen (CTLA-4), programmed cell death 1 (PD-1), lymphocyte-activated gene-3 (LAG-3), and T cell immunoglobin and mucin domain 3 (TIM-3). Activation of these inhibitory receptors by ligands expressed by tumour cells and cells within the immunosuppressive TME (Box 1) aggravates the cell exhaustion phenotype, thereby compromising CAR-T function over time. Although anti-CTLA-4, anti-PD-1, anti-PD-L1, and anti-LAG-3 monoclonal antibodies

TABLE 1

Overview of clinical trials using CRISPR–Cas9- or TALEN-engineered immune cell therapies for the treatment of cancer.

NCT identifier	Disease	Cell source	Genetic engineering/CAR target	Refs
CRISPR				
NCT02793856	Non-small cell lung cancer	Autologous	PD-1-knockout T cells	[7]
NCT03747965	Mesothelin-positive solid tumours (pancreatic cancer, ovarian cancer, cholangiocarcinoma)	Autologous	PD-1-knockout mesothelin-directed CAR-T cells	
NCT03545815	Mesothelin-positive solid tumours	Autologous	TCR and PD-1-knockout mesothelin-directed CAR-T cells	
NCT03044743	EBV-associated malignancies	Autologous	PD-1-knockout EBV-CTL	
NCT04037566	CD19-positive B cell leukaemia and lymphoma	Autologous	XYF19-knockout CD19-directed CAR-T	
NCT03690011	T cell leukaemia and lymphoma	Autologous	CD7-directed CAR/28zeta CAR-T cells	
NCT03399448 (terminated)	Multiple myeloma, melanoma, synovial sarcoma, myxoid/round cell liposarcoma	Autologous	TRAC, TRBC, and PD-1-knockout NY-ESO-directed T cells (NYCE T cells)	[6]
NCT03166878	CD19-positive B cell leukaemia and lymphoma	Allogeneic	B2M and TCR-knockout CD19-directed BBζ CAR-T (UCART019)	
NCT03398967	B cell leukaemia and lymphoma	Allogeneic	Dual CD19 and CD20- or CD19 and CD22-directed CAR-T	
NCT04035434	CD19-positive non-Hodgkin's lymphoma (CARBON trial)	Allogeneic	CD19-directed CAR-T (CTX110™)	[54]
NCT04244656	Multiple myeloma	Allogeneic	BCNA-directed CAR-T (CTX120™)	
NCT04502446	T or B cell malignancies	Allogeneic	CD70-directed T cells (CTX130™)	
NCT04438083	Renal cell carcinoma	Allogeneic	CD70-directed T cells (CTX130™)	
NCT04557436	CD19-positive B-ALL	Allogeneic	CD52, TRAC knockout CD19 CAR-T (TT52CAR19)	
NCT04637763	CD19-positive non-Hodgkin lymphoma	Allogeneic	PD-1 knockout CD19-directed CAR-T (CB010A)	
NCT04227015	B cell leukaemia and lymphoma	Allogeneic	CD52, TRAC knockout CD19/CD22-directed CAR-T (CTA101 UCAR-T)	[55]
NCT03752541	Multiple myeloma	Allogeneic	TRAC, HLA-I knockout BCMA-directed UCAR T	
NCT04629729	B cell lymphoma, precursor B-ALL, CLL	Allogeneic iPSCs	1XX CD19-targeting CAR-T (FT819)	[56]
TALEN				
NCT04142619	Multiple myeloma	Allogeneic	CS1, SLAMF7-directed CAR-T (UCARTCS1)	
NCT04150497	CD22-positive B-ALL	Allogeneic	TRAC, CD52 knockout CD22-directed CAR-T (UCART22)	
NCT03190278	AML	Allogeneic	TCR, CD52 knockout CD123-directed (UCART123)	
NCT02746952, NCT02808442, NCT03229876	CD19-positive B cell ALL	Allogeneic	TRAC, CD52 knockout CD19-directed CAR-T (UCART19)	[13]
NCT03939026	CD19-positive B cell or follicular lymphoma	Allogeneic	TRAC, CD52 CD19-directed CAR-T (ALLO-501)	
NCT04416984	CD19-positive B cell lymphoma	Allogeneic	TRAC, CD52 CD19-directed CAR-T (ALLO-501A)	
NCT04093596	Multiple myeloma	Allogeneic	TRAC, CD52 BCMA-directed CAR-T (ALLO-715)	

[a]Abbreviations: AML, acute myeloid leukaemia; CTL, cytotoxic T lymphocyte; CLL, chronic lymphocytic leukemia; EBV, Epstein–Barr virus; iPSC, induced pluripotent stem cell.

(mAbs) have proved successful in the clinic to reverse T cell exhaustion [15], these pharmacological approaches can cause adverse effects and toxicity that are exacerbated when these antibodies are used in combination. Thus, although there is clinical evidence that suppression of inhibitory molecules using mAbs to enhance effector function and persistence of engineered T cells can improve progression-free and overall survival in patients, there are some safety concerns because both mAbs and CAR-T cells can lead to life-threatening cytokine storms. Genetic removal of these checkpoint inhibitors on therapeutic T cells could alleviate this risk.

The PD-1–PD-L1 axis is the most characterised inhibitory signalling pathway and, therefore, is a prominent target in CAR-T immunotherapies. Rupp *et al.* demonstrated that PD-L1 expression on tumour cells impaired CAR-T cell-mediated killing *in vitro* as well as tumour clearance *in vivo*. However, CRISPR–Cas9-induced knock out of *PDCD1*, which encodes PD-1, from CAR-T cells mitigated this effect and enhanced antitumour efficacy in a subcutaneous tumour xenograft model [16]. Liu *et al.* reported that CRISPR–Cas9-mediated double-knockout (*TRAC* and *B2M*) and triple-knockout (*TRAC*, *B2M*, and *PDCD1*) CAR-T cells had antitumour activity *in vitro*, and this was most significant with triple-knockout CAR-T cells [17]. Similarly, Ren *et al.* found that double knockout (*TRBC* and *B2M*) and triple knockout (*TRBC*, *B2M*, and *PDCD1*) reduced alloreactivity *in vitro*, and showed enhanced cell engraftment, proliferation, and antitumour function *in vivo*. Antitumour efficacy was increased by *PDCD1* disruption compared with double-knockout CAR-T cells [18]. Fas ligand (CD95L), which is present in the TME, promotes apoptosis and the terminal differentiation of cytotoxic T cells, thereby limiting the efficacy of CAR-T cell therapy. CRISPR–Cas9-mediated knock out of CD95 (the receptor of FasL), TRAC, and PDCD1 attenuated CD95–CD95L-dependent activation-induced cell death (AICD) and prolonged allogeneic CAR-T cell survival *in vitro* and *in vivo* [19]. Although these authors attempted a quadruple knockout to include inactivation of CTLA-4, gene editing efficiency was low. This highlights the importance of optimised delivery of CRISPR–Cas9 reagents for efficient manufacture of functional CAR-T cells that require multiple genetic engineering events. Recent reports from clinical

Box 1 CAR-T cells and the immunosuppressive TME.

CAR-T cells and the immunosuppressive TME.CAR-T cell-based immunotherapies are initially efficacious in treating CD19-positive haematological malignancies, but CAR-T cells targeting a variety of other tumour-associated antigens expressed by solid tumours have failed to yield reproducible or effective clinical responses. Many factors have contributed to these disappointing clinical results, including poor T cell expansion and persistence, variable antigen expression, physical difficulties infiltrating the heterogeneous solid tumour environment, and challenges navigating and prevailing in the hostile TME. A diverse range of inhibitory and inflammatory mediators abrogate engineered T cell function and proliferation within a complex and dynamic system. Although PD-1 and CTLA-4 have become the backbone of inhibitory receptor blockade strategies and are being targeted in CAR-T cells, alternative checkpoint molecules, such as LAG-3, TIM-3, VISTA, TIGIT, BTLA, and B7-H3, are also being evaluated in preclinical and early clinical trials for mitigating resistance to immunotherapy [57]. However, comprehensive modulation of the complex mechanisms and signalling pathways that underly T cell exhaustion is likely needed to complement single and dual checkpoint inhibition and improve clinical outcome.

Transforming growth factor (TGF)-β is one molecule known to have immunosuppressive properties. Tang *et al.* demonstrated that genetic knock out of TGFβ receptor 2 promoted CAR-T cell survival and proliferation in tumour xenograft models, and this was associated with increased anti-tumour efficacy [58]. Pharmacological or short hairpin (sh)RNA-mediated inhibition of the adenosine A2A receptor on CAR-T cells relieved adenosine-mediated immunosuppression of these cells in a hypoxic tumour environment [59], and CRISPR–Cas9-mediated knockout of diacylglycerol kinase (DGK) increased resistance to TGFβ and prostaglandin E2 inhibitory molecules, ultimately improving tumour infiltration and effector function of CAR-T cells in a xenograft glioblastoma model [60].

Box 2 NK CARs.

While most immunotherapies are heavily focussed on cytotoxic T cells, other cell types that comprise the milieu of the TME have also been exploited to promote tumour clearance. NK cells engineered with a CAR showed a promising safety profile by not causing GvHD, CRS, or immune cell-associated neurotoxicity. In a proof-of-principle, preclinical study, Gang *et al.* demonstrated that CD19-directed ML NK-CAR cells were effective in treating B cell lymphoma both *in vitro* and *in vivo*, thereby providing another strategy for addressing haematological malignancy [61]. In the solid tumour context, Pomeroy *et al.* demonstrated that further fine-tuning with genetic disruption of PD-1 or ADAM17 significantly enhanced effector function, cytokine production, and cancer cell killing of engineered NK cells *in vitro*, and PD-1-knockout NK cells reduced tumour burden and increased survival in a murine model of ovarian cancer. However, the authors acknowledged that functional effects were dampened *in vivo*; therefore, simultaneous editing of multiple regulatory genes would be required to confer more effective response [62].

trials indicate that autologous T cells that carry CRISPR–Cas9-mediated genetic modifications designed to improve persistence and efficacy can be safely infused [6,7]. Further data are awaited to see whether these alterations translate to prolonged response rates in treated patients.

Aside from improving CAR-T cell efficacy, genetic engineering can be used to limit the toxicities seen in some patients treated with these therapies. Cytokine release syndrome (CRS; also known as a cytokine storm) and immune effector cell-associated neurotoxicity syndrome (ICANS) are common adverse effects of CAR-T cell therapy, which pose a safety risk to patients and require careful intervention and management [1,2,13,20]. Although the understanding of what drives these responses is in its infancy, CRISPR–Cas9-mediated knockout of the gene encoding granulocyte macrophage colony-stimulating factor (GM-CSF) abrogated CAR-T cell-induced CRS and neuroinflammation, and enhanced CAR-T cell activity in xenograft models [21]. This preliminary work indicates that further genetic fine-tuning could mitigate the risk of cell therapy toxicities. Alternatively, engineered natural killer (NK) cells could offer safety advantages over T cells, although CAR-NK cells would still require multiple genetic edits (Box 2). As the number of genes subject to genetic engineering increases, so do the risks of off-target editing elsewhere in the genome.

Gene editing and off-target effects

Gene-editing efficiency, specificity, and safety are paramount to the application of genetically modified cells in the clinical setting. An important concern is the incidence of off-target effects, including unintended mutagenesis in the genome. These have the potential to drive genomic instability and cell death, disrupt essential genes, inactivate tumour-suppressor genes, or activate oncogenes. Researchers have addressed this issue through rational and optimised guide RNA (gRNA) design, protein engineering, and development of high-fidelity nuclease variants [22–24]. Off-target detection strategies, such as GUIDE-seq [25], Digenome-seq [26], or CIRCLE-seq [27], determined that, although low, the occurrence of gRNA-dependent off-target effects remains present and measurable [6,7].

On-target editing using CRISPR–Cas9 or other nuclease-based gene-editing tools that introduce DNA double-strand breaks (DSBs) can activate p53-dependent damage response pathways. In cells with a low tolerance to DNA damage, this can result in cell cycle arrest or apoptosis, whereas in cells that survive and repair the DSBs, there is a risk that the less specific DNA repair pathways could result in large insertions or deletions (indels), duplications, inversions, or translocations, or result in complex chromosomal rearrangements [28–30]. The occurrence of such genomic aberrations increases in the context of multiplex editing, where concurrent DSBs yield multiple free DNA ends that are amenable to repair and annealing in a variety of different configurations. This is also relevant to genetic knockout strategies that use multiple gRNAs targeting the same gene of interest

Box 3 Prime editing.

The latest development in gene editing is referred to as prime editing [63]. Anzalone *et al.* described a gene-editing platform comprising a reverse transcriptase (RT) fused to a Cas9 nickase and that requires a modified prime-editing gRNA (pegRNA) to make insertions and deletions in the absence of DNA DSBs. Prime editors trigger the DNA repair machinery to synthesise a sequence of up to 44 new base pairs (bp) using the pegRNA, or to delete up to 80 bp. They also facilitate all possible single nucleotide transitions and transversions. Although this technology offers incredible versatility, further development is required to overcome limitations associated with DNA-based delivery, and to address the lower performance and higher unintended on-target indel formation compared with BEs used for the introduction of the same transition mutations. Prime editing is still in its infancy but the rapid development of gene-editing technologies combined with the concerted effort to translate these to the clinic will likely bring this powerful and complementary new player to the field of cell and gene therapies.

for more effective gene disruption [17]. Therefore, the risk of complex rearrangements presents a major barrier to the widespread use of CRISPR–Cas9 in therapeutic approaches. A recent report of autologous immunotherapy using CRISPR–Cas9 to perform simultaneous disruption of four loci *TRAC, TRBC1, TRBC2*, and *PDCD1*, noted detectable chromosomal translocations in the resulting CAR T cell products [6]. These unintended outcomes were measurable despite low on-target editing, suggesting that improvements in reagent delivery and efficacy could yield even higher levels of translocation. Two preclinical studies using TALEN-mediated dual editing of CAR-T cells similarly reported chromosomal rearrangement and translocation events in the resulting product, highlighting the inherent risks associated with programmable nuclease-mediated DSBs [10,11].

As more targets are identified and improved delivery strategies and reagents enable high levels of on-target editing in human primary cells, the inherent safety risks and toxicities associated with the stochastic and off-target outcomes of DSBs and triggering imprecise DNA repair pathways become more prominent and more complex to detect or mitigate. Thus, gene-editing technologies that do not require DNA DSBs, such as base editing or prime editing (Box 3), offer a potentially safer alternative to genetically improve the next generation of cell-based immunotherapies.

Gene editing without DSB

Base editing, pioneered by two research groups led by David Liu (Harvard University) and Akihiko Kondo (Kobe University), and from the research group led by Shengkan Jin (Rutgers University), exploits DNA mismatch and base excision repair pathways to facilitate single-nucleotide alteration without introducing a DSB or requiring a DNA repair template [31–33]. Since their inception in 2016, base-editing technologies have been subject to intense innovation and optimisation efforts to advance technical capabilities and precision. Numerous iterations and variants of base editors (BEs) with improved specificity, purity, targeting scope, and high on-target editing efficiency are making base editing a strong contender in the development of future gene and cell therapies.

BEs comprise two main components: a catalytically impaired form of a Cas nuclease for programmable DNA binding; and a single-stranded effector protein, typically a deaminase enzyme, that mediates chemical alteration of the target nucleotide within a narrow editing window. This novel strategy, which David Liu called 'precision chemical surgery', is mediated by two categories of BEs; namely cytidine BEs (CBEs), which catalyse the conversion of C-G base pairs to T-A base pairs; and adenine BEs (ABEs), which catalyse the conversion of A-T to G-C. In the context of CBEs, APOBEC family members or activation-induced deaminase (AID) are used to deaminate C to T via a uridine intermediate [31,32], whereas synthetically engineered tRNA adenosine deaminase (TadA) in ABEs deaminates A to G via an inosine intermediate [34].

To avoid the risks associated with CRISPR–Cas9-mediated DSB for the purpose of gene knockout, base editing can be used to introduce premature stop codons in coding regions of target genes by specifically converting CAA, CAG, CGA, or TGG codons into stop codons, thereby facilitating more precise, safe, and controllable gene inactivation compared with CRISPR–Cas9 [35,36]. Functional gene knockout can also be achieved through BE-mediated disruption of highly conserved splice donor (exon|GT-intron) and splice acceptor (intron-AG|exon) sites [37,38]. In light of the increasing number of edits that will be required to generate more effective allogeneic cell immunotherapies, BEs offer the possibility to knock out multiple key genes simultaneously to promote cell therapy persistence and efficacy, while avoiding the risk of translocations or other DNA rearrangements.

Multiplex base editing has been used in the generation of allogeneic CAR-T cells. In the context of engineering hepatitis B virus (HBV) antigen-targeting CAR-T cells, Preece *et al.* used a CBE to introduce premature stop codons to disrupt the endogenous TCR, and demonstrated increased cytokine production and antigen-specific functional integrity in models of hepatocellular carcinoma compared with CAR-T cells in which the endogenous TCR remained active [39]. Using an ABE, Gaudelli *et al.* carried out multiplexed editing of *B2M*, *CIITA*, and *TRAC* in human primary T cells to reduce the expression of MHC class I and II molecules and the TCR, respectively, with editing efficiencies > 98% at each target locus [40]. A CBE system was used to knock out *TRAC*, *B2M*, and *PDCD1* in CAR-T cells targeting CD19 [38]. These cells retained robust cytokine functionality and displayed efficient target cell killing *in vitro*, with no evidence of translocation between the three target loci [38]. This contrasted with CRISPR–Cas9-edited cells, which showed up to 2% translocation frequencies, including between the three target genes and a predicted *PDCD1* off-target site. These data highlight the compounding and parabolic relationship between on- and off-target DSBs and potential translocation outcomes.

Despite the favourable comparison between CRISPR–Cas9 and BEs in terms of large genomic rearrangements, studies with BEs have raised concerns of gRNA-independent single nucleotide variations in DNA and RNA in *ex vivo* cultured cells and two-cell-stage embryos when APOBEC deaminases are used [40–44].

This off-target editing could have detrimental effects on cell function in a clinical setting; therefore, unbiased, genome- and transcriptome-wide safety profiling of BEs is under thorough evaluation. Concerns have been addressed via strategic alterations in the rat APOBEC1 domain to decrease kinetics of single-strand DNA deamination (e.g., SECURE' variants harbouring R33A or [R33A, K34A] mutations) [41,43]; through the use of more precise rat APOBEC1 homologues; using alternative deaminase effectors such as AID; or through using engineered ABEs that are less prone to promiscuous RNA editing or gRNA-independent DNA editing [42,45–49]. Although the development of next-generation CBEs, such as BE4 with RrA3F [wt, F130L], AmAPOBEC1, SsAPOBEC3B [wt, R54Q], or PpAPOBEC1 [wt, H122A, R33A] has minimised the occurrence of gRNA-independent off-target deamination without compromising on-target editing activity [47], the delivery method used to introduce base-editing components into a cell is also a crucial determinant of its safety profile [38,40]. Widespread use of plasmid-based expression of BEs for research purposes presents a worst-case scenario and an exaggerated mutational profile that is largely dissipated by more transient expression of therapeutically relevant delivery modalities, such as mRNA or ribonucleoprotein (RNP) complexes [7,38–40,46,49]. More specifically, Villiger *et al.* recently demonstrated that high rates of transcriptome-wide C-to-U editing in HEK293T cells transfected with a CBE-expressing plasmid were substantially reduced following mRNA delivery of the same CBE, supporting the hypothesis that prevalent off-target RNA editing is dependent on APOBEC1 overexpression. More notably, the authors showed that transient CBE mRNA delivery using lipid nanoparticles led to on-target correction of disease phenotype *in vivo* but no detectable off-target deamination of RNA nor genomic DNA in hepatocytes [49]. In human primary T cells, Webber *et al.* and Gaudelli *et al.* found no increase in unintended C-to-T or A-to-G editing in RNA or DNA following mRNA delivery of coBE4 mRNA [38] or ABE8 (ABE8.17-m or ABE8.20-m) [40], respectively. Taken together, the development of deaminase variants with an improved safety profile coupled with the use of transient delivery modalities *in vitro* and *in vivo* has largely mitigated the occurrence of unintended off-target effects. It is with more confidence that BEs will enter the clinical realm. The base editing discoveries of David Liu and colleagues were used to spin out Beam Therapeutics, a clinical-focused base-editing company. Press releases from Beam Therapeutics indicate that they have used their BEs in preclinical correction of disease-causing mutations underlying Glycogen Storage Disease Type Ia (GSDIa) [50], alpha-1 antitrypsin deficiency (Alpha-1) [51], and sickle cell anaemia (BEAM-101, BEAM-102) [52]. Beam Therapeutics are also developing base-edited allogeneic CAR-T cells (BEAM-201) to treat patients with T cell acute lymphoblastic leukaemia (T-ALL) [53]. This press release indicates that their CBE mediated 96–99% editing efficiencies across four target loci and that 85% of cells expressed the CD7 CAR. Beam estimated that 77% of modified T cells have all five genetic modifications with no evidence of genomic rearrangements or activation of key p53 signalling pathways. It is reported that these cells show robust cytokine production and cancer cell killing *in vitro* and *in vivo*, with resistance to immunosuppressive pathway signalling and fratricide (the killing of T cells by T cells) [53]. Formal peer review and publication of these high editing efficiencies and on-target T-ALL killing should add weight to the argument that base editing is a viable route forward in the next generation of cell therapies.

Concluding remarks

Early-stage trials and FDA approval of a growing number of autologous CAR-T therapies have provided evidence for the feasibility, safety, and efficacy of engineered cell immunotherapies for the treatment of haematological malignancies. However, these successes have not yet translated to the solid tumour environment. Additionally, the paradigm of individualised, autologous therapy faces manufacturing challenges and limited efficacy in the long term. Genetic fine-tuning could improve the persistence, efficacy, and scope of CAR-T cell therapies, but will require simultaneous introduction of multiple genomic alterations. Base editing has emerged as an ideal gene-editing technology to achieve this while avoiding the safety risks of traditional CRISPR–Cas9-mediated gene editing. Further work is required to optimise and demonstrate the safety and efficacy of BEs for use in the clinic, and a concerted effort from governing bodies, clinicians, and researchers will be required to streamline regulatory processes in this era of rapidly advancing genetic engineering. However, the breakneck speed of BE development combined with the promising results to date herald the exciting contribution that BEs could make in the treatment of cancer and other complex diseases.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: I declare that I am an employee of PerkinElmer's Horizon Discovery, a biotechnology company that drives the application of gene editing and gene modulation within the global life science market, supporting scientists on the path from research to therapy.

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