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What a Clinician Needs to Know about Genome Editing: Status, Opportunities, and Application to Immunology

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Abstract

During the past twenty years, gene editing has emerged as a novel form of gene therapy. Since the publication of the first potentially therapeutic gene editing platform for genetic disorders, increasingly sophisticated editing technologies have been developed. As with viral vector mediated gene addition, inborn errors of immunity (IEIs) are excellent candidate diseases for a corrective autologous haematopoietic stem cell gene editing strategy. Here, we present an overview of different gene editing technologies that are currently being studied for IEIs and the recent progress moving these technologies to clinical benefit.

Keywords: gene editing, inborn errors of immunity, CRISPR/Cas, prime editing, base editing

General introduction

Inborn errors of immunity (IEIs) have been at the heart of gene therapy since the first successful treatment of a patient, Ashanthi de Silva, with adenosine deaminase deficient severe combined immunodeficiency (ADA-SCID) in 1990. Over the intervening decades, we have seen the approaches pioneered in SCID disorders

applied to many immunological, metabolic and haematological inherited diseases with transformative results. More recently, interest has focused on gene editing technologies as potential therapeutic tools, which offer precise correction of genetic mutations in situ. Although gene editing holds great promise for IEIs, this is yet to be realised with no clinical trials underway at present. The achievements reported using the CRISPR/Cas editing system in CAR T cell therapies and sickle cell disease (SCD) are remarkable; however, these approaches rely on gene knock down rather than gene correction. Limited efficiency of targeted gene correction, particularly in haematopoietic stem cells (HSCs), is a major challenge for the field, but steady progress is being made. Here we discuss the status of gene editing for IEIs and provide an overview of current and future technologies which will hopefully reach the clinic in the coming years.

Gene editing platforms

The concept of gene editing is based on the creation of a targeted double or single strand break in the DNA by an endonuclease. Upon creation of the break, the cell has two main repair pathways. Firstly, non-homologous end-joining (NHEJ) is the preferred but error prone pathway, which results in the creation of small insertions and deletions (indels). The second pathway, homology directed repair (HDR), requires a homologous donor and results in integration of the donor template. By incorporating the intact genetic sequence of interest, this pathway can result in correction of mutations or integration of the corrective transgene as a whole. In both cases, following HDR, gene expression remains under the control of the endogenous promotor and, if relevant, additional regulatory elements, which is essential in certain diseases.

Developments in gene editing are progressing at a rapid pace. In early years, double strand breaks (DSBs) in the DNA were created using Zinc-finger nucleases (ZFNs) [1, 2] and transcription activator-like effector (TALE) nucleases (TALENs) [3, 4]. ZFNs and TALENs consist of a nonspecific nuclease domain that is bound to a DNA-binding protein that is sequence specific and guides the nuclease to the targeted locus where the nuclease subsequently creates the DSB. More recently, in 2012, the CRISPR/Cas system was discovered [5]. In contrast to ZFNs and TALENs

the Cas endonuclease is guided by an RNA guide sequence, the guide RNA (gRNA), to a targeted locus. The generation of short gRNA sequences is relatively easy, quick and affordable in comparison to the ZFN and TALEN mediated approaches and the generic Cas endonuclease has become widely commercially available.

We will focus here on the use of gene editing for the treatment of immunological disorders, covering several platform approaches (Figure 1). In its most simple form, creation of a DSB without the introduction of a homology donor results in small mutations through **NHEJ**. This approach can be used to knock out a pathological dominant active genetic element, such as a gain of function mutation. Alternatively, when a homology donor is introduced simultaneously with the creation of the DSB, HDR can occur. When a corrective cDNA is incorporated in the homology donor cassette, site specific **gene insertion** will lead to functional correction of disease-causing mutations throughout the gene. Similarly, the homology donor can contain a shorter corrective sequence. Upon integration a mutation in the targeted area can be corrected in this manner. This form of **gene correction** can be useful for diseases with a single recurrent point mutation.

More recently, alternative Cas nucleases that create a break in only one of the DNA strands have been developed for editing. Fusion of these Cas9 ‘nickases’, or Cas9n, to a deaminase have led to the development of **base editing**. Following the targeted single stranded break created by the Cas9n, the deaminase removes an amino group from the targeted DNA base. Subsequently, DNA mismatch repair mechanisms or DNA replication yield a single nucleotide base edit. In this manner a C to T or A to G single nucleotide base edit can be accomplished using a Cytosine or Adenine base editor respectively. Again, in the case of a dominant point mutation, base editing provides a promising technique. Even more recently, **prime editing** techniques have been developed. This technique also uses Cas9n, which is, in this case, fused to a reverse transcriptase and a special guide RNA, the prime-editing guide RNA (pegRNA). Besides the guide sequence, the pegRNA also contains the reverse transcriptase primer. This sequence acts as template for the reverse transcriptase and contains the desired edit. Prime editing is more versatile than base editing as it can be used to introduce small insertions, deletions and any base-to-base conversion and hence may be of interest for a broader group of diseases.

Current developments by platform

NHEJ based approaches

Upon the creation of a DSB in the DNA, the dominant repair pathway in both dividing and non-dividing cells is NHEJ. During this process, the DSB ends are ligated in an error-prone manner, with a high chance of introducing indels, culminating in knockout of gene expression. Hence, in diseases that are caused by a pathological dominant mutation, gene knockout following the creation of a DSB with gene editing techniques could be a successful therapeutic approach.

SCD and Transfusion-dependent beta-Thalassemia (TDT). In SCD and TDT, increased expression of γ-globin and subsequent restoration of foetal haemoglobin synthesis reduces morbidity and mortality. BCL11A is a transcription factor that represses γ-globin expression in erythroid cells. Hence, downregulating *BCL11A* expression by CRISPR/Cas9 mediated knockout of its erythroid enhancer in HSCs restores γ-globin synthesis and increases synthesis of foetal haemoglobin [6]. Initial data from two clinical trials show that this editing approach is an effective and safe treatment option for SCD and TDT [7].

HIV. CCR5 is a key co-receptor for HIV-1 entry of immune cells but it is not essential for the survival and function of these immune cells. These two characteristics make CCR5 an ideal target for knockout through editing to create HIV resistance. Indeed, naturally occurring CCR5 null cells are resistant to HIV-1. An autologous approach in which CCR5 edited CD4+ T-cells were infused into HIV seropositive patients, showed protection of edited cells from HIV mediated T-cell lysis and a delay to viral rebound during analytical antiretroviral therapy interruption [8, 9]. However, a T-cell based therapy is not a permanent curative treatment option and recurrent infusions are likely to be necessary. Therefore, studies also focus on editing HSCs as a cure. *In vivo* results showed long-term engraftment of successfully edited HSCs, leading to HIV-1 resistance [10]. However, in a recent case report describing a patient receiving CCR5 edited HSCs, editing rates were too low to provide cure of the HIV-1 infection [11]. Further research is aimed at improving safety and efficacy of autologous HSC based gene therapy for HIV infection [12].

Gene knockout in HSCs through editing has the potential to be a treatment option for certain IEIs, but studies looking into this approach are scarce and, when performed, at early pre-clinical stages.

Severe congenital neutropenia (SCN). SCN is an interesting disease from a gene editing perspective and multiple different approaches have been attempted. Over half of the SCN cases are caused by an autosomal dominant mutation in the *ELANE* gene, which encodes for neutrophil elastase. Patients can be treated with regular G-CSF injections, but 15% respond poorly and treated patients are at increased risk of developing MDS/AML [13]. As the disease is autosomal dominant, knockout of the mutated *ELANE* allele, in theory, will result in restored production of neutrophils. However, this approach may generate unedited, monoallelic edited (of mutated or wildtype allele), or bi-allelic edited cells. Therefore, in the case of an autosomal dominant disease such as SCN, editing is associated with the potential risk of creating a novel pathogenic mutated allele through the creation of indels when the wildtype allele is targeted. For SCN, it is expected that this risk is very limited, as null alleles are not pathogenic and mutations in the wildtype allele likely would be tolerated. Hence, CRISPR/Cas9 mediated knockout of *ELANE* was attempted and resulted in restored production of functional neutrophils *in vitro* [14]. Cells with bi-allelic changes seemed to have a natural survival advantage and no newly introduced autosomal dominant mutations were observed [14].

Other IEIs that may profit from a knockout approach include diseases that are caused by a gain of function mutation. For example, CRISPR mediated knockout for STAT1 gain-of-function is being studied currently (personal correspondence E. Morris); it is not known what percentage of cells would need to have the gain-of-function allele knocked out for clinical benefit, as a remaining population of leukocytes with dominant-active STAT1 may still cause symptoms.

Targeted gene insertion

Targeted insertion of the corrective cDNA as novel therapy for IEIs is studied extensively and can be of therapeutic value for many monogenetic immunologic disorders even if a large number of different pathogenic mutations distributed along the length of the gene have been described. By creating a DSB at an early position

in the gene of interest and subsequent integration of the corrective transgene, all mutations downstream of the DSB will be corrected, while gene expression remains under the regulation of the endogenous promotor and other regulatory elements. One major challenge is achieving sufficient levels of correction in the cells of interest to make the treatment clinically relevant. Research mainly focusses on editing of HSCs, which would lead to durable correction. However, toxicity issues and low editing rates in the true naïve stem cell population have often led to lower editing rates than required. Particularly, editing rates have been shown to drop in *in vivo* studies compared to *in vitro* observations. Safety of gene editing is another challenge and off-target activity of the nucleases poses a potential risk. We will describe the progress made in editing for various IEIs and the different approaches that have been attempted to address the above outlined challenges.

SCIDs. As with more conventional viral mediated gene addition approaches, SCID disorders were a first target for gene editing platforms due to the strong selective advantage of corrected cells and relatively low levels of HSC correction required for clinical benefit.

X-SCID has been a popular disease model and proof-of-concept for therapeutic gene editing has been demonstrated across ZFNs, [15, 16] TALENs [17] and CRISPR/Cas platforms [16, 18, 19] as well as using nuclease free adeno-associated virus (AAV) to direct repair [20]. In 2014, Genovese et al. demonstrated the ability to perform targeted gene editing in human HSCs from healthy donors and X-SCID patients [15]. The group used two ZFN-based approaches; one targeting the IL2RG locus and one targeting the AAVS1 safe site harbour with delivery of the corrective donor template by non-integrating lentivirus (integrase deficient lentivirus, IDLV). A safe harbour locus is a place in the genome which allows for expression of an inserted transgene without the risk of affecting surrounding endogenous genes. Integrating a transgene in a safe harbour locus is an alternative to targeted integration at the endogenous locus. In this case, the transgene is not under the control of the endogenous promotor and other regulatory elements. Genovese et al. showed that even at modest levels of correction, edited patient HSCs engrafted in an immunodeficient NSG mouse model gave rise to functional T-cells. Following on from this, the group developed a humanised X-SCID mouse model and through mixed chimerism studies

established that ~10% correction was required to fully correct disease phenotype, providing a target threshold for editing efficiency [16].

With optimised protocols, editing rates in human HSCs and long-term repopulating cells have improved bringing these approached one step closer to, but not yet in the clinic. Ex-vivo HSC lentiviral gene therapy for X-SCID is proving effective and increasingly safe [21, 22] so time will tell if a gene editing strategy can provide superior outcomes. Other forms of SCID where level of correction and gene expression regulation may be more crucial are also being tackled using editing platforms. RAG2 SCID is a good example of this, where proof of concept for CRISPR/Cas mediated targeted correction resulting in functional lymphoid reconstitution has been published using patient derived iPSC and HSCs [23, 24].

X-linked Hyper IgM Syndrome (XHIM or CD40 ligand deficiency). XHIM is a classic example of an IEI that benefits from a site-specific gene editing approach. The disease is due to defects in the *CD40L* gene on the X-chromosome and absent CD40L expression on T lymphocytes results in aberrant communication with B lymphocytes via CD40 that impairs immunoglobulin class switch recombination. Due to lack of signaling through CD40 on other immune cells such as dendritic cells and monocytes/macrophages, patients have a combined immunodeficiency presenting with *Pneumocystic jirovecii* pneumonias, complicated cryptosporidial biliary tract infections, central nervous system infections, and susceptibility to malignancies and autoimmunity [25].

In the 1990's, two groups demonstrated the efficacy of CD40L cDNA gene addition using gamma retroviral vectors in a mouse model of XHIM. Despite successful immunologic reconstitution, a large proportion of mice in both studies developed abnormal lymphoproliferation with some progressing to frank lymphomas due to constitutive and dysregulated CD40L expression on T lymphocytes [26, 27]. These studies highlighted the tightly regulated nature of CD40L expression and subsequent work investigated the use of lentiviral vectors to deliver CD40L cDNA under control of a 1.3kb fragment of the endogenous proximal promoter. While this achieved near-physiologic expression of CD40L on T lymphocytes, there was no further work in primary HSC or murine models [28].

Site-specific gene editing for XHIM was first demonstrated in primary T lymphocytes using TALEN mRNA targeting the 5'UTR and delivery of a codon-optimized cDNA cassette followed by either the endogenous 3'UTR or Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence using AAV6 [29]. Gene-modified, patient-derived T cells showed restored CD40L expression with normal binding to CD40 as measured by flow cytometry. Shortly thereafter, both TALENs and CRISPR/Cas9 were shown to efficiently target gene modification in primary T-cells and HSCs [30]. The feasibility of gene editing for XHIM has also been demonstrated by other groups focusing on the clinical translation of T-cell editing for this disease [31].

Immune dysregulation polyendocrinopathy X-linked (IPEX) syndrome. IPEX syndrome is a severe primary immune regulatory disorder due to mutations in the *FOXP3* gene that result in regulatory T (Treg) cell dysfunction and recalcitrant multiorgan autoimmunity. Similar to many other IEIs, allogeneic HSC transplantation (HSCT) is the only available cure and provides evidence that gene therapy with autologous transplant may also be curative. As IPEX patients typically present with significant end organ damage, pre-transplant conditioning is generally associated with higher morbidity, and the potential with gene therapy for fewer complications associated with reduced conditioning and lack of risk of Graft versus Host Disease with autologous transplant presents an attractive alternative.

Gene modification as a therapeutic approach for IPEX has utilised either lentiviral based gene addition or CRISPR/Cas9 gene editing approaches in either CD4+ T-cells converted into Treg-like cells or in primary HSC. Using a lentiviral vector containing FOXP3 cDNA under control of the human elongation factor EF1α promoter, CD4+ T-cells can be converted into Treg-like cells with stable FOXP3 expression and suppressive functions *in vitro* and *in vivo*. There is currently a Phase 1 dose-escalation clinical trial open at Stanford (NCT05241444) for the administration of FOXP3 lentiviral (LV) vector modified autologous T-cells in IPEX syndrome. Interestingly, the same FOXP3 LV has been shown to be inappropriate in primary HSC, as constitutive FOXP3 expression can alter the engraftment potential of HSCs as well as the differentiation of T lymphocytes [32]. Instead, an LV construct containing the endogenous *FOXP3* promoter and three conserved *FOXP3*-specific regulatory elements (CNS 1-3), cDNA, and endogenous 3'UTR has been shown to

exhibit more physiologic expression and function both *in vitro* and in the scurfy mouse model [33].

CRISPR/Cas9 mediated gene insertion of a corrective FOXP3 template delivered by AAV6 has also been demonstrated in Treg cells isolated from IPEX syndrome patients [34]. While FOXP3 expression was restored under control of endogenous promoter elements, the difficulty of collecting sufficient numbers of peripheral Tregs from affected patients makes this approach less clinically feasible. As an alternative, Honaker et al. utilised TALEN and CRISPR/Cas9 to integrate the constitutive MND promoter just upstream of the FOXP3 coding region [35]. Converted Treg-like cells demonstrated suppressive activity *in vitro* and in *in vivo* models of inflammatory disease. In HSCs, targeted integration of the FOXP3 cDNA has been achieved by utilizing CRISPR/Cas9 targeting exon 1 and AAV6 virus [34]. In contrast to LV transduced HSC constitutively expressing FOXP3, gene edited cells maintained their differentiation capability as assessed by colony-forming unit assays and engraftment in immunodeficient mice.

Overall, there remain multiple approaches of gene modification involving both LV vectors and site-specific nucleases in both T-cells and HSC that may become effective treatment options for IPEX.

Wiskott-Aldrich syndrome (WAS). WAS is an X-linked primary immunodeficiency caused by defects of WASp, expressed in haematopoietic cells and a regulator of actin cytoskeleton. Patients suffer from microthrombocytopaenia, severe eczema, recurrent infections, and have an increased risk of developing lymphoid malignancies [36]. Allogeneic HSCT is curative, but associated with high morbidity and mortality rates when mismatched donors are used [36]. Current clinical trials using LV mediated gene addition with the WAS promotor driving *WAS* expression in autologous HSCs showed promising results, with survival rates of 91% up to 9 years post-treatment [37]. Despite the fact that multilineage engraftment resulted in clinical improvement, platelet counts remained subnormal in LV treated WAS patients [37-40].

Pre-clinical studies are focusing on an editing approach for WAS. Physiological gene expression might result in a more natural pattern of correction in all involved lineages, including platelets. Initial proof-of-concept studies confirmed the feasibility

of targeted *WAS* gene insertion at the WAS locus, which resulted in physiological WASp expression levels in patient-derived iPSCs [41, 42]. More recently, high rates of targeted gene insertion at the *WAS* locus of up to 60% were achieved in human HSCs using CRISPR/Cas9 based gene editing. WASp expression was restored to physiological levels and correction of functional defect in myeloid and lymphoid cells was observed. In addition, *in vitro* results suggested that targeted integration was successful in megakaryocytic progenitors with similar rates to those detected in WAS HSCs. Platelets derived from edited WAS HSCs expressed WASp at levels comparable to their wildtype counterparts. Finally, *in vivo* studies showed successful engraftment of edited HSCs while differentiation potential was preserved [43].

X-linked agammaglobulinemia (XLA). XLA is caused by mutations in the gene encoding the Bruton's agammaglobulinemia tyrosine kinase (*BTK*) protein, which is essential for the development of mature B lymphocytes. As a result, patients have low levels of immunoglobulins, increasing the risk of recurrent and severe infections greatly. Immunoglobulin replacement therapy (IRT) improves patient quality of life and life expectancy, but is expensive and patients remain at an increased risk of infections. Currently, allogeneic HSCT is not considered standard of care for XLA, due to associated toxicity, but a less risky autologous procedure has the potential to provide a cure. XLA is another example of a disease for which a site-specific gene editing approach is preferable, as tight regulation of BTK expression is required; low levels of BTK expression might lead to less efficient signaling and may not restore B lymphopoiesis, while overexpression of BTK is correlated with some types of B lymphoid leukemias [44, 45]. LV-based gene addition has been studied for XLA. However, mimicking endogenous levels of BTK protein expression has proven to be challenging. Low levels of expression in human B-cells were observed when LV constructs containing the endogenous BTK promoter were used [46]. While on the other hand, use of a strong viral promotor, SFFV, driving BTK expression resulted in polyclonal erythroid myeloproliferation *in vivo [47]*. LV based gene therapy in which BTK expression is driven by the human EFS promotor, or B-cell specific CD19 promotor led to partial restoration of BTK expression levels in BTK deficient B-cells [47]. More recently, a study showed that addition of a ubiquitous chromatin opening element (UCOE) upstream of the BTK promotor and a codon optimized BTK cDNA

restored BTK expression in a lineage specific manner to sub-endogenous levels, but mimicking endogenous expression patterns and restoring B-cell development a mouse model [45].

Using a CRISPR/Cas9 platform and AAV donor, a pre-clinical study showed that targeted integration of *BTK* cDNA alone did not result in endogenous BTK expression levels in BTK deficient cell lines [48]. Increasing the AAV6 vector dose, resulted in increased rates of editing, but also reduced viability and expansion of the treated cells likely due to cytotoxicity. Various modifications to the BTK donor cassette were made to improve BTK expression levels. Addition of a truncated BTK terminal intron and a WPRE to the donor cassette improved BTK expression in BTK deficient cell lines and in edited human CD34+ cells, reaching clinically relevant levels of integration and BTK expression [48]. Neither LV nor CRISPR based therapies for BTK have reached the stage of clinical trials.

SCN. Alternative to the previously described knockout approach, targeted integration of the 4th exon of *ELANE* gene has been successfully demonstrated in SCN patient derived HSCs with edited HSCs successfully differentiating into functional neutrophils [49]. The gRNA used in this study targeted both the wildtype and mutant allele. Authors showed that 6% of the wildtype alleles contained indels [49]. Even though there is the previously described small risk of introducing new autosomal dominant mutations, these mutations were not described to be pathogenic.

CTLA4 insufficiency. CTLA4 insufficiency is caused by heterozygous germline mutations in the *CTLA4* gene. CTLA4 is a negative immune regulator that is expressed on regulatory T-cells and conventional T-cells upon activation. CTLA4 insufficiency leads to immune dysregulation due to reduced immune suppression by regulatory T-cells. As the disease is primarily mediated through the lymphoid compartment, T-cell gene therapy, as opposed to HSC gene therapy, may offer a cure. Correcting T-cells has various advantages over HSC gene therapy. First of all, T-cells are more readily available through non-mobilized apheresis. In addition, conditioning regimens required prior to autologous infusion are much less toxic compared to the regimens used for autologous HSC transplantation. Furthermore, as T-cells are terminally differentiated cells, the consequences of introducing

unintended mutations, for example by off-target activity of a nuclease, are limited. Also, higher editing efficiencies tend to be obtained in T-cells, with less toxicity observed. On the other hand, successful editing of HSCs, followed by successful engraftment with preservation of self-renewal and multilineage differentiation capacity, may provide a permanent cure. However, data are showing that if sufficient numbers of central and effector memory T-cells are modified and transferred, T-cells can also persist long-term following infusion [50].

An editing approach for CTLA4 insufficiency proved successful in T-cells, resulting in restoration of CTLA4 expression, with similar expression patterns to healthy control cells [51], confirming preserved endogenous regulation. Furthermore, successfully edited T-cells isolated from CTLA4 insufficient patients functioned normally, and corrected murine T-cells prevented mice from developing lymphoproliferative disease *in vivo* [51].

X-linked lymphoproliferative (XLP). XLP disease is caused by deficiency of SAP (Slam-associated protein) caused by mutations in the *SH2D1A* gene. Disease manifestations include haemophagocytic lymphohistiocytosis, dysgammagolulinemia, an increased risk of developing lymphoma and autoimmunity. Similar to CTLA4 insufficiency, in XLP T-cell dysregulation plays an important role in the disease pathophysiology. Therefore, both a T-cell and HSC approach could be of value. Furthermore, SAP is an important signaling molecule and, as with CD40L and BTK, SAP expression is tightly regulated. As a result, SAP gene addition may be associated with certain risks, such as autoimmunity. Nevertheless, in a proof-of-concept study, lentiviral mediated SAP gene transfer led to restoration of cellular and humoral responses in SAP deficient mice, without the occurrence of adverse effects [52]. A potentially safer T-cell approach, avoiding the risk of ectopic SAP expression, is moving to clinical trial. Infusion of SAP corrected T-cells restored humoral immunity in SAP deficient mice. *In vitro* LV mediated SAP gene transfer into SAP patient derived T-cells restored both humoral and cytotoxic function. Furthermore, corrected SAP patient T-cells were capable of inducing tumor regression in an EBV-LCL lymphoma tumor model in NSG mice [53].

Besides a gene addition approach, gene editing of T-cells for XLP has also been studied. Results showed that SAP expression could be restored to endogenous

levels in T-cells upon successful integration of *SH2D1A* cDNA at the *SH2D1A* locus, restoring SAP-dependent immune functions in XLP patient T-cells [54].

X-linked MAGT1 deficiency with increased susceptibility to Epstein–Barr virus (EBV) infection and N-linked glycosylation defect (XMEN). Achieving editing rates that are clinically relevant in the primitive HSC population is a major challenge and in many *in vivo* studies, editing rates drop significantly compared to prior results obtained *in vitro*. Various mechanisms most likely underly these observations. Firstly, HDR occurs mainly during the S/G2 cell cycle phase. More primitive HSC populations are, however, quiescent (G_0), and hence more likely to undergo NHEJ. Furthermore, HSCs are sensitive to DSBs, impairing their ability to engraft and self-renew.

Brault et al. have tried to improve editing rates and the engraftment potential of edited HSCs while studying gene editing for XMEN disease. XMEN disease is caused by MAGT1 deficiency and is associated with lymphomas. The authors show that upon AAV transduction of HSCs, a strong DNA damage response (DDR) occurs. This DDR has negative effects, inducing apoptosis, cell death and cell-cycle arrest, severely impacting engraftment potential of HSCs. Transient suppression of TP53-binding protein 1 (53BP1) dampens the DDR temporarily, improving engraftment potential. In addition, transient p53 inhibition forces cell-cycle progression, improving editing efficiency. Results showed good levels of engraftment of edited cells and high levels of targeted integration, which were persistent in engrafted human CD45+ cells that had kept their differentiation potential [55].

Chronic Granulomatous Disease (CGD). CGD is caused by decreased activity of phagocyte NADPH oxidase, a complex consisting of 5 proteins, leading to impaired production of reactive oxygen species. Patients suffer from severe recurrent infections, granulomatous inflammation and inflammatory bowel disease. X-linked CGD is the most common form, affecting approximately 65% of patients, and is caused by a mutation in the *CYBB* gene resulting in a defective or absent gp91-phox protein. In autosomal recessive forms of CGD, any of the remaining 4 proteins of the complex are affected, most commonly p47-phox, which is encoded by the *NCF1* gene.

Initial attempts at HSC gene therapy for X-CGD through viral mediated gene addition were unsuccessful due to silencing of transgene expression and insertional mutagenesis causing myelodysplasia [56-58]. Later studies used a safer self-inactivating LV vector that contained a chimeric promotor to preferentially drive transgene expression at high levels in myeloid cells [59, 60]. This vector is currently used in clinical trials, with promising initial results showing 78% patient survival, no CGD-related infections post-treatment, and discontinuation of antibiotic prophylaxis in 67% of patients [61].

Targeted integration of *CYBB* cDNA at the *CYBB* locus ensures that *CYBB* expression remains under the control of endogenous regulatory elements and thus avoids the risks associated with aberrant production of ROS in corrected HSCs. This editing approach has shown restoration of gp91-phox and ROS production by phagocytes both in X-CGD iPSC and primary patient HSCs [62, 63], with elements in the first intron being essential for endogenous levels of pg91-phox production. Similar to studies in XMEN disease, transient inhibition of NHEJ through temporary inhibition of the NHEJ-promoting DNA repair protein 53BP1, resulted in a clear increase of targeted integration in HSCs [63].

As an alternative approach, De Ravin et al. optimized targeted integration at the AAVS1 safe harbour locus in HSCs and tested out their protocol using CGD as disease model [64]. Using their optimized delivery protocol for ZFN mRNA electroporation and AAV6 delivery, the authors show that insertion of gp91-phox cDNA driven by the MND promotor resulted in 15% gp91-phox protein expression in CGD patient derived HSCs *in vitro*. The MND-gp91 corrected CGD HSCs were functional. Corrected CGD patient HSCs were able to engraft in NSG mice with persistent, albeit at low frequency, gp91 expression in engrafted human CD45+ cells [64].

Again, similar to studies performed in X-CGD, Klatt et al. integrated a therapeutic phox-47 transgene at the AAVS1 safe harbour locus. This time, cell-type specific promoters, namely the myeloid specific miR223, CatG/cFes and MRP8 promotors were tested. MRP8 promotor silencing occurred through high CpG methylation, but the other two promotors resulted in therapeutically relevant levels of phox47 in corrected iPSC-derived myeloid cells [65].

A final interesting editing approach for p47-CGD encompasses the correction of a 2-nt deletion (ΔGT) from the GTGT start of exon 2 of the *NCF1* gene. ΔGT results in a frameshift and a premature stopcodon and is the most common mutation in p47-CGD patients, with >80% of the p47-CGD patients being homozygous for ΔGT *NCF1*. Interestingly, *NCF1* has two pseudogenes, *NCF1B* and *NCF1C*, in the vicinity in which the same ΔGT mutation in exon 2 is constitutive. Pseudogenes are elements in the DNA that resemble a functional gene, but are nonfunctional through mutation(s), making the pseudogene incapable of coding for a functional protein. Targeting the ΔGT mutation, will lead to correction of *NCF1* or either of the pseudogenes. Merling et al. pursued this approach using ZFNs and an rAAV2 donor cassette and showed restoration of p47-phox expression and oxidase function in differentiated p47phox patient derived iPSCs. Furthermore, correction of a pseudogene alone, in p47-CGD patient derived iPSCs that contained a different mutation, also resulted in restoration of phox47 expression and cell function, thus showing that targeted correction of a pseudogene alone can correct a monogenic disorder [66].

Newer platform approaches

Gene Correction

Instead of targeted integration of the full cDNA of the gene of interest, gene editing can be used to correct a point mutation. This approach can be of interest as treatment for diseases in which a single causative point mutation is present in the majority of the patients. An advantage of this approach is that the required HDR donor sequence is short and hence alternatives to a viral HDR donor can be used such as a single-stranded DNA oligonucleotide donor (ssODN), evading the risk of off-target integration and avoiding the intensive engineering that is associated with viral donors. The ssODN consists of two homology arms, one of which contains the desired edit. Besides the above described knockout approach, targeted gene correction is studied as treatment for SCD [67, 68].

SCN. Mutation specific gene correction in the *ELANE* gene is not an approach that is clinically relevant, as over 200 disease causing mutations, spread all along the length of the gene have been described in patients. However, by targeting the

mutated allele specifically, the wildtype allele will not be targeted, avoiding the previously mentioned risk of introducing new pathogenic dominant mutations. In a proof-of-principle study, it was shown that targeted correction of a mutation in exon 4 could be achieved, with editing rates up to 56%, while keeping the wildtype allele intact. This restored neutrophil differentiation *in vivo* and *in vitro* and resulted in restoration of function in the repaired neutrophils *in vitro* [49].

CGD. C676T substitution in exon 7 of the *CYBB* gene is the most common mutation described in X-CGD patients, accounting for 6% of the cases. The mutation results in a premature stop codon and an inactive gp91-phox protein. Targeted correction of the C676T substitution, using the CRISPR/Cas platform and an ssODN donor, resulted in restoration of gp91-phox expression in approximately one third of X-CGD HSC-derived myeloid cells with partial restoration of cell function. The authors showed similar rates of HDR gene repair in various CD34+ HSC subpopulations, including primitive progenitor cells. The edited cells were able to engraft in NSGs successfully. As is common, a decrease in gene repair rates were observed when comparing pre-transplant data to post-transplant rates, but analysis of mouse peripheral blood showed stable gene repair levels over time as indicated by gp91-phox expression levels in human CD45+ myeloid cells derived from gene-corrected P1 CD34+ HSCs *[69]*.

Base editing

In the past few years, newer gene editing approaches have been developed that can make more precise genomic changes than those produced using nucleases, such as CRSPR/Cas9 or ZFNs or TALENS. Base editing uses the DNA localization activity of the CRISPR Cas9 protein to position an enzyme capable of deaminating single cytosine or adenine bases at the target genomic site [70, 71]. The deaminated nucleotides are then converted to thymidine or guanidine bases respectively, reverting C:G base-pairs to A:T or vice versa.

CD3δ SCID. Adenine base editing was recently shown to be able to correct a stop codon mutation (TAG) in the *CD3D* gene that is a rare cause of SCID, but occurs with high incidence in a Mennonite population living in Canada and Mexico [72]. By deaminating the A opposite the T of the stop codon, the TAG stop codon is reverted

to the wild-type CAG encoding an arginine in the CD3δ protein. Adenine base editing of CD3δ SCID patient bone marrow CD34+ HSC corrected the *CD3D* mutation with high efficiency; the edited HSC had normal T lymphopoiesis capacity, as assayed in an Artificial Thymic Organoid system.

Prime editing

While base editing is efficient and precise, it is only capable of reverting single base pair mutations. A next iteration of editing, Prime Editing, can "write" into the genome sequence changes of 5-15 bases in length at a precise location [73].

CGD. One IEI being approached by Prime Editing is p47 autosomal recessive CGD due to the previously described 2 base-pair deletion. Prime editing can insert the two missing bases and restore the reading frame for the p47 protein [74].

Even newer editing approaches use the sequence-specific DNA recombination of bacteriophage recombinases and transposases to insert whole cDNA-size DNA sequences [75]. This approach can be used for disorders where there is a wide number of different mutations in the responsible gene across different patients; it is similar to using Cas9 nuclease and homologous donor to insert a gene, but does not produce a double-strand DNA break. Thus, the toolbox of editing strategies is rapidly advancing and may allow essentially any genetic mutation to be repaired precisely, allowing autologous HSCT to be used without the need for immune suppression and risks of GVHD seen in allogeneic HSCT.

Concluding remarks

Gene editing holds great promise for IEIs, and development of more efficient and potentially safer techniques is rapid. Despite much work in the area, most work is still focused on pre-clinical studies aimed at improving efficiency of gene editing delivery systems, targeting long term repopulating haematopoietic stem cell progenitors and increasing overall correction efficiency. The high cost of such potential therapies is extremely relevant and many groups are also working on developing approaches which reduce manufacturing costs, ideally resulting in

improved access to trials and therapies. Once these challenges have been overcome, successful scale-up studies showing good safety and efficacy will hopefully lead to translation of these promising techniques into clinical trials in the near future.

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Conflicts of Interest

There are no conflicts of interest.

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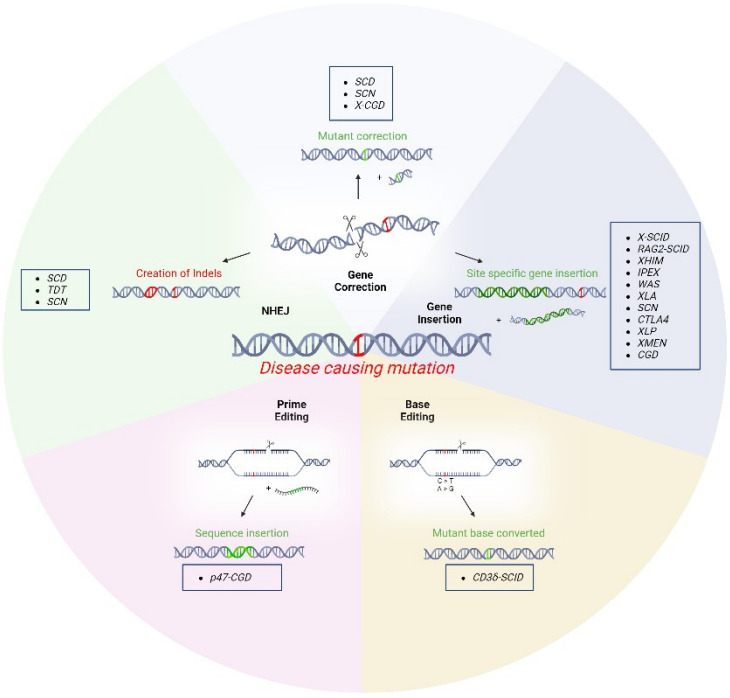

Figure 1. Schematic of the 5 different gene editing platforms. Creation of a targeted double or single strand DNA break lies at the basis of gene editing. **NHEJ** is the most dominant, but error prone DNA repair pathway a cell uses to repair a double strand DNA break (DSB). NHEJ results in the creation of small insertions and deletions, leading to gene knockout. This approach can be used to knockout a pathologic dominant gene. **Gene correction** occurs after homology directed repair (HDR) of a DSB. A homology donor, containing the corrective gene sequence in one of the homology arms is used to drive HDR. This approach can be used to repair a single point mutation that is causative in the majority of the cases. **Gene insertion** is also based on the occurrence of HDR of a DSB. In contrast to gene correction, the whole corrected cDNA sequence is present in the homology donor and inserted at the targeted locus. This approach can be used to repair various different mutations in monogenic disorders. **Base editing** follows the creation of a targeted single strand DNA break created by a modified Cas9 endonuclease, Cas9 nickase (Cas9n). The Cas9n is fused to a deaminase, which effectuates the single nucleotide base edit. This approach can be used to repair a dominant point mutation. **Prime editing** also is based on the creation of a targeted single strand DNA break created by Cas9n. A special prime-editing guide RNA contains a sequence that acts as template for repair. This approach can be used to repair a variety of small insertions, deletions and base substitution. Created with BioRender.com

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