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CRISPR/Cas-based Human T cell Engineering: Basic Research and Clinical Application

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A B S T R A C T

Engineering human T cells for the treatment of cancer, viral infections and autoimmunity has been a long-standing dream of many immunologists and hematologists. Although primary human T cells have been genetically engineered for decades, this process was challenging, time consuming and mostly limited to transgene insertions mediated by viral transduction. The absence of widely accessible tools to efficiently and precisely engineer T cells genetically in a targeted manner limited their applicability as a living drug. This fundamentally changed with the discovery of CRISPR/Cas9 and its adaptation to human T cells. CRISPR/Cas9 has made T cell engineering widely accessible and accelerated the development of engineered adoptive T cell therapies. Only 6 years after the discovery of CRISPR/Cas9 as a biotechnological tool the first CRISPR engineered T cells have been administered to patients with refractory cancers in a phase I clinical trial. Novel Cas proteins - natural and engineered ones - are rapidly emerging. These offer for instance increased flexibility, activity and/or specificity. Moreover, sophisticated protein engineering and fusions of Cas with deaminases or reverse transcriptases enable genomic DNA editing without the need for a double strand cut. Thus, the "CRISPR tool box" for experimental use as well as for novel therapeutic approaches is rapidly expanding. In this review, we will summarize the current state of CRISPR/Cas-based engineering in human T cells for basic research and its clinical applications.

How to "crispr" human T cells?

The basic principle of most genome engineering technologies relies on the introduction of DNA double strand breaks (DSBs) at a specific gene region in a highly controlled manner. Zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) have been the engineering tools of choice for a long time. These classical designer nuclease systems are based on the engineering and correct orientation of two DNA-binding protein domains each fused to one FokI DNA single-strand cleavage domain to introduce a DSB (reviewed in: [1–3]). However, the challenging design and protein engineering process for two designer nucleases to introduce one single DSB limits their throughput and their accessibility for experimental editing. In 2012, it was discovered that a clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) system, which acts as an integral part of the adaptive immune system in many bacteria and archaea, is a programmable RNA-guided DNA endonuclease. The fundamental difference to ZFNs or TALENs is that the cutting specificity is determined by the guide RNA (gRNA), not the protein, which enables simple redirecting of the endonuclease to almost any site in the genome [4,5]. Shortly after, the functionality of CRISPR/Cas9 in different mammalian cell types including human cell lines could be proven, establishing CRISPR/Cas9 as a novel cell engineering tool [6–8].

To introduce DSBs the DNA endonuclease Cas9 forms a complex with a gRNA, which consists of a heteroduplex of two individual RNAs - the CRISPR RNA (crRNA) and the trans-activating CRISPR RNA (tracrRNA). The active Cas9:crRNA:tracrRNA complex scans the DNA for a protospacer adjacent motif (PAM) and the DNA sequence complementary to the approximately 20 nt long protospacer sequence encoded in the crRNA. The widely used *Streptococcus pyogenes* Cas9 (SpCas9) requires the relatively short PAM sequence NGG (N stands for any nucleotide), which is frequently found in the genome. Once the complex finds its target sequence the bound Cas9:crRNA:tracrRNA complex introduces a DSB approximately 3 nt upstream of the PAM sequence [4,5]. Cas9 is composed of two distinct nuclease domains to introduce the DSB – the HNH-like nuclease domain which cuts the DNA strand complementary to the protospacer sequence (target strand) and the RuvC-like nuclease domain that cleaves the opposite, non-target strand (Cas9 structure and detailed cutting mechanism reviewed in: [9]). To simplify this system even further Jinek et al. successfully reduced the bacterial three

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component system to two components by fusing the crRNA and the tracrRNA to a so-called single-guide RNA (sgRNA) [4].

Initially it has been challenging to introduce Cas9 into human T cells. Optimized workflows, however, allow the quick and streamlined introduction of Cas9 into human T cells using viral transduction or applying Cas9 mRNA, plasmid or recombinant protein using non-viral methods, most commonly electroporation [10–17]. Alternative non-viral methods such as cell-penetrating peptide-conjugated Cas9, liposomes or nanoparticles in different formulations have been successful in cell lines, but have not been adapted to T cell editing so far [18–20].

Duration of intracellular Cas9 expression is an important parameter determining efficacy and specificity of a given genome edit. Viral transduction of human T cells allows the constitutive expression of Cas9 [12–14]. This methodology can be attractive to functionally interrogate gene regulatory elements, but the constitutive expression of Cas9 has several drawbacks for therapeutic editing [13,14]. The random integration of the viral genome into the DNA of the host cells as well as the prolonged expression can result in the accumulation of off-target mutations and an increased risk of oncogenesis [21,22]. Furthermore, the risk of immunologic rejection is increased [23–25]. In contrast, rapid cellular degradation of Cas9 protein (24-48 h) or mRNA (several days) greatly reduces the risk for off-target indels [15,26]. Plasmids encoding for Cas9 are not commonly used as the achieved editing efficiencies in human T cells are relatively low [11,16,17]. Moreover, toxicity is much higher than with mRNA or protein delivery, presumably triggered by an innate immune response of T cells towards double-stranded DNA (dsDNA) [17,27,28]. In contrast, combined electroporation of Cas9 mRNA and free, non-complexed sgRNA is well tolerated by T cells and results in reproducible, efficient editing (Fig. 1; Fig. 2A). However, non-complexed gRNAs are easily degraded by RNAses, an effect that can be significantly reduced by chemical RNA modifications on both termini [11].

Cas9 ribonucleoproteins (Cas9 RNPs) are composed of a recombinant Cas9 protein carrying nuclear localization signal (NLS)-sequences complexed with *in vitro* transcribed or chemically synthesized sgRNAs. The Cas9 RNP-complexes are relatively stable, which can result in high editing rates (Fig. 1; Fig. 2A) [29]. The efficiency of Cas9 RNP electroporation can be further enhanced by adding anionic compounds such as dsDNA, single-stranded DNA (ssDNA) or polyglutamic acid (PGA) and thereby increasing the solubility of Cas9 [29,30]. Conveniently, the assembled Cas9 RNPs can be stored at 4°C for several days or lyophilized with polyglutamic acid (PGA) for long-term storage [29].

Lentiviral transduction of human T cells with Cas9 has been initially proven to be rather challenging, presumably because of the large size of SpCas9 resulting in low viral titers and therefore low transduction rates, which limited this approach to a few successful experiments [12,13]. Recently a second generation of lentiviral transfer plasmids with reduced size have been developed for highly efficient expression of different Cas9 variants in human T cells (Fig. 2C) [14]. Alternatively, Cas9 RNPs have been packaged in engineered lentiviral particles pseudotyped with the CD4-tropic HIV-1 envelope glycoprotein to specifically target Cas9 RNPs to human $CD4^+$ T cells [31].

Once the Cas9:gRNA complex has introduced a DNA DSB, different cellular DNA repair pathways are activated. The “classical” pathways include non-homologous end-joining (NHEJ) pathway which can result in gene knock-outs (KO) and homology directed repair (HDR) allowing the generation of gene knock-ins (KI) [32,33]. To a lesser extent alternative DNA repair pathways such as the Fanconi anemia (FA) or the microhomology-mediated end joining (MMEJ) pathway contribute to

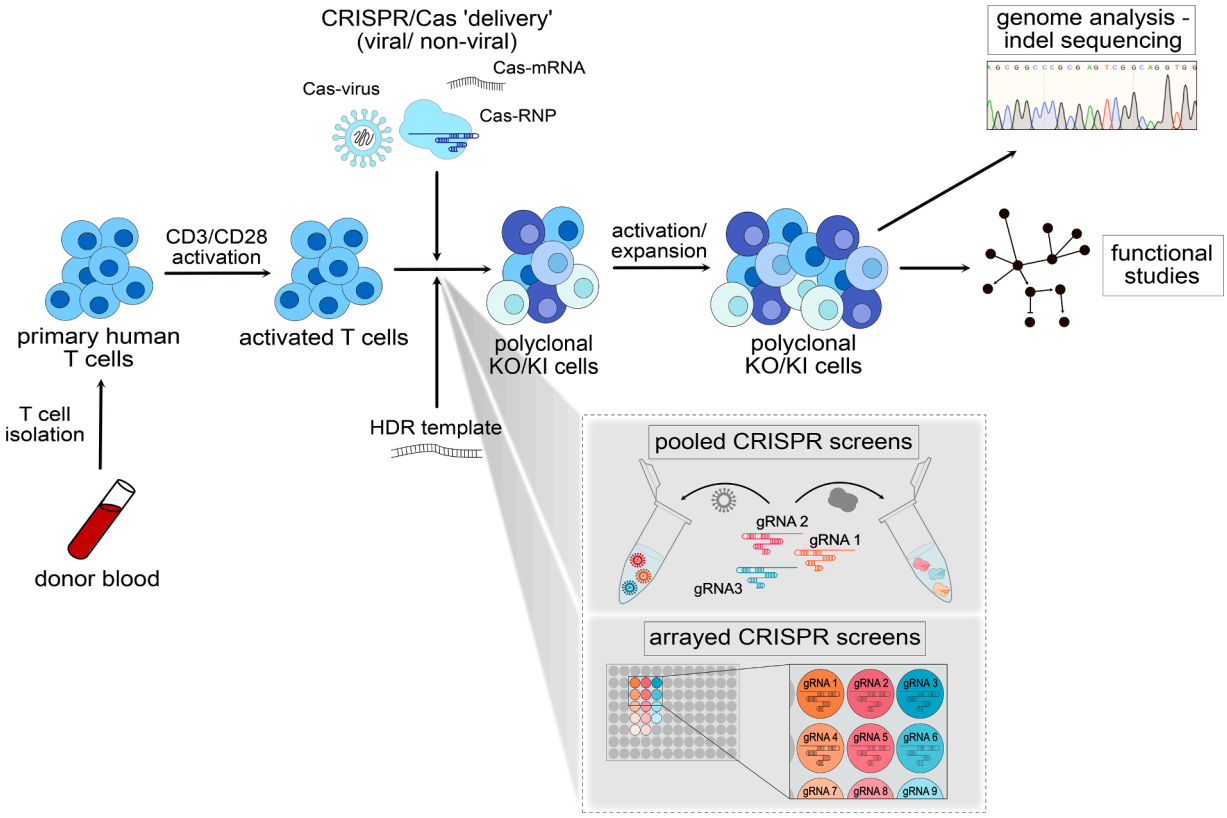

Fig. 1. Workflow of CRISPR/Cas-based editing in human T cells.
Following isolation of primary human T cells out of human blood and CD3/CD28 activation, the Cas enzyme is introduced into the cells together with the sgRNA using viral or non-viral methods. For HDR-mediated KI, templates are additionally added. The validation of successful genetic perturbation and phenotypic changes are determined by sequencing analysis, immunostaining, or functional studies. In addition, T cells can be assessed in pooled and arrayed screens for high/medium-throughput loss- or gain-of function studies.

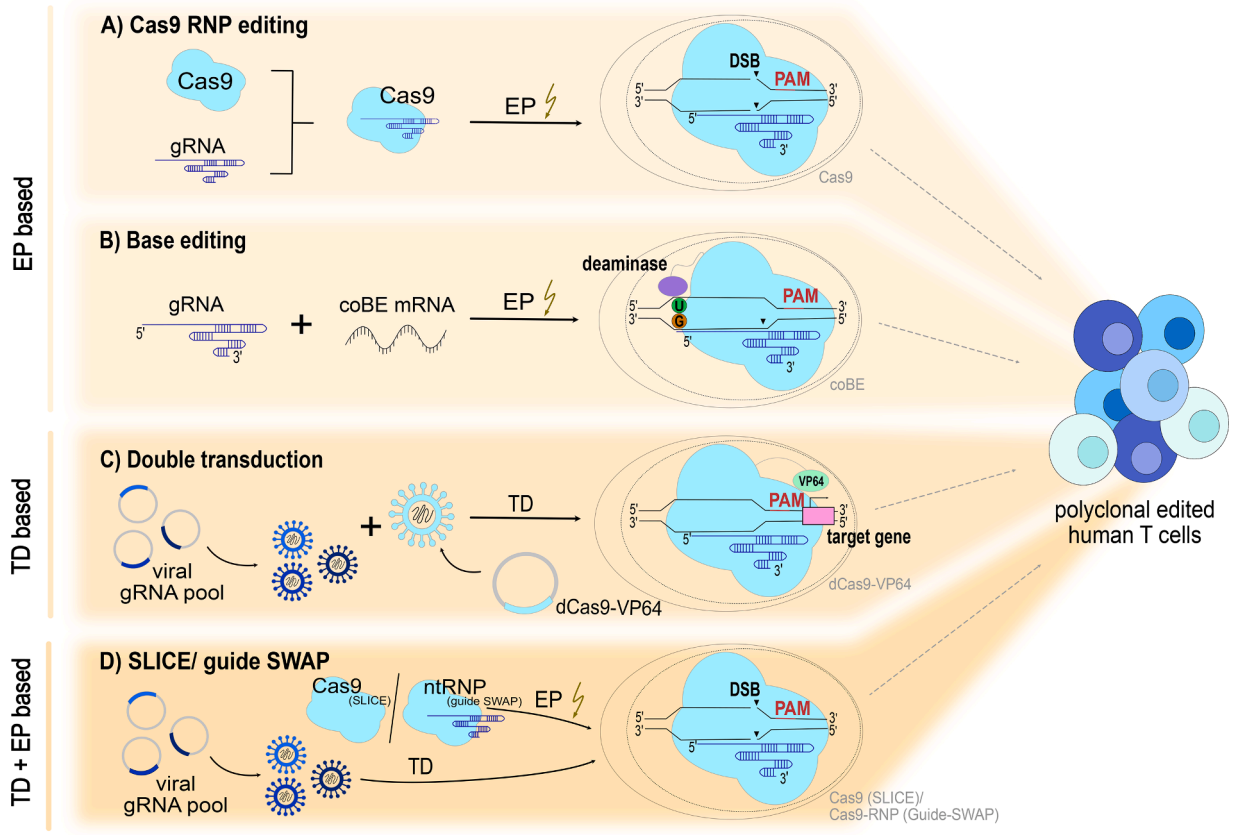

Fig. 2. Editing systems applied in human T cells using CRISPR/Cas.
A) Cas9 ribonucleoprotein (RNP)-based editing: After hybridizing of the gRNA with Cas9 protein the generated Cas9 RNP is transferred in T cells via electroporation (EP). The Cas9 nuclease cuts 3 bp upstream of the PAM sequence and induces a double strand break (DSB), that is followed by endogenous repair mechanisms resulting in knock-out or knock-in edited T cells.
B) Base editing: Base editors (BEs) consist of a Cas9 nickase fused to a deaminase which converts one targeted nucleotide to another without the need for a DSB and a homology directed repair template (HDRT). To transfer base editors to human lymphocytes the highest editing efficiency was achieved by EP of gRNA and the mRNA of codon-optimized BEs (coBE).
C) Double transduction (TD): Optimized lentiviral backbones enable the lentiviral transduction of dCas9-effector enzymes together with viral gRNA pools.
D) SLICE/guide SWAP: To perform large-scale screens in human T cells SLICE and guide SWAP have been developed. Here, transduction and EP are combined, as a gRNA pool is lentivirally transduced into the cells and Cas9 protein (SLICE) or non-targeting Cas9 RNPs (guide SWAP) are introduced via electroporation into human T cells.

the DNA repair outcome [34–36]. However, the contribution of these alternative DNA repair pathways to the DNA repair outcome following a Cas9-induced DSB is not well characterized yet but MMEJ can dominate over HDR [33,37].

Generation of knock-out human T cells: Introducing indels

NHEJ is a simple and fast mechanism to re-join the cut DNA-ends and is active throughout the cell cycle. However, this repair pathway is inherently error-prone resulting in the frequent loss or addition of nucleotides leading to mutations, also called insertions-deletions ("indels"). Indels can induce frameshifts, pre-mature stop-codons, nonsense mediated mRNA decay or dysfunctional protein resulting in an efficient ablation of the gene of interest [38].

The editing efficiency and outcome can be quickly analyzed by amplicon PCR of the target region followed by Sanger or next generation sequencing (NGS) [10,16]. The sequencing results can be submitted to several online tools to determine editing efficiency (Sanger: TIDE (https://tide.nki.nl), ICE (https://ice.synthego.com/#/); NGS: CRISPResso (https://crispresso.pinellolab.partners.org/submission)) [39,40].

By mixing different Cas9 RNPs the simultaneous perturbation of multiple genes or increase in KO efficiency can be achieved [41–43]. A crucial parameter for successful T cell editing with Cas9 RNPs is the activation status of the cells and an electroporation program optimized for the respective T cell stimulation. Seki et al. could nicely show that naïve or anti-CD3/28-stimulated T cells need distinct T cell electroporation protocols for efficient editing and optimal cell survival [43].

Generation of knock-in human T cells: Replacing and inserting DNA-sequences

In contrast to NHEJ, HDR relies on homology directed repair templates (HDRTs) for DNA repair. Normally, the corresponding allele acts as template but by adding exogenous DNA templates this repair pathway can be co-opted for the controlled replacement or introduction of novel DNA sequences into the genome. HDR enables the targeted insertion of DNA sequences, from point mutations to entire cDNAs enabling for example to fluorescently tag proteins or to repair disease-causing single nucleotide polymorphisms [44,45]. Importantly, the HDR machinery is only active throughout late S/G2-phase during the cell cycle [33,46]. HDR KI efficiencies are usually lower than NHEJ-mediated indels. In addition, the result of an HDR experiment is a mix of cells with unedited cells, NHEJ repaired cells as well as cells that contain the precise desired insertion [10,44]. To select KI T cells the simultaneous KI of reporter genes besides the mutation or gene of interest is an attractive strategy. For experimental editing KI of "classical" fluorescent proteins are

frequently used [44]. For therapeutic editing truncated growth factor receptors which are inactive and importantly not immunogenic, can be applied [47,48]. However, successful HDR events can also be detected by amplicon PCR combined with Sanger/NGS sequencing technologies [39,40]. Beyond that, targeted locus amplification (TLA) enables the detection of desired as well as undesired HDR integration sites by proximity ligation [49,50].

Current therapeutic KI editing strategies often rely on gain of function phenotypes or the introduction of artificial receptors such as T cell receptors (TCRs) or chimeric antigen receptors (CARs), for which a successful monoallelic KI can be sufficient [44,51,52]. However, if homozygous KIs are desired the zygosity cannot easily be controlled. The KI of different HDRT encoding for different non-immunogenic reporters and the subsequent flow cytometry-based sorting of double-positive cells is one possibility. This method requires the generation of two HDRTs and only a small fraction of cells will acquire both KIs [44].

The transfer of the HDRTs into the cells can be achieved via viral transduction or by electroporation (Fig. 1) [10,44,45,53]. Adeno associated virus (AAV)-encoded HDRTs are highly efficient at introducing large KI constructs into cells [51,53].

In viral-free systems HDRTs are either encoded on plasmids or applied as single-stranded or dsDNA fragments that can easily be generated by PCR or ordered as synthetic fragments [10,44]. ssDNA donors have the advantage of reducing cell toxicity compared to double-stranded constructs [29,44,54]. Generally, non-viral constructs can be developed more rapidly and therefore provide increased flexibility over virus-based HDRTs and are likely safer for patients, although they tend to result in lower HDR efficiencies especially with large HDRTs.

To achieve higher KI rates a multitude of strategies has been tested and developed. Parameters that heavily influence the success involve strength of the applied T cell stimulation, the general survival of the KI T cells, the applied gRNA as well as HDRT design itself [29,44]. Furthermore, KI efficiency can be improved by enhancing the transport of the HDRT into the nucleus. The addition of truncated Cas9 target sequences (tCTSs) on both ends of the HDRT allows the Cas9 to directly bind to the HDRT without cutting it. Thus, all components are shuttled together into the nucleus with the help of the NLS-sequences of the bound Cas9 protein [29,54].

Besides the optimization of HDRT design other publications focused their KI strategies on skewing DNA repair pathway choices upon Cas9-induced DSBs, as for instance the synchronization of the HDR with the cell cycle progression or the inhibition of the NHEJ pathway using small molecules or RNAi-mediated knock-down [34,46,54-56]. A multitude of different inhibitors have been applied to inhibit key enzymes for successful NHEJ. However, the effect of these inhibitors seems to be partially cell type specific [34]. Wienert et al., reported that the FA DNA repair pathway also plays a crucial role during HDR with ds- and ssDNA donors. In human T cells treated with the FA-pathway-targeting cycle 7-related protein kinase inhibitor XL413 HDR events could be boosted [34]. Furthermore, the DNA-PK inhibitors NU7441 and M3814 and the HDAC class I/II Inhibitor Trichostatin A (TSA) have been reported to increase HDR frequency in human T cells [54].

Thus, targeted precise engineering of single nucleotides up to a few thousand base pairs is feasible. The field is very dynamic and a multitude of approaches are being developed to further increase KI efficiencies.

S. pyogenes Cas9 and beyond

The most popular Cas9 enzyme is still the “originally” published SpCas9 due to its relatively short and frequently appearing PAM sequence. However, SpCas9 also has certain limitations including its size and blunt end cutting, the strictly defined PAM sequence, the risk of off-target mutations and its limited success in the functional dissection of gene regulatory elements. Therefore, immediately after the discovery of SpCas9 other bacterial species were systematically investigated for CRISPR/Cas proteins with genome editing potential and Cas variants were designed to extend the CRISPR tool box.

With regards to the size, the Cas9 of *Staphylococcus aureus* (SaCas9) has the advantage of being significantly smaller (124kDa) compared to SpCas9 (160 kDa) allowing easier lentiviral transduction also in human T cells [57,58]. In addition, SaCas9 can also be applied by SaCas9 RNP electroporation [59]. However, the high complexity of the PAM sequence of SaCas9 (NNGRRT; whereas R is A or G) results in a limited set of suitable gRNAs [57–59].

Cas9 enzymes with an expanded PAM repertoire can be beneficial to introduce and functionally validate predicted disease-causing mutations since these may reside in regions that are inaccessible to SpCas9 (e.g. AT rich regions). Cas9 enzymes were engineered to recognize different PAM sequences, to increase their activity while preserving or increasing their specificity. Joung and colleagues engineered SpCas9 to recognize NGA and NAG PAMs and SaCas9 to bind towards NNNRRT PAMs [60,61]. Also engineered SpCas9 variants that can bind NRNH PAMs (H is A, C or T) have been developed [62]. This approach got even further extended to so-called “SpRY” SpCas9, which recognized NRN and to a lesser extent NYN PAMs (where Y is C or T). Thus, this SpCas9 variants are now near “PAMless”, i.e. there are very few restrictions as to where such nucleases can cut the genome [63].

In addition to that, an important feature of Cas9 is its blunt end cutting which is a major determinant of DNA repair outcome. The controlled introduction of “sticky” DSBs with staggered DNA overhangs was thought to be a boosting factor for HDR. Cas12a (previously Cpf1) isolated out of *Lachnospiraceae* or *Acidaminococcus sp.* is a single RNA-guided endonuclease lacking a tracrRNA and introducing a staggered DSB. The PAM for this alternative editing system is TTTV, where "V" is an A, C, or G, which results in reduced numbers of available gRNAs compared to SpCas9 [64]. To extend the applicability of Cas12a, variants with extended PAM recognition and higher on-target activity have been engineered [65]. However, whether Cas12a or Cas9-mediated HDR is more efficient will also largely depend on the target locus.

One strategy of reducing the risk of off-target mutation was the development of so-called Cas9 nickases. They carry alanine substitutions at key catalytic residues within the nuclease domains: the RuvC mutant D10A produces a nick on the targeting strand while the HNH mutant H840A generates a nick on the non-targeting strand [6,66]. To introduce a DSB in an alternative manner two Cas9 nickase:gRNA complexes must act together in the correct orientation. This system can greatly increase specificity while minimizing off-target effects. However, this system is also more challenging because both Cas9 nickases need to be available in one cell and act simultaneously to introduce a DSB [6,44,66-68].

The genetic dissection of gene regulatory elements remains challenging because of their combinatorial nature. Therefore, nuclease-dead Cas9 (dCas9) is often used as a programmable shuttle protein to bring effector molecules of choice to a specific genomic region [67,69]. These effector domains include among others transcriptional repressors, activators and DNA-demethylases to target gene regulatory elements for experimental dissection and functional validation (reviewed in: [70, 71]). These constructs are either applied as plasmids into human T cells or by viral transduction [13,14,72]. Plasmids, which have a sufficiently long half-life in T cells to result in gene regulatory changes, need a certain amount of “input cell material” because of the DNA toxicity [27, 28]. Lentiviral transduction resulting in constitutive expression of dCas9-effectors enables now to perform screens interrogating gene regulatory elements in human T cells [14].

In general, Cas9 nickases- and dCas9-based approaches massively expand the CRISPR tool box, but also increase the complexity of designing efficient gRNAs and need significantly more time and resources for optimization.

Analyzing and controlling the specificity of CRISPR/Cas mediated genome editing

To further reduce the risk of off-target effects several high-fidelity Cas9 nucleases with altered nuclease activity have been engineered based on screens or rational design [73–80]. Additionally, strategies for optimal gRNA design and careful monitoring of off-target mutations can increase the specificity of CRISPR editing [81,82].

The first generation of high-fidelity (HIFI) Cas9 proteins, eSpCas9 (1.1) and SpCas9-HF1, have alanine substitutions to weaken the interaction between the HNH/RuvC groove and the non-target strand, or the interaction with the DNA phosphate backbone of the target strand [73, 74]. In HypaCas9, mutations in the recognition lobe 3 (REC3) domain increase the proof-reading capacities of the enzymes [75]. The next generation of HIFI Cas9 enzymes including evoCas9, xCas9, Sniper-Cas9, HIFI Cas9 and Cas9R63A/768A were selected using different unbiased screening systems [76–80]. More recently, structure-based analysis of mismatch surveillance led to the development of an engineered Cas9 variant with further reduced off-target DNA cleavage [83]. To benchmark engineered HIFI Cas9 variants Kim et al. (2020) systematically compared and ranked several of these Cas9 enzymes. The overall activity was highest in the "wildtype" SpCas9 followed by Sniper-Cas, eSpCas9(1.1), SpCas9-HF, HypaCas9 /xCas9 and evoCas9. The cutting specificity, however, is inversely correlated to the on-target activity with evoCas9 ranked highest followed by HypaCas9/SpCas9-H, eSpCas9(1.1), xCas9, Sniper-Cas9 and the original SpCas9 [84]. The lower on-target performance of highly engineered Cas9 variants can most likely be explained with a significantly lower intrinsic cleavage rate [85].

In general, gRNAs can tolerate some mismatches which increases the risk of off-targets. However, the location of the mismatch matters. The first 8 to 12 PAM-proximal base pairs of the protospacer sequence form a seed region where mutations are less tolerated compared to mismatches in the PAM-distal region [4]. To select gRNAs with an optimal on-target efficiency and safety profile different prediction and scoring systems have been developed based on experimental data and computational analysis [81,82]. Besides that, truncated 16 bps-long gRNA protospacer sequences result in fewer off-targets due to an increased sensitivity to mismatches [86]. Thus, optimization of a particular gRNA can increase its specificity for a desired edit.

For the detection of off-target mutations, biased and unbiased approaches have been applied, each with varying sensitivity and associated costs. Amplicon sequencing of predicted off-target sites is easy to implement, but is highly dependent on the computational algorithm used to identify the potential off-target binding and narrows the analysis to a limited set of genetic sites with a strong homology to the actual cut site. In addition, such approaches usually do not account for possible interindividual sequence variations. Whole genome sequencing (WGS) on the other hand is an unbiased, but expensive method, and it is still under debate which read depth would be necessary to sufficiently pick up rare mutations [87,88]. Therefore, to assess potential off-target mutations on an unbiased, genome-wide level but still with a reasonably high resolution, several specific sequencing methods have been developed. SITE-seq (selective enrichment and identification of tagged genomic DNA ends by sequencing), Guide-seq and CIRCLE-seq (circularization for *in vitro* reporting of cleavage effects by sequencing) are biochemical methods to detect cut-sites by labeling or by separating DNA-fragments with off-target cuts based on global, genomic DNA [89–91]. Alternatively, ONE-seq (OligoNucleotide enrichment and sequencing) uses a computationally designed and chemically synthesized DNA library to reduce the "background noise" of presumably irrelevant sequences [92]. Discover-seq and ChIP-dCas9 are chromatin immunoprecipitation (ChIP)-based methods of bound dCas9 RNPs or cell intrinsic proteins of the DNA repair machinery to isolate DNA fragments with DSBs [93,94]. In addition, CAST-seq (chromosomal aberrations analysis by single targeted linker-mediated PCR sequencing) enables us to determine the frequency of unintended chromosomal rearrangements using low input cell material [95].

These more sophisticated methods are not standard for experimental applications and often require higher input material which can preclude their use in primary cell types. Future development will show which gold standards will be established for therapeutic applications by researchers in partnership with the regulatory authorities.

Genome editing without DNA double strand break: Base and prime editing

While most genome engineering applications rely on a dsDNA cut, this activity bears the risk for chromosomal rearrangements, particularly with multiplexed editing [95,96]. Therefore, alternative approaches that do not rely on dsDNA cleavage were developed. Cas9 nickases were used as programmable vehicles to develop "base editors", i.e. fusion proteins that enzymatically and irreversibly convert one targeted nucleotide to another without the need for a dsDNA break or a HDRT. Tethering a cytidine deaminase to a Cas9 nickase converts cytidine to uridine resulting in a cytosine (C) to thymine (T) conversion [97]. Although this is limited to a narrow space of a few nucleotides, it can result in high efficiency and a very defined genome editing outcome (Fig. 2B). In particular, uncontrolled indels can be strongly reduced. Subsequent extensive engineering enabled the creation of molecules that allow adenine (A) to guanine (G) conversion [98]. Thus, base editors mediate direct chemical modifications of the target nucleobases in living cells. Importantly, they avoid the need for cleavage of the nucleic acid backbone. Although base editors are also RNA-programmable, the possible editing is more restricted since base editors not only have PAM restrictions but the attached deaminase can also only act in a limited "editing window". However, much like the rapid development of many different versions of nucleases for DNA cutting, the development of improved and diverse base editors occurs at a very high pace. For instance, dual editors which can mediate both cytosine and adenine base conversions [99,100] and base editors tethered to alternative Cas nickases (e.g. Cas12a, xCas9) were rapidly engineered [65]. These molecules have different PAM restrictions and therefore broaden the number of nucleotides in the genome that are accessible for base editing. In addition, more recently a near PAM-less SpRY Cas9 molecule was engineered that is compatible with high fidelity Cas variants and can be used for cytosine as well as adenine base editing [63]. Thus, many base editor molecules are available to insert targeted small DNA modifications in the majority of the human genome. However, the editing depends on the actual sequence context and is therefore more restricted compared to HDR for instance, where an exogenous DNA sequence can be freely designed and precisely inserted. Furthermore, within the base editing "sequence window" also additional off-target mutations have been reported [101]. In addition, base editors are even larger than the Cas proteins themselves which can render their delivery challenging. In human T cells however, base editors delivered as mRNA, particularly codon-optimized (coBE) or engineered virus-like particles can have very high editing efficiencies and high T cell viability [101,102] (Fig. 2B). For molecular details, overviews of the existing base editors and guidance to choose base editors for specific applications we refer to recent reviews [103–105].

About half of the known pathogenic genetic variants are due to single-nucleotide variants [105]. Therefore, base editors are highly interesting for the correction of such diseases, but also for other applications. They can be used for genetic screens as well as to inactivate genes by introducing premature stop codons or by affecting RNA splicing [106–109]. Moreover, the possibility to install targeted nucleotide changes without the need for dsDNA cleavage poises base editing for multiplexed genome modifications because the risk for chromosomal translocations is likely lower than with multiplexed HDR. Indeed, multiplexed base editing of human T cells can be highly efficient with >90% editing efficiency at 3 genes simultaneously using base editor mRNA

electroporation [102]. Since this format is potentially scalable and combined with the features of high efficiency multiplexing, it is very likely that base editors will become important for T cell research and T cell-based therapeutics.

In an effort to overcome the need for a dsDNA cut or introduction of a HDRT as well as to enhance editing efficacy and precision, prime editing was developed as another promising approach applying CRISPR/Cas9 in human cells. Using a reverse transcriptase (RT) fused to Cas9 nickase genetic information is directly copied from a prime editing gRNA (pegRNA) into the target locus [110]. The pegRNA serves not only to guide the prime editor to the locus of interest but also serves as the RT template. The functionality of this tool was successfully verified in HEK293T cells and human T cells. C to G conversions with up to 7.5 % editing efficiency were introduced here [111]. While initial editing efficiencies were rather low, engineered pegRNAs increase prime editing efficiencies [112]. Thus, prime editing holds great promise for safer engineered T cell therapies in the future but needs future improvements to achieve sufficient KI efficiencies.

CRISPR screens in human T cells

CRISPR screens allow to quickly dissect and validate sets of genes - from a very focused selection up to unbiased whole genome screens. We distinguish thereby between arrayed screens in 96 or 384 well plate-format and pooled screens in only one vessel. Arrayed screens have a low to medium throughput and are more labor-intensive but allow for direct and in-depth phenotyping with multiple readouts and transcriptome profiling. Arrayed Cas9 RNP screens have been applied to interrogate HIV host factors in human CD4+ T cells or to identify novel transcriptional regulators in Treg cell function [113,114]. Pooled screening platforms can be pursued with a higher throughput and have the advantage that fewer batch effects occur. However, pooled screens need further validation steps of candidate genes identified in the initial screen as also multiple edits in individual cells can occur. Moreover, pooled screens are mostly limited to single parameter readouts based on sortable marker proteins or cell proliferation (Fig. 1).

Ting et al. describe Guide Swap as a novel pooled enrichment and depletion screening approach in primary human CD4+ T cells. Non-targeting Cas9 RNPs are electroporated following lentiviral transduction of a sgRNA library targeting over 2500 genes. Intracellularly, the non-targeting sgRNAs are then “swapped” by the lentivirally transduced targeting sgRNAs. After isolation of genomic DNA, the encoded sgRNA abundance in the selected T cell population can be analyzed via next-generation sequencing. Guide Swap was applied for genome-wide functional screenings of coding sequences, splice sites and regulatory regions. This approach and the gained insight could have implications in regenerative medicine and applied immunology [115].

The same year Shifrut et al. established sgRNA lentiviral infection followed by Cas9 protein electroporation (SLICE) as an alternative pooled screening approach, which is based on lentiviral sgRNA transduction with electroporation of Cas9 protein alone. Here, genome-wide loss-of-function and gain-of-function screens were conducted in primary human cells. The SLICE approach could be used to identify key negative regulators of TCR signaling-dependent cell proliferation providing a promising set of candidate genes to improve cellular functionality. Importantly, ablation of several of those genes such as RASA2 and SOCS1/TCEB2 led to a significantly increased tumor cell clearance *in vitro* [116]. Both approaches, Guide Swap and SLICE, have the potential to identify gene targets for the modulation of T cell functions for T cell immunotherapies (Fig. 2D).

Pooled Cas9 RNP editing is an alternative strategy for medium throughput screens in primary human T cells. To this end, a mixture of different Cas9 RNPs is directly electroporated into the cells. In this case, no lentiviral sgRNA construct can act as a barcode, however by sorting cell populations of interest and isolating genomic DNA the actual gene-ablating indels can be identified by multiplexed amplicon PCR followed by next-generation sequencing. This method allows the direct correlation of a phenotypic change after KO with the actual frame-shift mutation. This approach enabled the identification of HIVEP2, a transcription factor regulating regulatory T cell identity [114].

To allow high-throughput characterization of CRISPR KO T cells based on a set of flow cytometry markers but also on a global, unbiased transcriptomic level, pooled KO CRISPR editing platforms and single cell RNA-seq (scRNA-seq) have been combined. The identification of individual sgRNAs within the polyadenylated mRNA pool of each individual cell is achieved by either combining sgRNA expression with an RNA Polymerase II (Pol II) transcribed barcode (Perturb-seq [117], CRISP-seq [118]) or by capturing the sgRNA directly as a Pol II transcript and using the protospacer sequence as a barcode (CROP-seq [116,119]).

All of the screening approaches described above are based on targeted gene deletions. More recently pipelines for low- and medium-throughput pooled KI screens using nonviral HDRT libraries were also optimized for primary human T cells. A set of DNA-barcoded candidate HDRTs encoding for synthetic co-inhibitory receptor chimeras were integrated into the endogenous T cell receptor alpha (*TRAC)* locus of primary human T cells using Cas9 RNPs and screened for increased T cell fitness. Pooled KI screens identified the benefit of the synthetic chimeric receptor TGFβR2/4-1BB to increase the cancer cell killing capacity of tumor-infiltrating lymphocytes *in vitro* and *in vivo* [120]. This discovery platform has the potential to identify synthetic receptors that can overcome immunosuppressive tumor microenvironments in the future.

Recently, the development of arrayed and pooled CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) screens has been established in human T cells. The limiting requirement of continuous dCas9 expression was overcome by transducing human T cells with an optimized minimal lentiviral plasmid encoding for the transcriptional activator dCas9-VP64 or transcriptional repressor dCas9-KRAB together with a sgRNA lentivirus library. The authors thereby uncovered a remodeling of the T cell activation response with an increase in pro-inflammatory cytokines by synthetically overexpressing the transcription factor FOXQ1 [14] (Fig. 2C).

In summary, medium to large-scale CRISPR-Cas9 screens provide attractive pipelines to quickly and efficiently dissect gene networks in human T cells. The results not only provide a better understanding of human T cell biology but can also directly be relevant to select future clinical candidate targets for diverse therapeutic approaches.

Clinical translation of CRISPR/Cas-modified human T cells

While CRISPR/Cas has already revolutionized academic and industry research, its clinical application is still in its infancy. For therapeutic purposes, additional aspects need to be considered and some parameters may be weighted differently. Safety is of utmost importance and therefore rigorous assessment of on-target and off-target editing is critical. Although multiple assays have been developed and are applied during the development of gene edited cells for therapeutic purpose, there are no established standards yet to quantify undesired editing. Nevertheless, first-in-human safety studies with CRISPR/Cas engineered hematopoietic stem cells and T cells were approved by regulatory authorities and already show promising results [42,121]. Owing to the fear of uncontrolled proliferation and toxicity of adoptively transferred T cells, a number of strategies have been developed to induce suicide genes and engage safety switches upon the administration of commercially available drugs, as for example rituximab or rapamycin [122–125]. In addition to safety, the reagents have to be produced under good manufacturing practice (GMP) and the editing processes have to be scaled to large numbers of cells.

From the plethora of different Cas endonucleases, SpCas9 is the most advanced one for clinical application. Lentiviral expression of CRISPR/Cas9 and the risk for insertional mutagenesis with DNA-based delivery of CRISPR increases the risk of off-target effects [22,126]. In contrast, CRISPR/Cas9 RNPs have multiple advantages, because they are

immediately functional with a high efficiency and they have a limited editing window due to their rapid degradation. Thus, the above mentioned engineered HIFI Cas9 RNPs are particularly attractive for future clinical application due to their efficacy, precision and short editing activity. Nevertheless, off-target edits may induce unintended mutagenesis and gene rearrangements with the potential to alter function or oncogenic transformation of the therapeutic cell. The risk for chromosomal translocations is higher with multiplexed approaches including DSBs than with newer approaches without DSBs, as base editors [98]. However, they are more recently developed and might bear yet unidentified risks.

Importantly, *ex vivo* editing of human T cells allows for sufficient evaluation of the cell's functionality and for a reliable quality control before adoptive transfer into patients. In the next few years safety data for the various genome engineering approaches will inform future developments.

Immunogenicity is another concern regarding the CRISPR/Cas components. The most frequently used Cas9 molecules originate from *Streptococcus pyogenes* and *Staphylococcus aureus*, which belong to the most common bacterial commensals in humans. Therefore, within a healthy population approximately three-quarters show a humoral and the majority a cellular anti-Cas9 immune response [23–25]. However, the clinical significance of the anti-Cas9 response in *ex vivo* manipulated cells is possibly negligible because of the transient Cas9 expression during the *ex vivo* culturing period. However, a preexisting immune response may limit gene therapy efficacy following DNA-based delivery methods as well as in *in vivo* studies with long-term expression of Cas9. Therefore, the relevance of anti-Cas9 immunity needs to be evaluated in future trials.

For clinical application, CRISPR/Cas9 is particularly being explored to generate next-generation cell therapies in the treatment of malignancies. The first-in-human phase I clinical trial (NCT NCT03399448) of multiplexed CRISPR/Cas9 gene-edited T cells was performed only six years after its discovery in three patients with either multiple myeloma or advanced sarcoma. Autologous T cells were engineered to express a cancer-specific transgenic TCR, along with multiplexed Cas9 RNP-mediated inactivation of the TCR (targeting TCR α- and β-chain) to prevent mispairing with the transgenic TCR, as well as KO of PD-1 to circumvent T cell exhaustion. The authors detected a single mutation in 40% of the infused engineered T cells, and a double or triple mutation in 20% and 10%, respectively. The off-target hits varied among the three loci, with the highest frequency being observed at the TRBC locus. In addition, owing to the multiplexed editing, chromosomal translocation was identified in all cell products. However, a proportional decrease of affected cells was observed during expansion in the manufacturing period, as well as *in vivo*, suggesting no survival advantage of the engineered cells [42]. In the same year, a second pilot study (NCT02793856) described the adoptive transfer of autologous CRISPR-induced PD-1 KO T cells in twelve patients with late-stage lung cancer. In this study Cas9 and PD-1-targeting gRNA has been introduced via plasmid electroporation which resulted in editing efficiencies of 8-30% [127]. The studies demonstrate feasibility and safety of the gene-edited cell product with proof of cell persistence. No major toxicities were stated, however both reports observed minor off-target effects, whose relevance cannot be conclusively assessed also considering the limited number of patients with late-stage cancer disease.

Alongside the rapid development of CRISPR/Cas9 as a novel biotechnology tool, a new era of cell-based therapies has initiated with the success of human T cells expressing a synthetic CAR in haemato-oncology [128]. To date, numerous preclinical investigations explore CRISPR/Cas9's potential to improve CAR-T cell function, longevity and persistence *in vivo*. Using autologous T cells in cancer patients, however, has a number of disadvantages, such as variability in successful manufacturing, potential T cell dysfunction due to the underlying disease or previous lines of therapies, or limited and delayed availability owing to the long manufacturing process. Therefore, major efforts in the field are focusing on generating allogeneic "off-the-shelf" CAR T cells from healthy donors to overcome these disadvantages [129]. Allogeneic T cells, however, potentially elicit an alloimmune response and graft-versus-host disease (GvHD). Thus, CRISPR/Cas9-mediated editing is increasingly used to circumvent these detrimental side effects, and to explore novel strategies to enhance anti-tumor efficacy as well as to prevent T cell exhaustion in the immunosuppressive environment of solid tumors. To date, there are numerous ongoing phase 1/2 clinical trials investigating allogeneic T cells with either single- or multiplexed manipulation with CRISPR/Cas9 (ClinicalTrials.gov Identifier: NCT04637763, NCT04244656, NCT04438083, NCT04557436, NCT03398967, NCT03166878, NCT04035434). The targeted molecules for gene inactivation include HLA class I and II to prevent alloimmune response, the TCR complex to avoid GvHD and increase cell persistence *in vivo* as well as silencing inhibitory receptors of T cell activation (e.g. PD1) to increase anti-tumor efficacy (clinicaltrials.gov). Further approaches of CRISPR/Cas9 in clinical trials include the induction of drug resistance to potential immunotherapies such as CD52-depleting agents (NCT04557436), or deleting the CAR-specific antigen on the therapeutic cells to prevent fratricide (e.g. in $CD5^+$ and $CD7^+$ hematological malignancies) (ISRCTN19144142) [130]. Moreover, tumor infiltrating lymphocytes or virus-specific T cells in virus-induced malignancies are manipulated *ex vivo* to knock-out inhibitory regulators of T cells (NCT03044743, NCT04426669).

The field of gene- and cell-therapy is also extensively studying therapeutic applications of HDR-mediated CRISPR/Cas9 editing, in particular to repair disease-causing mutations. Although clinical application is still awaiting, promising pre-clinical studies by Roth et al. demonstrated feasibility of correcting a monogenic autoimmune mutation in the CD25RA locus with the help of Cas9 RNPs and a dsDNA HDRT to restore T cell function *in vitro* [44]. Furthermore, FOXP3 function was reconstituted in T cells of immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) patients with diverse FOXP3 mutations using Cas9 RNPs and a rAAV6-FOXP3 HDRT [45]. A proof-of-principle for FOXP3 repair was previously shown in a mouse model of a singular FOXP3 mutation [131]. A further promising application of HDR-mediated KI in T cells, is the site-specific integration of larger transgenes, as for instance a CAR or TCR into the TCR locus, allowing a physiologic genetic regulation of the transgene with increased T cell functionality and reduced T cell exhaustion [44,50-52,132].

Besides NHEJ and HDR-mediated CRISPR/Cas9 modifications, base editing is also rapidly being developed for clinical application. Preclinical studies in T cells investigate multiplexed gene modifications in human allogeneic CAR-T cells with base editors. The absence of the DSB reduces off-target effects and allows it to target multiple loci more safely in comparison to the classic CRISPR/Cas9 system [102].

Outlook

Technical limitations in the field of CRISPR/Cas-based T cell engineering are rapidly being overcome and novel cell engineering tools will enable us to quickly dissect genes as well as gene regulatory elements in T cell biology and function. In the future, improved CRISPR/Cas-based T cell engineering tools will allow for higher efficiency and precision, for the highly controlled insertion/conversion of individual bases as well as large stretches of DNA with optimized safety profiles. Using gene correction and reprogramming of the genomic code, T cells are increasingly becoming programmable vehicles that execute complex tasks and will open avenues to novel curative therapeutic strategies for the treatment of cancer, autoimmunity and viral infections. Importantly, tools to repair disease-causing mutations are now on-hand.

T cells as an easily accessible cell type are at the forefront of these cell-engineering developments. Making such novel therapies accessible to a wide range of patients requires efficient large-scale GMP-grade manufacturing procedures and clear guidelines for safety criteria. Finally, in the long run costs will need to be controlled in order to offer

such therapies to many patients and also for regions with less sophisticated medical infrastructures.

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Conflict of interest statement

KS has a research agreement with HTG Molecular Diagnostics and is an inventor on two patent applications related to CRISPR-engineered human T cells. LTJ is a co-founder of, holds equity in and has a sponsored research agreement with Cimeio Therapeutics AG. LTJ is an inventor on patent applications related to engineered hematopoietic cells.

References

[1] T. Cathomen, J.K. Joung, Zinc-finger nucleases: the next generation emerges, Mol Ther 16 (7) (2008) 1200–1207.
[2] J.K. Joung, J.D. Sander, TALENs: a widely applicable technology for targeted genome editing, Nat Rev Mol Cell Biol 14 (1) (2013) 49–55.
[3] T.I. Cornu, C. Mussolino, T. Cathomen, Refining strategies to translate genome editing to the clinic, Nat Med 23 (4) (2017) 415–423.
[4] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, E. Charpentier, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science 337 (6096) (2012) 816–821.
[5] G. Gasiunas, R. Barrangou, P. Horvath, V. Siksnys, Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria, Proc Natl Acad Sci U S A 109 (39) (2012) E2579–E2586.
[6] L. Cong, F.A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P.D. Hsu, X. Wu, W. Jiang, L.A. Marraffini, F. Zhang, Multiplex genome engineering using CRISPR/Cas systems, Science 339 (6121) (2013) 819–823.
[7] P. Mali, L. Yang, K.M. Esvelt, J. Aach, M. Guell, J.E. DiCarlo, J.E. Norville, G. M. Church, RNA-guided human genome engineering via Cas9, Science 339 (6121) (2013) 823–826.
[8] M. Jinek, A. East, A. Cheng, S. Lin, E. Ma, J. Doudna, RNA-programmed genome editing in human cells, Elife 2 (2013) e00471.
[9] F. Jiang, J.A. Doudna, CRISPR-Cas9 Structures and Mechanisms, Annu Rev Biophys 46 (2017) 505–529.
[10] K. Schumann, S. Lin, E. Boyer, D.R. Simeonov, M. Subramaniam, R.E. Gate, G. E. Haliburton, J.Y. Chun, J.A. Bluestone, J.A. Doudna, Generation of knock-in primary human T cells using Cas9 ribonucleoproteins, Proceedings of the National Academy of Sciences 112 (33) (2015) 10437–10442.
[11] A. Hendel, R.O. Bak, J.T. Clark, A.B. Kennedy, D.E. Ryan, S. Roy, I. Steinfeld, B. D. Lunstad, R.J. Kaiser, A.B. Wilkens, R. Bacchetta, A. Tsalenko, D. Dellinger, L. Bruhn, M.H. Porteus, Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells, Nat Biotechnol 33 (9) (2015) 985–989.
[12] X. Chen, L. Kozhaya, C. Tastan, L. Placek, M. Dogan, M. Horne, R. Abblett, E. Karhan, M. Vaeth, S. Feske, D. Unutmaz, Functional Interrogation of Primary Human T Cells via CRISPR Genetic Editing, J Immunol 201 (5) (2018) 1586–1598.
[13] R. Nasrallah, C.J. Imianowski, L. Bossini-Castillo, F.M. Grant, M. Dogan, L. Placek, L. Kozhaya, P. Kuo, F. Sadiyah, S.K. Whiteside, M.R. Mumbach, D. Glinos, P. Vardaka, C.E. Whyte, T. Lozano, T. Fujita, H. Fujii, A. Liston, S. Andrews, A. Cozzani, J. Yang, S. Mitra, E. Lugli, H.Y. Chang, D. Unutmaz, G. Trynka, R. Roychoudhuri, A distal enhancer at risk locus 11q13.5 promotes suppression of colitis by Treg cells, Nature 583 (7816) (2020) 447–452.
[14] R. Schmidt, Z. Steinhart, M. Layeghi, J.W. Freimer, R. Bueno, V.Q. Nguyen, F. Blaeschke, C.J. Ye, A. Marson, CRISPR activation and interference screens decode stimulation responses in primary human T cells, Science 375 (6580) (2022) eabj4008.
[15] H.X. Zhang, Y. Zhang, H. Yin, Genome Editing with mRNA Encoding ZFN, TALEN, and Cas9, Mol Ther 27 (4) (2019) 735–746.
[16] P.K. Mandal, L.M. Ferreira, R. Collins, T.B. Meissner, C.L. Boutwell, M. Friesen, V. Vrbanac, B.S. Garrison, A. Stortchevoi, D. Bryder, K. Musunuru, H. Brand, A. M. Tager, T.M. Allen, M.E. Talkowski, D.J. Rossi, C.A. Cowan, Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9, Cell Stem Cell 15 (5) (2014) 643–652.
[17] F. Knipping, M.J. Osborn, K. Petri, J. Tolar, H. Glimm, C. von Kalle, M. Schmidt, R. Gabriel, Genome-wide Specificity of Highly Efficient TALENs and CRISPR/Cas9 for T Cell Receptor Modification, Mol Ther Methods Clin Dev 4 (2017) 213–224.
[18] S. Ramakrishna, A.B. Kwaku Dad, J. Beloor, R. Gopalappa, S.K. Lee, H. Kim, Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA, Genome Res 24 (6) (2014) 1020–1027.
[19] H.X. Wang, Z. Song, Y.H. Lao, X. Xu, J. Gong, D. Cheng, S. Chakraborty, J.S. Park, M. Li, D. Huang, L. Yin, J. Cheng, K.W. Leong, Nonviral gene editing via CRISPR/Cas9 delivery by membrane-disruptive and endosomolytic helical polypeptide, Proc Natl Acad Sci U S A 115 (19) (2018) 4903–4908.
[20] E.Y. Cho, J.Y. Ryu, H.A.R. Lee, S.H. Hong, H.S. Park, K.S. Hong, S.G. Park, H. P. Kim, T.J. Yoon, Lecithin nano-liposomal particle as a CRISPR/Cas9 complex delivery system for treating type 2 diabetes, J Nanobiotechnology 17 (1) (2019) 19.
[21] P. Akcakaya, M.L. Bobbin, J.A. Guo, J. Malagon-Lopez, K. Clement, S.P. Garcia, M.D. Fellows, M.J. Porritt, M.A. Firth, A. Carreras, T. Baccega, F. Seeliger, M. Bjursell, S.Q. Tsai, N.T. Nguyen, R. Nitsch, L.M. Mayr, L. Pinello, Y. M. Bohlooly, M.J. Aryee, M. Maresca, J.K. Joung, In vivo CRISPR editing with no detectable genome-wide off-target mutations, Nature 561 (7723) (2018) 416–419.
[22] M. Kosicki, S.S. Rajan, F.C. Lorenzetti, H.H. Wandall, Y. Narimatsu, E. Metzakopian, E.P. Bennett, Dynamics of Indel Profiles Induced by Various CRISPR/Cas9 Delivery Methods, Prog Mol Biol Transl Sci 152 (2017) 49–67.
[23] A. Li, M.R. Tanner, C.M. Lee, A.E. Hurley, M. De Giorgi, K.E. Jarrett, T.H. Davis, A.M. Doerfler, G. Bao, C. Beeton, W.R. Lagor, AAV-CRISPR Gene Editing Is Negated by Pre-existing Immunity to Cas9, Mol Ther 28 (6) (2020) 1432–1441.
[24] C.T. Charlesworth, P.S. Deshpande, D.P. Dever, J. Camarena, V.T. Lemgart, M. K. Cromer, C.A. Vakulskas, M.A. Collingwood, L. Zhang, N.M. Bode, M.A. Behlke, B. Dejene, B. Cieniewicz, R. Romano, B.J. Lesch, N. Gomez-Ospina, S. Mantri, M. Pavel-Dinu, K.I. Weinberg, M.H. Porteus, Identification of preexisting adaptive immunity to Cas9 proteins in humans, Nat Med 25 (2) (2019) 249–254.
[25] D.L. Wagner, L. Amini, D.J. Wendering, L.M. Burkhardt, L. Akyuz, P. Reinke, H. D. Volk, M. Schmueck-Henneresse, High prevalence of Streptococcus pyogenes Cas9-reactive T cells within the adult human population, Nat Med 25 (2) (2019) 242–248.
[26] S. Kim, D. Kim, S.W. Cho, J. Kim, J.S. Kim, Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins, Genome Res 24 (6) (2014) 1012–1019.
[27] S. Su, B. Hu, J. Shao, B. Shen, J. Du, Y. Du, J. Zhou, L. Yu, L. Zhang, F. Chen, H. Sha, L. Cheng, F. Meng, Z. Zou, X. Huang, B. Liu, CRISPR-Cas9 mediated efficient PD-1 disruption on human primary T cells from cancer patients, Sci Rep 6 (2016) 20070.
[28] E. Kamali, F. Rahbarizadeh, Z. Hojati, M. Frodin, CRISPR/Cas9-mediated knockout of clinically relevant alloantigenes in human primary T cells, BMC Biotechnol 21 (1) (2021) 9.
[29] D.N. Nguyen, T.L. Roth, P.J. Li, P.A. Chen, R. Apathy, M.R. Mamedov, L.T. Vo, V. R. Tobin, D. Goodman, E. Shifrut, J.A. Bluestone, J.M. Puck, F.C. Szoka, A. Marson, Polymer-stabilized Cas9 nanoparticles and modified repair templates increase genome editing efficiency, Nat Biotechnol 38 (1) (2020) 44–49.
[30] C.D. Richardson, G.J. Ray, N.L. Bray, J.E. Corn, Non-homologous DNA increases gene disruption efficiency by altering DNA repair outcomes, Nat Commun 7 (2016) 12463.
[31] J.R. Hamilton, C.A. Tsuchida, D.N. Nguyen, B.R. Shy, E.R. McGarrigle, C. R. Sandoval Espinoza, D. Carr, F. Blaeschke, A. Marson, J.A. Doudna, Targeted delivery of CRISPR-Cas9 and transgenes enables complex immune cell engineering, Cell Rep 35 (9) (2021), 109207.
[32] J.D. Sander, J.K. Joung, CRISPR-Cas systems for editing, regulating and targeting genomes, Nat Biotechnol 32 (4) (2014) 347–355.
[33] C. Xue, E.C. Greene, DNA Repair Pathway Choices in CRISPR-Cas9-Mediated Genome Editing, Trends Genet 37 (7) (2021) 639–656.
[34] B. Wienert, D.N. Nguyen, A. Guenther, S.J. Feng, M.N. Locke, S.K. Wyman, J. Shin, K.R. Kazane, G.L. Gregory, M.A.M. Carter, F. Wright, B.R. Conklin, A. Marson, C.D. Richardson, J.E. Corn, Timed inhibition of CDC7 increases CRISPR-Cas9 mediated templated repair, Nat Commun 11 (1) (2020) 2109.
[35] S. Nakade, T. Tsubota, Y. Sakane, S. Kume, N. Sakamoto, M. Obara, T. Daimon, H. Sezutsu, T. Yamamoto, T. Sakuma, K.T. Suzuki, Microhomology-mediated end-joining-dependent integration of donor DNA in cells and animals using TALENs and CRISPR/Cas9, Nat Commun 5 (2014) 5560.
[36] M. van Overbeek, D. Capurso, M.M. Carter, M.S. Thompson, E. Frias, C. Russ, J. S. Reece-Hoyes, C. Nye, S. Gradia, B. Vidal, J. Zheng, G.R. Hoffman, C.K. Fuller, A.P. May, DNA Repair Profiling Reveals Nonrandom Outcomes at Cas9-Mediated Breaks, Mol Cell 63 (4) (2016) 633–646.
[37] C.D. Yeh, C.D. Richardson, J.E. Corn, Advances in genome editing through control of DNA repair pathways, Nat Cell Biol 21 (12) (2019) 1468–1478.
[38] M.W. Popp, L.E. Maquat, Leveraging Rules of Nonsense-Mediated mRNA Decay for Genome Engineering and Personalized Medicine, Cell 165 (6) (2016) 1319–1322.
[39] E.K. Brinkman, T. Chen, M. Amendola, B. van Steensel, Easy quantitative assessment of genome editing by sequence trace decomposition, Nucleic Acids Res 42 (22) (2014) e168.
[40] K. Clement, H. Rees, M.C. Canver, J.M. Gehrke, R. Farouni, J.Y. Hsu, M.A. Cole, D.R. Liu, J.K. Joung, D.E. Bauer, L. Pinello, CRISPResso2 provides accurate and rapid genome editing sequence analysis, Nat Biotechnol 37 (3) (2019) 224–226.
[41] J.F. Hultquist, K. Schumann, J.M. Woo, L. Manganaro, M.J. McGregor, J. Doudna, V. Simon, N.J. Krogan, A. Marson, A Cas9 ribonucleoprotein platform for functional genetic studies of HIV-host interactions in primary human T cells, Cell reports 17 (5) (2016) 1438–1452.
[42] E.A. Stadtmauer, J.A. Fraietta, M.M. Davis, A.D. Cohen, K.L. Weber, E. Lancaster, P.A. Mangan, I. Kulikovskaya, M. Gupta, F. Chen, L. Tian, V.E. Gonzalez, J. Xu, I.

Y. Jung, J.J. Melenhorst, G. Plesa, J. Shea, T. Matlawski, A. Cervini, A.L. Gaymon, S. Desjardins, A. Lamontagne, J. Salas-Mckee, A. Fesnak, D.L. Siegel, B.L. Levine, J.K. Jadlowsky, R.M. Young, A. Chew, W.T. Hwang, E.O. Hexner, B.M. Carreno, C.L. Nobles, F.D. Bushman, K.R. Parker, Y. Qi, A.T. Satpathy, H.Y. Chang, Y. Zhao, S.F. Lacey, C.H. June, CRISPR-engineered T cells in patients with refractory cancer, Science 367 (2020), 6481.

[43] A. Seki, S. Rutz, Optimized RNP transfection for highly efficient CRISPR/Cas9-mediated gene knockout in primary T cells, J Exp Med 215 (3) (2018) 985–997.

[44] T.L. Roth, C. Puig-Saus, R. Yu, E. Shifrut, J. Carnevale, P.J. Li, J. Hiatt, J. Saco, P. Krystofinski, H. Li, Reprogramming human T cell function and specificity with non-viral genome targeting, Nature 559 (7714) (2018) 405–409.

[45] M. Goodwin, E. Lee, U. Lakshmanan, S. Shipp, L. Froessl, F. Barzaghi, L. Passerini, M. Narula, A. Sheikali, C.M. Lee, G. Bao, C.S. Bauer, H.K. Miller, M. Garcia-Lloret, M.J. Butte, A. Bertaina, A. Shah, M. Pavel-Dinu, A. Hendel, M. Porteus, M. G. Roncarolo, R. Bacchetta, CRISPR-based gene editing enables FOXP3 gene repair in IPEX patient cells, Sci Adv 6 (19) (2020) eaaz0571.

[46] S. Lin, B.T. Staahl, R.K. Alla, J.A. Doudna, Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery, Elife 3 (2014) e04766.

[47] P.J. Paszkiewicz, S.P. Frassle, S. Srivastava, D. Sommermeyer, M. Hudecek, I. Drexler, M. Sadelain, L. Liu, M.C. Jensen, S.R. Riddell, D.H. Busch, Targeted antibody-mediated depletion of murine CD19 CAR T cells permanently reverses B cell aplasia, J Clin Invest 126 (11) (2016) 4262–4272.

[48] R.L. Rutishauser, C.D.T. Deguit, J. Hiatt, F. Blaeschke, T.L. Roth, L. Wang, K. A. Raymond, C.E. Starke, J.C. Mudd, W. Chen, C. Smullin, R. Matus-Nicodemos, R. Hoh, M. Krone, F.M. Hecht, C.D. Pilcher, J.N. Martin, R.A. Koup, D.C. Douek, J. M. Brenchley, R.P. Sekaly, S.K. Pillai, A. Marson, S.G. Deeks, J.M. McCune, P. W. Hunt, TCF-1 regulates HIV-specific CD8+ T cell expansion capacity, JCI Insight 6 (3) (2021).

[49] P.J. de Vree, E. de Wit, M. Yilmaz, M. van de Heijning, P. Klous, M.J. Verstegen, Y. Wan, H. Teunissen, P.H. Krijger, G. Geeven, P.P. Eijk, D. Sie, B. Ylstra, L. O. Hulsman, M.F. van Dooren, L.J. van Zutven, A. van den Ouweland, S. Verbeek, K.W. van Dijk, M. Cornelissen, A.T. Das, B. Berkhout, B. Sikkema-Raddatz, E. van den Berg, P. van der Vlies, D. Weening, J.T. den Dunnen, M. Matusiak, M. Lamkanfi, M.J. Ligtenberg, P. ter Brugge, J. Jonkers, J.A. Foekens, J. W. Martens, R. van der Luijt, H.K. van Amstel, M. van Min, E. Splinter, W. de Laat, Targeted sequencing by proximity ligation for comprehensive variant detection and local haplotyping, Nat Biotechnol 32 (10) (2014) 1019–1025.

[50] T.R. Muller, S. Jarosch, M. Hammel, J. Leube, S. Grassmann, B. Bernard, M. Effenberger, I. Andra, M.Z. Chaudhry, T. Kauferle, A. Malo, L. Cicin-Sain, P. Steinberger, T. Feuchtinger, U. Protzer, K. Schumann, M. Neuenhahn, K. Schober, D.H. Busch, Targeted T cell receptor gene editing provides predictable T cell product function for immunotherapy, Cell Rep Med 2 (8) (2021), 100374.

[51] J. Eyquem, J. Mansilla-Soto, T. Giavridis, S.J. van der Stegen, M. Hamieh, K. M. Cunanan, A. Odak, M. Gonen, M. Sadelain, Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection, Nature 543 (7643) (2017) 113–117.

[52] K. Schober, T.R. Müller, F. Gökmen, S. Grassmann, M. Effenberger, M. Poltorak, C. Stemberger, K. Schumann, T.L. Roth, A. Marson, Orthotopic replacement of T-cell receptor α-and β-chains with preservation of near-physiological T-cell function, Nature biomedical engineering 3 (12) (2019) 974–984.

[53] K.S. Gwiazda, A.E. Grier, J. Sahni, S.M. Burleigh, U. Martin, J.G. Yang, N.A. Popp, M.C. Krutein, I.F. Khan, K. Jacoby, M.C. Jensen, D.J. Rawlings, A.M. Scharenberg, High Efficiency CRISPR/Cas9-mediated Gene Editing in Primary Human T-cells Using Mutant Adenoviral E4orf6/E1b55k "Helper" Proteins, Mol Ther 24 (9) (2016) 1570–1580.

[54] B.R. Shy, V. Vykunta, A. Ha, T.L. Roth, A. Talbot, D.N. Nguyen, Y.Y. Chen, F. Blaeschke, S. Vedova, M.R. Mamedov, J.-Y. Chung, H. Li, J. Wolf, T.G. Martin, L. Ye, J. Eyquem, J.H. Esensten, A. Marson, Hybrid ssDNA repair templates enable high yield genome engineering in primary cells for disease modeling and cell therapy manufacturing, bioRxiv (2021) 2021.09.02.458799.

[55] G. Li, D. Liu, X. Zhang, R. Quan, C. Zhong, J. Mo, Y. Huang, H. Wang, X. Ruan, Z. Xu, E. Zheng, T. Gu, L. Hong, Z. Li, Z. Wu, H. Yang, Suppressing Ku70/Ku80 expression elevates homology-directed repair efficiency in primary fibroblasts, Int J Biochem Cell Biol 99 (2018) 154–160.

[56] A. Shahryari, N. Moya, J. Siehler, X. Wang, I. Burtscher, H. Lickert, Increasing Gene Editing Efficiency for CRISPR-Cas9 by Small RNAs in Pluripotent Stem Cells, CRISPR J 4 (4) (2021) 491–501.

[57] Q. Xiao, S. Chen, Q. Wang, Z. Liu, S. Liu, H. Deng, W. Hou, D. Wu, Y. Xiong, J. Li, D. Guo, CCR5 editing by Staphylococcus aureus Cas9 in human primary CD4(+) T cells and hematopoietic stem/progenitor cells promotes HIV-1 resistance and CD4 (+) T cell enrichment in humanized mice, Retrovirology 16 (1) (2019) 15.

[58] F.A. Ran, L. Cong, W.X. Yan, D.A. Scott, J.S. Gootenberg, A.J. Kriz, B. Zetsche, O. Shalem, X. Wu, K.S. Makarova, E.V. Koonin, P.A. Sharp, F. Zhang, In vivo genome editing using Staphylococcus aureus Cas9, Nature 520 (7546) (2015) 186–191.

[59] Y. Wang, B. Wang, H. Xie, Q. Ren, X. Liu, F. Li, X. Lv, X. He, C. Cheng, R. Deng, J. Li, J. Zhao, Z. Song, F. Gu, Efficient Human Genome Editing Using SaCas9 Ribonucleoprotein Complexes, Biotechnol J 14 (7) (2019), e1800689.

[60] B.P. Kleinstiver, M.S. Prew, S.Q. Tsai, N.T. Nguyen, V.V. Topkar, Z. Zheng, J. K. Joung, Broadening the targeting range of Staphylococcus aureus CRISPR-Cas9 by modifying PAM recognition, Nat Biotechnol 33 (12) (2015) 1293–1298.

[61] B.P. Kleinstiver, M.S. Prew, S.Q. Tsai, V.V. Topkar, N.T. Nguyen, Z. Zheng, A. P. Gonzales, Z. Li, R.T. Peterson, J.R. Yeh, M.J. Aryee, J.K. Joung, Engineered CRISPR-Cas9 nucleases with altered PAM specificities, Nature 523 (7561) (2015) 481–485.

[62] S.M. Miller, T. Wang, P.B. Randolph, M. Arbab, M.W. Shen, T.P. Huang, Z. Matuszek, G.A. Newby, H.A. Rees, D.R. Liu, Continuous evolution of SpCas9 variants compatible with non-G PAMs, Nat Biotechnol 38 (4) (2020) 471–481.

[63] R.T. Walton, K.A. Christie, M.N. Whittaker, B.P. Kleinstiver, Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants, Science 368 (6488) (2020) 290–296.

[64] B. Zetsche, J.S. Gootenberg, O.O. Abudayyeh, I.M. Slaymaker, K.S. Makarova, P. Essletzbichler, S.E. Volz, J. Joung, J. van der Oost, A. Regev, E.V. Koonin, F. Zhang, Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system, Cell 163 (3) (2015) 759–771.

[65] B.P. Kleinstiver, A.A. Sousa, R.T. Walton, Y.E. Tak, J.Y. Hsu, K. Clement, M. M. Welch, J.E. Horng, J. Malagon-Lopez, I. Scarfo, M.V. Maus, L. Pinello, M. J. Aryee, J.K. Joung, Engineered CRISPR-Cas12a variants with increased activities and improved targeting ranges for gene, epigenetic and base editing, Nat Biotechnol 37 (3) (2019) 276–282.

[66] B. Shen, W. Zhang, J. Zhang, J. Zhou, J. Wang, L. Chen, L. Wang, A. Hodgkins, V. Iyer, X. Huang, W.C. Skarnes, Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects, Nat Methods 11 (4) (2014) 399–402.

[67] P. Mali, J. Aach, P.B. Stranges, K.M. Esvelt, M. Moosburner, S. Kosuri, L. Yang, G. M. Church, CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering, Nat Biotechnol 31 (9) (2013) 833–838.

[68] F.A. Ran, P.D. Hsu, C.Y. Lin, J.S. Gootenberg, S. Konermann, A.E. Trevino, D. A. Scott, A. Inoue, S. Matoba, Y. Zhang, F. Zhang, Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity, Cell 154 (6) (2013) 1380–1389.

[69] K.M. Shakirova, V.Y. Ovchinnikova, E.B. Dashinimaev, Cell Reprogramming With CRISPR/Cas9 Based Transcriptional Regulation Systems, Front Bioeng Biotechnol 8 (2020) 882.

[70] X. Xu, L.S. Qi, A CRISPR-dCas Toolbox for Genetic Engineering and Synthetic Biology, J Mol Biol 431 (1) (2019) 34–47.

[71] J. Pulecio, N. Verma, E. Mejia-Ramirez, D. Huangfu, A. Raya, CRISPR/Cas9-Based Engineering of the Epigenome, Cell Stem Cell 21 (4) (2017) 431–447.

[72] C. Kressler, G. Gasparoni, K. Nordstrom, D. Hamo, A. Salhab, C. Dimitropoulos, S. Tierling, P. Reinke, H.D. Volk, J. Walter, A. Hamann, J.K. Polansky, Targeted De-Methylation of the FOXP3-TSDR Is Sufficient to Induce Physiological FOXP3 Expression but Not a Functional Treg Phenotype, Front Immunol 11 (2020), 609891.

[73] I.M. Slaymaker, L. Gao, B. Zetsche, D.A. Scott, W.X. Yan, F. Zhang, Rationally engineered Cas9 nucleases with improved specificity, Science 351 (6268) (2016) 84–88.

[74] B.P. Kleinstiver, V. Pattanayak, M.S. Prew, S.Q. Tsai, N.T. Nguyen, Z. Zheng, J. K. Joung, High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects, Nature 529 (7587) (2016) 490–495.

[75] J.S. Chen, Y.S. Dagdas, B.P. Kleinstiver, M.M. Welch, A.A. Sousa, L.B. Harrington, S.H. Sternberg, J.K. Joung, A. Yildiz, J.A. Doudna, Enhanced proofreading governs CRISPR-Cas9 targeting accuracy, Nature 550 (7676) (2017) 407–410.

[76] A. Casini, M. Olivieri, G. Petris, C. Montagna, G. Reginato, G. Maule, F. Lorenzin, D. Prandi, A. Romanel, F. Demichelis, A. Inga, A. Cereseto, A highly specific SpCas9 variant is identified by in vivo screening in yeast, Nat Biotechnol 36 (3) (2018) 265–271.

[77] J.H. Hu, S.M. Miller, M.H. Geurts, W. Tang, L. Chen, N. Sun, C.M. Zeina, X. Gao, H.A. Rees, Z. Lin, D.R. Liu, Evolved Cas9 variants with broad PAM compatibility and high DNA specificity, Nature 556 (7699) (2018) 57–63.

[78] J.K. Lee, E. Jeong, J. Lee, M. Jung, E. Shin, Y.H. Kim, K. Lee, I. Jung, D. Kim, S. Kim, J.S. Kim, Directed evolution of CRISPR-Cas9 to increase its specificity, Nat Commun 9 (1) (2018) 3048.

[79] C.A. Vakulskas, D.P. Dever, G.R. Rettig, R. Turk, A.M. Jacobi, M.A. Collingwood, N.M. Bode, M.S. McNeill, S. Yan, J. Camarena, C.M. Lee, S.H. Park, V. Wiebking, R.O. Bak, N. Gomez-Ospina, M. Pavel-Dinu, W. Sun, G. Bao, M.H. Porteus, M. A. Behlke, A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells, Nat Med 24 (8) (2018) 1216–1224.

[80] M. Bratovic, I. Fonfara, K. Chylinski, E.J.C. Galvez, T.J. Sullivan, S. Boerno, B. Timmermann, M. Boettcher, E. Charpentier, Bridge helix arginines play a critical role in Cas9 sensitivity to mismatches, Nat Chem Biol 16 (5) (2020) 587–595.

[81] J.G. Doench, N. Fusi, M. Sullender, M. Hegde, E.W. Vaimberg, K.F. Donovan, I. Smith, Z. Tothova, C. Wilen, R. Orchard, H.W. Virgin, J. Listgarten, D.E. Root, Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9, Nature Biotechnology 34 (2) (2016) 184.

[82] J.G. Doench, E. Hartenian, D.B. Graham, Z. Tothova, M. Hegde, I. Smith, M. Sullender, B.L. Ebert, R.J. Xavier, D.E. Root, Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation, Nat Biotechnol 32 (12) (2014) 1262–1267.

[83] J.P.K. Bravo, M.S. Liu, G.N. Hibshman, T.L. Dangerfield, K. Jung, R.S. McCool, K. A. Johnson, D.W. Taylor, Structural basis for mismatch surveillance by CRISPR-Cas9, Nature (2022).

[84] N. Kim, H.K. Kim, S. Lee, J.H. Seo, J.W. Choi, J. Park, S. Min, S. Yoon, S.R. Cho, H. H. Kim, Prediction of the sequence-specific cleavage activity of Cas9 variants, Nat Biotechnol 38 (11) (2020) 1328–1336.

[85] M.S. Liu, S. Gong, H.H. Yu, K. Jung, K.A. Johnson, D.W. Taylor, Engineered CRISPR/Cas9 enzymes improve discrimination by slowing DNA cleavage to allow release of off-target DNA, Nat Commun 11 (1) (2020) 3576.

[86] Y. Fu, J.D. Sander, D. Reyon, V.M. Cascio, J.K. Joung, Improving CRISPR-Cas nuclease specificity using truncated guide RNAs, Nat Biotechnol 32 (3) (2014) 279–284.
[87] A. Veres, B.S. Gosis, Q. Ding, R. Collins, A. Ragavendran, H. Brand, S. Erdin, C. A. Cowan, M.E. Talkowski, K. Musunuru, Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing, Cell Stem Cell 15 (1) (2014) 27–30.
[88] T. Kaeuferle, T.A. Stief, S. Canzar, N.N. Kutlu, S. Willier, D. Stenger, P. Ferrada-Ernst, N. Habjan, A.E. Peters, D.H. Busch, T. Feuchtinger, Genome-wide off-target analyses of CRISPR/Cas9-mediated T-cell receptor engineering in primary human T cells, Clin Transl Immunology 11 (1) (2022) e1372.
[89] P. Cameron, C.K. Fuller, P.D. Donohoue, B.N. Jones, M.S. Thompson, M. M. Carter, S. Gradia, B. Vidal, E. Garner, E.M. Slorach, E. Lau, L.M. Banh, A. M. Lied, L.S. Edwards, A.H. Settle, D. Capurso, V. Llaca, S. Deschamps, M. Cigan, J.K. Young, A.P. May, Mapping the genomic landscape of CRISPR-Cas9 cleavage, Nat Methods 14 (6) (2017) 600–606.
[90] S.Q. Tsai, Z. Zheng, N.T. Nguyen, M. Liebers, V.V. Topkar, V. Thapar, N. Wyvekens, C. Khayter, A.J. Iafrate, L.P. Le, M.J. Aryee, J.K. Joung, GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases, Nat Biotechnol 33 (2) (2015) 187–197.
[91] S.Q. Tsai, N.T. Nguyen, J. Malagon-Lopez, V.V. Topkar, M.J. Aryee, J.K. Joung, CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets, Nat Methods 14 (6) (2017) 607–614.
[92] K. Petri, D.Y. Kim, K.E. Sasaki, M.C. Canver, X. Wang, H. Shah, H. Lee, J.E. Horng, K. Clement, S. Iyer, S.P. Garcia, J.A. Guo, G.A. Newby, L. Pinello, D.R. Liu, M.J. Aryee, K. Musunuru, J. Keith Joung, V. Pattanayak, Global-scale CRISPR gene editor specificity profiling by ONE-seq identifies population-specific, variant off-target effects, bioRxiv (2021) 2021.04.05.438458.
[93] B. Wienert, S.K. Wyman, C.D. Richardson, C.D. Yeh, P. Akcakaya, M.J. Porritt, M. Morlock, J.T. Vu, K.R. Kazane, H.L. Watry, L.M. Judge, B.R. Conklin, M. Maresca, J.E. Corn, Unbiased detection of CRISPR off-targets in vivo using DISCOVER-Seq, Science 364 (6437) (2019) 286–289.
[94] X. Wu, D.A. Scott, A.J. Kriz, A.C. Chiu, P.D. Hsu, D.B. Dadon, A.W. Cheng, A. E. Trevino, S. Konermann, S. Chen, R. Jaenisch, F. Zhang, P.A. Sharp, Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells, Nat Biotechnol 32 (7) (2014) 670–676.
[95] G. Turchiano, G. Andrieux, J. Klermund, G. Blattner, V. Pennucci, M. El Gaz, G. Monaco, S. Poddar, C. Mussolino, T.I. Cornu, M. Boerries, T. Cathomen, Quantitative evaluation of chromosomal rearrangements in gene-edited human stem cells by CAST-Seq, Cell Stem Cell 28 (6) (2021), 1136-1147 e5.
[96] E. Brunet, M. Jasin, Induction of Chromosomal Translocations with CRISPR-Cas9 and Other Nucleases: Understanding the Repair Mechanisms That Give Rise to Translocations, Adv Exp Med Biol 1044 (2018) 15–25.
[97] A.C. Komor, Y.B. Kim, M.S. Packer, J.A. Zuris, D.R. Liu, Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage, Nature 533 (7603) (2016) 420–424.
[98] N.M. Gaudelli, A.C. Komor, H.A. Rees, M.S. Packer, A.H. Badran, D.I. Bryson, D. R. Liu, Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage, Nature 551 (7681) (2017) 464–471.
[99] X. Zhang, B. Zhu, L. Chen, L. Xie, W. Yu, Y. Wang, L. Li, S. Yin, L. Yang, H. Hu, H. Han, Y. Li, L. Wang, G. Chen, X. Ma, H. Geng, W. Huang, X. Pang, Z. Yang, Y. Wu, S. Siwko, R. Kurita, Y. Nakamura, L. Yang, M. Liu, D. Li, Dual base editor catalyzes both cytosine and adenine base conversions in human cells, Nat Biotechnol 38 (7) (2020) 856–860.
[100] J. Grunewald, R. Zhou, C.A. Lareau, S.P. Garcia, S. Iyer, B.R. Miller, L.M. Langner, J.Y. Hsu, M.J. Aryee, J.K. Joung, A dual-deaminase CRISPR base editor enables concurrent adenine and cytosine editing, Nat Biotechnol 38 (7) (2020) 861–864.
[101] S. Banskota, A. Raguram, S. Suh, S.W. Du, J.R. Davis, E.H. Choi, X. Wang, S. C. Nielsen, G.A. Newby, P.B. Randolph, M.J. Osborn, K. Musunuru, K. Palczewski, D.R. Liu, Engineered virus-like particles for efficient in vivo delivery of therapeutic proteins, Cell 185 (2) (2022) 250–265, e16.
[102] B.R. Webber, C.L. Lonetree, M.G. Kluesner, M.J. Johnson, E.J. Pomeroy, M. D. Diers, W.S. Lahr, G.M. Draper, N.J. Slipek, B.A. Smeester, K.N. Lovendahl, A. N. McElroy, W.R. Gordon, M.J. Osborn, B.S. Moriarity, Highly efficient multiplex human T cell engineering without double-strand breaks using Cas9 base editors, Nat Commun 10 (1) (2019) 5222.
[103] A.V. Anzalone, L.W. Koblan, D.R. Liu, Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors, Nat Biotechnol 38 (7) (2020) 824–844.
[104] H.A. Rees, D.R. Liu, Base editing: precision chemistry on the genome and transcriptome of living cells, Nat Rev Genet 19 (12) (2018) 770–788.
[105] E.M. Porto, A.C. Komor, I.M. Slaymaker, G.W. Yeo, Base editing: advances and therapeutic opportunities, Nat Rev Drug Discov 19 (12) (2020) 839–859.
[106] R.E. Hanna, M. Hegde, C.R. Fagre, P.C. DeWeirdt, A.K. Sangree, Z. Szegletes, A. Griffith, M.N. Feeley, K.R. Sanson, Y. Baidi, L.W. Koblan, D.R. Liu, J.T. Neal, J. G. Doench, Massively parallel assessment of human variants with base editor screens, Cell 184 (4) (2021) 1064–1080, e20.
[107] C. Kuscu, M. Parlak, T. Tufan, J. Yang, K. Szlachta, X. Wei, R. Mammadov, M. Adli, CRISPR-STOP: gene silencing through base-editing-induced nonsense mutations, Nat Methods 14 (7) (2017) 710–712.
[108] M. Gapinske, A. Luu, J. Winter, W.S. Woods, K.A. Kostan, N. Shiva, J.S. Song, P. Perez-Pinera, CRISPR-SKIP: programmable gene splicing with single base editors, Genome Biol 19 (1) (2018) 107.
[109] M.G. Kluesner, W.S. Lahr, C.L. Lonetree, B.A. Smeester, X. Qiu, N.J. Slipek, P. N. Claudio Vazquez, S.P. Pitzen, E.J. Pomeroy, M.J. Vignes, S.C. Lee, S.P. Bingea, A.A. Andrew, B.R. Webber, B.S. Moriarity, CRISPR-Cas9 cytidine and adenosine base editing of splice-sites mediates highly-efficient disruption of proteins in primary and immortalized cells, Nat Commun 12 (1) (2021) 2437.
[110] A.V. Anzalone, P.B. Randolph, J.R. Davis, A.A. Sousa, L.W. Koblan, J.M. Levy, P. J. Chen, C. Wilson, G.A. Newby, A. Raguram, D.R. Liu, Search-and-replace genome editing without double-strand breaks or donor DNA, Nature 576 (7785) (2019) 149–157.
[111] K. Petri, W. Zhang, J. Ma, A. Schmidts, H. Lee, J.E. Horng, D.Y. Kim, I.C. Kurt, K. Clement, J.Y. Hsu, L. Pinello, M.V. Maus, J.K. Joung, J.J. Yeh, CRISPR prime editing with ribonucleoprotein complexes in zebrafish and primary human cells, Nat Biotechnol (2021) https://www.nature.com/articles/s41587-021-00901-y.
[112] J.W. Nelson, P.B. Randolph, S.P. Shen, K.A. Everette, P.J. Chen, A.V. Anzalone, M. An, G.A. Newby, J.C. Chen, A. Hsu, D.R. Liu, Engineered pegRNAs improve prime editing efficiency, Nat Biotechnol (2021).
[113] J.F. Hultquist, K. Schumann, J.M. Woo, L. Manganaro, M.J. McGregor, J. Doudna, V. Simon, N.J. Krogan, A. Marson, A Cas9 Ribonucleoprotein Platform for Functional Genetic Studies of HIV-Host Interactions in Primary Human T Cells, Cell Rep 17 (5) (2016) 1438–1452.
[114] K. Schumann, S.S. Raju, M. Lauber, S. Kolb, E. Shifrut, J.T. Cortez, N. Skartsis, V. Q. Nguyen, J.M. Woo, T.L. Roth, Functional CRISPR dissection of gene networks controlling human regulatory T cell identity, Nature Immunology 21 (11) (2020) 1456–1466.
[115] P.Y. Ting, A.E. Parker, J.S. Lee, C. Trussell, O. Sharif, F. Luna, G. Federe, S. W. Barnes, J.R. Walker, J. Vance, M.Y. Gao, H.E. Klock, S. Clarkson, C. Russ, L. J. Miraglia, M.P. Cooke, A.E. Boitano, P. McNamara, J. Lamb, C. Schmedt, J. L. Snead, Guide Swap enables genome-scale pooled CRISPR-Cas9 screening in human primary cells, Nat Methods 15 (11) (2018) 941–946.
[116] E. Shifrut, J. Carnevale, V. Tobin, T.L. Roth, J.M. Woo, C.T. Bui, P.J. Li, M. E. Diolaiti, A. Ashworth, A. Marson, Genome-wide CRISPR Screens in Primary Human T Cells Reveal Key Regulators of Immune Function, Cell 175 (7) (2018) 1958–1971, e15.
[117] A. Dixit, O. Parnas, B. Li, J. Chen, C.P. Fulco, L. Jerby-Arnon, N.D. Marjanovic, D. Dionne, T. Burks, R. Raychowdhury, B. Adamson, T.M. Norman, E.S. Lander, J. S. Weissman, N. Friedman, A. Regev, Perturb-Seq: Dissecting Molecular Circuits with Scalable Single-Cell RNA Profiling of Pooled Genetic Screens, Cell 167 (7) (2016) 1853–1866, e17.
[118] D.A. Jaitin, A. Weiner, I. Yofe, D. Lara-Astiaso, H. Keren-Shaul, E. David, T. M. Salame, A. Tanay, A. van Oudenaarden, I. Amit, Dissecting Immune Circuits by Linking CRISPR-Pooled Screens with Single-Cell RNA-Seq, Cell 167 (7) (2016) 1883–1896, e15.
[119] P. Datlinger, A.F. Rendeiro, C. Schmidl, T. Krausgruber, P. Traxler, J. Klughammer, L.C. Schuster, A. Kuchler, D. Alpar, C. Bock, Pooled CRISPR screening with single-cell transcriptome readout, Nat Methods 14 (3) (2017) 297.
[120] T.L. Roth, P.J. Li, F. Blaeschke, J.F. Nies, R. Apathy, C. Mowery, R. Yu, M.L. T. Nguyen, Y. Lee, A. Truong, J. Hiatt, D. Wu, D.N. Nguyen, D. Goodman, J. A. Bluestone, C.J. Ye, K. Roybal, E. Shifrut, A. Marson, Pooled Knockin Targeting for Genome Engineering of Cellular Immunotherapies, Cell 181 (3) (2020) 728–744, e21.
[121] H. Frangoul, D. Altshuler, M.D. Cappellini, Y.S. Chen, J. Domm, B.K. Eustace, J. Foell, J. de la Fuente, S. Grupp, R. Handgretinger, T.W. Ho, A. Kattamis, A. Kernytsky, J. Lekstrom-Himes, A.M. Li, F. Locatelli, M.Y. Mapara, M. de Montalembert, D. Rondelli, A. Sharma, S. Sheth, S. Soni, M.H. Steinberg, D. Wall, A. Yen, S. Corbacioglu, CRISPR-Cas9 Gene Editing for Sickle Cell Disease and beta-Thalassemia, N Engl J Med 384 (3) (2021) 252–260.
[122] A. Hotblack, E.K. Kokalaki, M.J. Palton, G.W. Cheung, I.P. Williams, S. Manzoor, T.I. Grothier, A. Piapi, V. Fiaccadori, P. Wawrzyniecka, H.A. Roddy, G. Agliardi, C. Roddie, S. Onuoha, S. Thomas, S. Cordoba, M. Pule, Tunable control of CAR T cell activity through tetracycline mediated disruption of protein-protein interaction, Sci Rep 11 (1) (2021) 21902.
[123] B. Philip, E. Kokalaki, L. Mekkaoui, S. Thomas, K. Straathof, B. Flutter, V. Marin, T. Marafioti, R. Chakraverty, D. Linch, S.A. Quezada, K.S. Peggs, M. Pule, A highly compact epitope-based marker/suicide gene for easier and safer T-cell therapy, Blood 124 (8) (2014) 1277–1287.
[124] M. Stavrou, B. Philip, C. Traynor-White, C.G. Davis, S. Onuoha, S. Cordoba, S. Thomas, M. Pule, A Rapamycin-Activated Caspase 9-Based Suicide Gene, Mol Ther 26 (5) (2018) 1266–1276.
[125] G. Giordano-Attianese, P. Gainza, E. Gray-Gaillard, E. Cribioli, S. Shui, S. Kim, M. J. Kwak, S. Vollers, A.J. Corria Osorio, P. Reichenbach, J. Bonet, B.H. Oh, M. Irving, G. Coukos, B.E. Correia, A computationally designed chimeric antigen receptor provides a small-molecule safety switch for T-cell therapy, Nat Biotechnol 38 (4) (2020) 426–432.
[126] M. Kosicki, K. Tomberg, A. Bradley, Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements, Nat Biotechnol 36 (8) (2018) 765–771.
[127] Y. Lu, J. Xue, T. Deng, X. Zhou, K. Yu, L. Deng, M. Huang, X. Yi, M. Liang, Y. Wang, H. Shen, R. Tong, W. Wang, L. Li, J. Song, J. Li, X. Su, Z. Ding, Y. Gong, J. Zhu, Y. Wang, B. Zou, Y. Zhang, Y. Li, L. Zhou, Y. Liu, M. Yu, Y. Wang, X. Zhang, L. Yin, X. Xia, Y. Zeng, Q. Zhou, B. Ying, C. Chen, Y. Wei, W. Li, T. Mok, Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer, Nat Med 26 (5) (2020) 732–740.
[128] T. Anagnostou, I.B. Riaz, S.K. Hashmi, M.H. Murad, S.S. Kenderian, Anti-CD19 chimeric antigen receptor T-cell therapy in acute lymphocytic leukaemia: a systematic review and meta-analysis, Lancet Haematol 7 (11) (2020) e816-e826.
[129] S. Depil, P. Duchateau, S.A. Grupp, G. Mufti, L. Poirot, Off-the-shelf' allogeneic CAR T cells: development and challenges, Nat Rev Drug Discov 19 (3) (2020) 185–199.

[130] S. Li, X. Wang, Z. Yuan, L. Liu, L. Luo, Y. Li, K. Wu, J. Liu, C. Yang, Z. Li, D. Wang, L. Shen, X. Ye, J. He, C. Han, Y. Wang, D. Zhang, Y. Dong, L. Fang, Y. Chen, M. Sersch, W.W. Cao, S. Wang, Eradication of T-ALL Cells by CD7-targeted Universal CAR-T Cells and Initial Test of Ruxolitinib-based CRS Management, Clin Cancer Res 27 (5) (2021) 1242–1246.

[131] M. Kornete, R. Marone, L.T. Jeker, Highly Efficient and Versatile Plasmid-Based Gene Editing in Primary T Cells, J Immunol 200 (7) (2018) 2489–2501.

[132] Y.D. Muller, L.M.R. Ferreira, E. Ronin, P. Ho, V. Nguyen, G. Faleo, Y. Zhou, K. Lee, K.K. Leung, N. Skartsis, A.M. Kaul, A. Mulder, F.H.J. Claas, J.A. Wells, J.A. Bluestone, Q. Tang, Precision engineering of an anti-HLA-A2 chimeric antigen receptor in regulatory T cells for transplant immune tolerance, bioRxiv (2021) 2021.08.16.456548.