Clinical Commentary Review

What a Clinician Needs to Know About Genome Editing: Status and Opportunities for Inborn Errors of Immunity

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During the past 20 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 are excellent candidate diseases for a corrective autologous hematopoietic stem cell gene editing strategy. Research on gene editing for inborn errors of immunity is still entirely preclinical, with no trials yet underway. However, with editing techniques maturing, scientists are investigating this novel form of gene therapy in context of an increasing number of inborn errors of immunity. Here, we present an overview of these studies and the recent progress moving these technologies closer to clinical benefit. **(J Allergy Clin Immunol Pract 2024;■:■-■)**

Key words: *Gene editing; Inborn errors of immunity; CRISPR/Cas; Prime editing; Base editing*

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 (SCID) in 1990. Over the intervening decades, we have seen the approaches pioneered in SCID disorders applied to many immunologic, metabolic, and hematological 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*. Similar to allogeneic hematopoietic stem cell transplantation (HSCT), autologous corrected HSCT, both conventional gene addition and gene editing, will not improve disease manifestations that are not caused by defects in the hematopoietic system, such as skeletal or neurological symptoms. However, the autologous nature of gene therapy holds certain advantages over allogeneic HSCT. First, gene therapy does not rely on the availability of a suitable allogeneic donor. Furthermore, there is no risk of graft versus host disease or alloreactivity posttreatment and a single-agent conditioning regimen will most likely suffice, because no lymphodepletion is necessary. For complex diseases in which tight regulation of protein expression is essential, gene editing has an additional advantage over conventional gene therapy as the endogenous regulation of gene expression is preserved with this approach. Although gene editing holds great promise for IEIs, this is yet to be realized with no clinical trials underway at present. Table I highlights some important aspects of conventional gene therapy and gene editing as potential treatment options for IEIs. To date, the achievements reported using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas editing system in chimeric antigen receptor 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 hematopoietic 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. We will also touch upon potential benefits of a gene editing approach in comparison to other treatment options, which will differ per disease.

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. On creation of the break, the cell has 2 main repair pathways. First, nonhomologous end-joining (NHEJ) is the preferred but error-prone pathway, which can result 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

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Abbreviations used
AAV- adeno-associated virus
BTK- Bruton's agammaglobulinemia tyrosine kinase
Cas9n- Cas9 nickase
cDNA- complementary DNA
CGD- chronic granulomatous disease
CTLA4- cytotoxic T-lymphocyte associated protein 4
CRISPR- clustered regularly interspaced short palindromic repeats
DSB- double-strand break
gRNA- guide RNA
HDR- homology-directed repair
HSC- hematopoietic stem cell
HSCT- hematopoietic stem cell transplantation
IEI- inborn error of immunity
indels- insertions and deletions
IPEX- immune dysregulation polyendocrinopathy enteropathy X-linked
iPSC- induced pluripotent stem cell
LV- lentiviral
NHEJ- nonhomologous end-joining
PI3K- phosphoinositide 3-kinase
SAP- Slam-associated protein
SCD- sickle cell disease
SCID- severe combined immunodeficiency
SCN- severe congenital neutropenia
TALEN- transcription activator–like effector nuclease
Treg- regulatory T
WAS- Wiskott-Aldrich syndrome
WASp- WAS protein
XMEN- X-linked MAGT1 deficiency with increased susceptibility to EBV-infection and N-linked glycosylation defect
XHIM- X-linked hyper-IgM
XLA- X-linked agammaglobulinemia
XLP- X-linked lymphoproliferative
ZFN- Zinc-finger nuclease

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 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 immunologic 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. However, it must be noted that if the gRNA does not specifically target the mutated allele only, this approach can lead to a full genetic knockout. Furthermore, even if specific knockout of a pathological-dominant active genetic element is achieved, haploinsufficiency of the respective gene might induce pathology. Alternatively, when a homology donor is introduced simultaneously with the creation of the DSB, HDR can occur. When a corrective complementary DNA (cDNA), containing the full remaining coding sequence, 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. On 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 1 of the DNA strands have been developed for editing. Fusion of these Cas9 "nickases," or Cas9n, to a deaminase has led to the development of *base editing*. Following creation of the targeted single-stranded break by 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, a *prime-editing* technique has been developed. This technique also uses Cas9n, which is, in this case, fused to a reverse transcriptase and a special gRNA, the prime-editing gRNA. Besides the guide sequence, the prime-editing gRNA 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 because 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

On the creation of a DSB in the DNA, the dominant repair pathway in both dividing and nondividing cells is NHEJ. During this process, the DSB ends are ligated in a more error-prone manner, which can lead to the introduction of indels and subsequent knockout of gene expression. In case of CRISPR/Cas-based editing, the gRNA will continue to bind to the intact target sequence, triggering the creation of a DSB. This process will come to a halt when the targeted sequence has been mutated as a result of the creation of indels or other aberrations. This eventually results in a high chance of introducing indels, culminating in high rates of knockout of gene expression. Hence, in diseases that are caused by a pathological- dominant mutation, gene knockout following the creation of a DSB using gene-editing techniques could be a successful therapeutic approach.

SCD and transfusion-dependent beta-thalassemia. In SCD and transfusion-dependent beta-thalassemia, increased expression of γ-globin and subsequent restoration of fetal hemoglobin 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 fetal hemoglobin.[6]

TABLE I. Comparison of some important characteristics of conventional gene therapy and gene editing

Facet	Conventional gene therapy	Gene editing
Technique specifications	• Gene expression driven by synthetic protomer that is incorporated in the vector	• Potential for physiological correction as the gene is repaired *in situ* with gene expression controlled by endogenous promoter
	• Risk of insertional mutagenesis	• Risk of off-target genotoxicity
Development and infrastructure	• Multiple gene therapy clinical trials currently active for various monogenic IEIs	• Research on targeted integration still in preclinical phase
	• Infrastructure for production and delivery of therapy established	• Infrastructure yet to be established
	• Long-term efficacy and safety data available	• Development and production of treatment associated with high costs

Initial data from 2 clinical trials show that this editing approach is an effective and safe treatment option for SCD and transfusion-dependent beta-thalassemia.[7] Indeed, the Medicines and Healthcare Products Agency in the United Kingdom and the United States Food and Drug Administration have recently approved Casgevy, the first CRISPR/Cas9-based therapy, for SCD and transfusion-dependent beta-thalassemia.

HIV. CCR5 is a key coreceptor for HIV-1 entry of immune cells, but it is not essential for the survival and function of these immune cells. These 2 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. Preclinical murine *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 (∼ 5%) 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 preclinical stages.

Severe congenital neutropenia. Severe congenital neutropenia (SCN) is an interesting disease from a gene editing perspective and multiple different approaches have been attempted. More than 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 myelodysplastic syndrome/acute myeloid leukemia.[13] Because 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 wild-type allele), or biallelic 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 wild-type allele is targeted. For SCN, it is expected that this risk is very limited, because null alleles are not pathogenic and mutations in the wild-type allele likely would be tolerated. Hence, CRISPR/Cas9-mediated knockout of *ELANE* was attempted, with editing rates varying between 27% and 94%, and resulted in restored production of functional neutrophils *in vitro*.[14] Cells with biallelic 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, but no studies have been published yet.

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 focuses on editing of HSCs, which would lead to durable correction. However, toxicity issues and low editing efficiency in the true naive stem cell population have often led to lower editing rates than required. Particularly, editing rates have been shown to drop in preclinical *in vivo* studies compared with *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.

Severe combined immunodeficiencies. 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[15] demonstrated the ability to

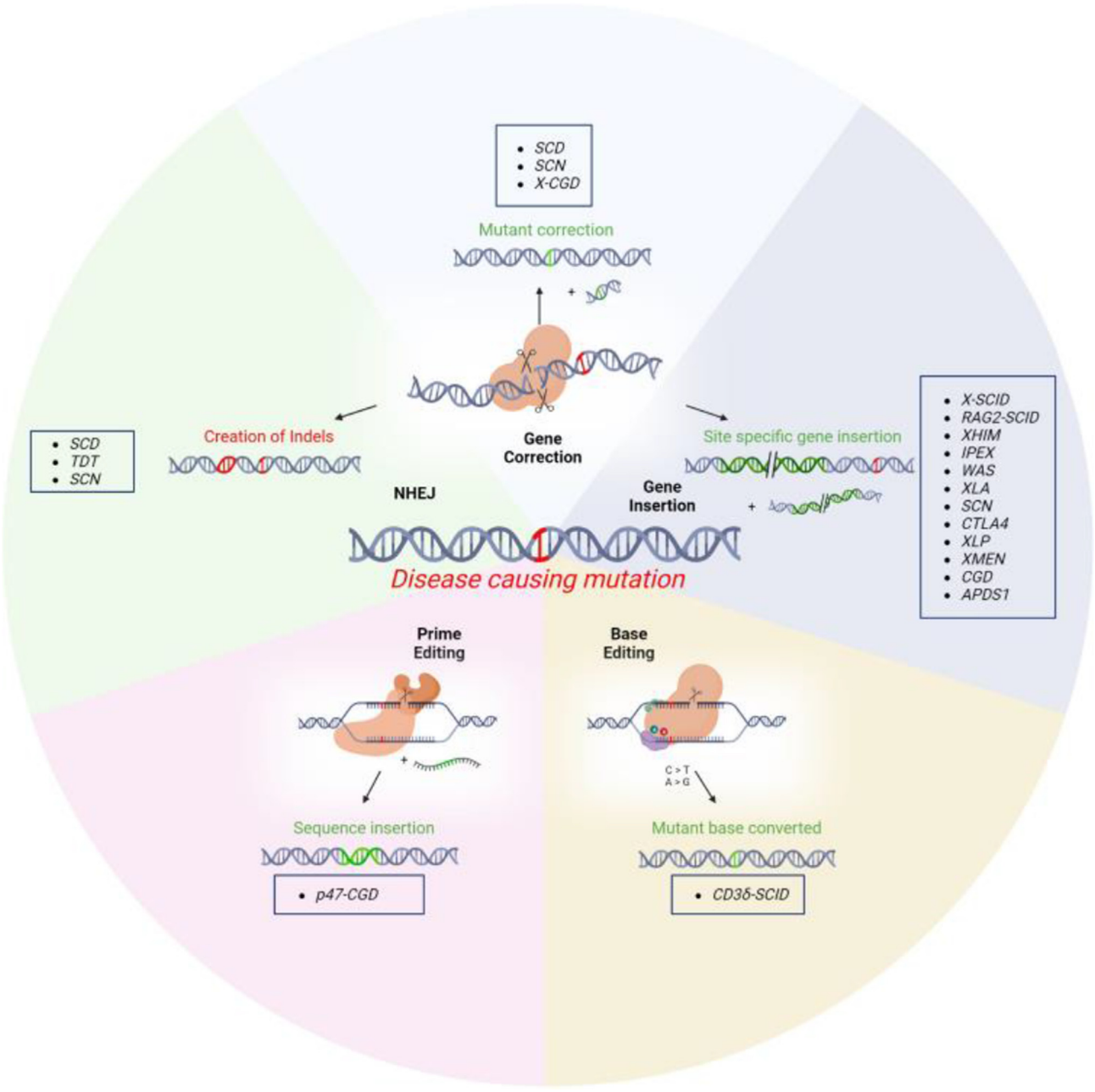

FIGURE 1. Schematic of the 5 different gene editing platforms. Creation of a targeted double- or single-strand DNA break lies at the basis of gene editing. *NHEJ* is the most dominant, but error-prone DNA repair pathway a cell uses to repair a DSB. NHEJ results in the creation of small indels, leading to gene knockout. This approach can be used to knock out a pathologic-dominant gene. *Gene correction* occurs after 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 most 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, 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 gRNA contains a sequence that acts as a template for repair. This approach can be used to repair various small insertions, deletions, and base substitution. *TDT*, Transfusion-dependent beta-thalassemia; *X-CGD*, X-linked CGD; *X-SCID*, X-linked SCID. Created with BioRender.com.

perform targeted gene editing in human HSCs from healthy donors and patients with X-SCID. The group used 2 ZFN-based approaches, one targeting the IL2RG locus and one targeting the AAVS1 safe site harbor with delivery of the corrective donor template by nonintegrating lentivirus (integrase-deficient lentivirus). A safe harbor locus is a place in the genome that allows for expression of an inserted transgene without the risk of affecting surrounding endogenous genes. Integrating a transgene in a safe harbor 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 humanized X-SCID mouse model and through mixed chimerism studies established that approximately 10% correction was required to fully correct disease phenotype, providing a target threshold for editing efficiency.[16]

With optimized 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. Promising results have been obtained by supplementing HSC culture medium with stem cell agonists, which improved levels of gene targeting in long-term repopulating HSCs both *in vitro* and *in vivo*.[21] Others have shown that transient inhibition of p53-binding protein 1, a protein that favors NHEJ, improved HDR rates in long-term engrafting $CD34^+$ cells.[22] On the basis of the observation that HDR is preferentially active in the S/G2 stages of cell cycle, other groups have investigated cell-cycle modulators to improve HDR rates in long-term HSCs.[23,24] *Ex vivo* HSC lentiviral (LV) gene therapy for X-SCID is proving effective and increasingly safe,[25,26] and so time will tell whether 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 induced pluripotent stem cells (iPSCs) and HSCs.[27,28]

X-linked hyper-IgM syndrome (or CD40 ligand deficiency). X-linked hyper-IgM (XHIM) syndrome 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. Because of 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.[29]

In the 1990s, 2 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.[30,31] These studies highlighted the tightly regulated nature of CD40L expression, and subsequent work investigated the use of LV vectors to deliver CD40L cDNA under control of a 1.3-kb fragment of the endogenous proximal promoter. Although this achieved near-physiologic expression of CD40L on T lymphocytes, there was no further work in primary HSC or murine models.[32]

Site-specific gene editing for XHIM was first demonstrated in primary T lymphocytes using TALEN mRNA targeting the 5′ untranslated region and delivery of a codon-optimized cDNA cassette followed by either the endogenous 3′ untranslated region or Woodchuck hepatitis virus posttranscriptional regulatory element sequence using AAV6.[33] 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, with a maximum rate of targeted integration of 16.6% and 27.4% in HSCs using a TALEN- or CRISPR-based approach, respectively.[34] 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.[35]

Immune dysregulation polyendocrinopathy X-linked syndrome. Immune dysregulation polyendocrinopathy X-linked (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 HSCT is the only available cure and provides evidence that gene therapy with autologous transplant may also be curative. Because patients with IPEX typically present with significant end-organ damage, pretransplant 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 used either LV-based gene addition or CRISPR/Cas9 gene-editing approaches in either $CD4^+$ T cells converted into Treg-like cells or in primary HSCs. Using an LV 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 preclinical *in vitro* and murine *in vivo* studies. There is currently a phase 1 dose-escalation clinical trial open at Stanford (NCT05241444) for the administration of FOXP3 LV vector−modified autologous T cells in IPEX syndrome. Interestingly, the same FOXP3 LV has been shown to be inappropriate in primary HSCs, because constitutive FOXP3 expression can alter the engraftment potential of HSCs as well as the differentiation of T lymphocytes.[36] Instead, an LV construct containing the endogenous *FOXP3* promoter and 3 conserved *FOXP3*-specific regulatory elements (CNS 1-3), cDNA, and endogenous 3' untranslated region has been shown to exhibit more physiologic expression and function both *in vitro* and in the scurfy mouse model.[37]

CRISPR/Cas9-mediated gene insertion of a corrective FOXP3 template delivered by AAV6 has also been demonstrated in Treg cells isolated from patients with IPEX syndrome.[38] Although FOXP3 expression was restored under control of endogenous promoter elements, the difficulty of collecting sufficient numbers of peripheral Treg cells from affected patients makes this approach less clinically feasible. As an alternative, Honaker et al[39]

used TALEN and CRISPR/Cas9 to integrate the constitutive MND promoter just upstream of the FOXP3 coding region. Converted Treg-like cells demonstrated suppressive activity *in vitro* and in murine *in vivo* models of inflammatory disease. In HSCs, targeted integration of the FOXP3 cDNA has been achieved by using CRISPR/Cas9 targeting exon 1 and AAV6 virus.[38] In contrast to LV-transduced HSCs 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 HSCs that may become effective treatment options for IPEX.

Wiskott-Aldrich syndrome. Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency caused by defects of WAS protein (WASp), expressed in hematopoietic cells and a regulator of actin cytoskeleton. Patients suffer from microthrombocytopenia, severe eczema, and recurrent infections, and have an increased risk of developing lymphoid malignancies.[40] Allogeneic HSCT is curative, but associated with high morbidity and mortality rates when mismatched donors are used.[40] 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 posttreatment.[41] Despite the fact that multilineage engraftment resulted in clinical improvement, platelet counts remained subnormal in LV-treated patients with WAS.[41-44]

Preclinical 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.[45,46] 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.[47] 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 those in their wild-type counterparts. Finally, murine *in vivo* studies showed successful engraftment of edited HSCs while differentiation potential was preserved.[47] Alternative systems for delivery of the Cas9 nuclease have also been studied. Using baboon envelope pseudotyped virus-like particles to deliver the Cas9 protein complexed with a gRNA, in combination with a donor-encoding AAV6 vector, led to knock-in rates up to 40% at the WAS target site in HSCs.[48]

X-linked agammaglobulinemia. X-linked agammaglobulinemia (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 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, because tight regulation of BTK expression is required; low levels of BTK expression might lead to less efficient signaling and may not restore B lymphopoiesis, whereas overexpression of BTK is correlated with some types of B lymphoid leukemias.[49,50] 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.[51] However, use of a strong viral promotor, SFFV, driving BTK expression resulted in polyclonal erythroid myeloproliferation in mice.[52] 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.[52] More recently, a study showed that addition of a ubiquitous chromatin opening element upstream of the BTK promotor and a codon-optimized BTK cDNA restored BTK expression in a lineage-specific manner to subendogenous levels, but mimicking endogenous expression patterns and restoring B-cell development a mouse model.[50]

Using a CRISPR/Cas9 platform and AAV donor, a preclinical study showed that targeted integration of *BTK* cDNA alone did not result in endogenous BTK expression levels in BTK- deficient cell lines.[53] 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 Woodchuck hepatitis virus posttranscriptional regulatory element 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.[53] Neither LV- nor CRISPR-based therapies for BTK have reached the stage of clinical trials.

Severe congenital neutropenia. Alternative to the previously described knockout approach, targeted integration of the fourth exon of *ELANE* gene has been successfully demonstrated in SCN patient–derived HSCs, with edited HSCs successfully differentiating into functional neutrophils.[54] The gRNA used in this study targeted both the wild-type and mutant allele. Authors showed that 6% of the wild-type alleles contained indels.[54] Even though there is the previously described small risk of introducing new autosomal-dominant mutations, these observed mutations were not described to be pathogenic.

CTLA4 insufficiency. Cytotoxic T-lymphocyte associated protein 4 (CTLA4) insufficiency is caused by heterozygous germline mutations in the *CTLA4* gene. CTLA4 is a negative immune regulator that is expressed on Treg cells and conventional T cells on activation. CTLA4 insufficiency leads to immune dysregulation due to reduced immune suppression by Treg cells. Because 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 nonmobilized apheresis. In addition, conditioning regimens required before autologous infusion are much less toxic compared with the regimens used for autologous HSC transplantation. Furthermore, because 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. However, 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.[55]

An editing approach for CTLA4 insufficiency proved successful in T cells, with editing rates of more than 60%, resulting in restoration of CTLA4 expression, with similar expression patterns to healthy control cells,[56] 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*.[56]

X-linked lymphoproliferative disease. X-linked lymphoproliferative (XLP) disease is caused by deficiency of Slam-associated protein (SAP), which is caused by mutations in the *SH2D1A* gene. Disease manifestations include hemophagocytic 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, LV-mediated SAP gene transfer led to restoration of cellular and humoral responses in SAP-deficient mice, without the occurrence of adverse effects.[57] 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-transformed lymphoblastoid cell line lymphoma tumor model in NSG mice.[58]

Besides a gene addition approach, gene editing of T cells for XLP has been studied. Results showed that SAP expression could be restored to endogenous levels in T cells on successful integration of *SH2D1A* cDNA at the *SH2D1A* locus, restoring SAP-dependent immune functions in XLP patient T cells.[59]

X-linked MAGT1 deficiency with increased susceptibility to EBV infection and N-linked glycosylation defect. As mentioned, achieving editing rates that are clinically relevant in the primitive HSC population is a major challenge, and in many preclinical murine *in vivo* studies, editing rates drop significantly compared with previous results obtained *in vitro*. Various mechanisms most likely underlie these observations. First, 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[60] have tried to improve editing rates and the engraftment potential of edited HSCs while studying gene editing for X-linked MAGT1 deficiency with increased susceptibility to EBV-infection and N-linked glycosylation defect (XMEN) disease. XMEN disease is caused by MAGT1 deficiency and is associated with lymphomas. The authors show that on AAV transduction of HSCs, a strong DNA damage response occurs. This DNA damage response has negative effects, inducing apoptosis, cell death, and cell-cycle arrest, severely impacting the engraftment potential of HSCs. Transient suppression of TP53-binding protein 1 dampens the DNA damage response temporarily, improving engraftment potential. In addition, transient p53 inhibition forces cell-cycle progression, improving editing efficiency to rates more than 60%. Murine studies showed good levels of engraftment of edited cells and high levels of targeted integration (63.1% ± 8.8% *in vivo* vs 62.3% ± 8% *in vitro*), which was persistent in engrafted human $CD45^+$ cells that had kept their differentiation potential.[60]

Activated phosphoinositide 3-kinase δ syndrome type 1. Activated phosphoinositide 3-kinase δ syndrome type 1 is caused by gain-of-function mutations within the *PIK3CD* gene, which encodes for the class IA phosphoinositide 3-kinase (PI3K) catalytic subunit p110δ. Class I PI3Ks are mainly expressed in leukocytes and are important for various cell functions. Disease phenotype is varied; most patients suffer from recurrent respiratory tract infections and chronic viral infections, but patients may also develop autoimmunity and chronic benign lymphoproliferation with increased risk of developing lymphomas.[61] In addition to the disease causing gain-of-function mutations, biallelic loss-of-function mutations in PIK3CD have been described in patients with combined immunodeficiency, highlighting the importance of regulation of PI3Kδ activity required for normal cell function.[62,63] In a recent proof-of-concept study for TALEN/AAV6-mediated PIK3CD gene correction, knock-in rates of up to 50% were achieved in activated phosphoinositide 3-kinase δ syndrome type 1 patient T cells, resulting in normalization of PI3K signaling as measured by phospho-AKT levels and correction of cytolytic CD8 T cell function.[64]

Chronic granulomatous disease. Chronic granulomatous disease (CGD) is caused by decreased activity of phagocyte nicotinamide adenine dinucleotide phosphate 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.[65-67] 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.[68,69] This vector is currently used in clinical trials, with promising initial results showing 78% patient survival, no CGD-related

infections posttreatment, and discontinuation of antibiotic prophylaxis in 67% of patients.[70]

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 reactive oxygen species in corrected HSCs. This editing approach has shown restoration of gp91-phox and reactive oxygen species production by phagocytes both in X-CGD iPSC and primary patient HSCs,[71,72] 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 TP53-binding protein 1 resulted in a clear increase of targeted integration in HSCs.[72]

As an alternative approach, De Ravin et al[73] optimized targeted integration at the AAVS1 safe harbor locus in HSCs and tested out their protocol using CGD as a disease model. 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.[73]

Again, similar to studies performed in X-CGD, Klatt et al[74] integrated a therapeutic phox-47 transgene at the AAVS1 safe harbor 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 2 promotors resulted in therapeutically relevant levels of phox47 in corrected iPSC-derived myeloid cells.[74]

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 patients with p47-CGD, with more than 80% of the patients with p47-CGD being homozygous for ΔGT *NCF1*. Interestingly, *NCF1* has 2 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[75] 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.[75]

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 most 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, evading the risk of off-target integration and avoiding the intensive engineering that is associated with viral donors. The single-stranded DNA oligonucleotide donor consists of 2 homology arms, one of which contains the desired edit. Besides the above-described knockout approach, targeted gene correction is studied as treatment for SCD.[76,77]

Severe congenital neutropenia. Mutation-specific gene correction in the *ELANE* gene is not an approach that is clinically relevant, because more than 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 wild-type 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 wild-type allele intact. This restored neutrophil differentiation *in vitro* and *in vivo* on HSC transplantation into humanized mice and resulted in restoration of function in the repaired neutrophils *in vitro*.[54]

Chronic granulomatous disease. C676T substitution in exon 7 of the *CYBB* gene is the most common mutation described in patients with X-CGD, 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 a single-stranded DNA oligonucleotide 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 was observed when comparing pretransplant data to posttransplant 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 $CD34^+$ HSCs.[78]

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.[79,80] 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.[81] 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 $CD34^+$ HSCs corrected the *CD3D* mutation with high efficiency; the edited HSCs had normal T lymphopoiesis capacity, as assayed in an Artificial Thymic Organoid system.

Prime editing

Although 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 to 15 bases in length at a precise location.[82]

Chronic granulomatous disease. 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 2 missing bases and restore the reading frame for the p47 protein.[83]

Even newer editing approaches use the sequence-specific DNA recombination of bacteriophage recombinases and transposases to insert whole cDNA-size DNA sequences.[84] 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 graft versus host disease 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 preclinical studies aimed at improving efficiency of gene-editing delivery systems, targeting long-term repopulating hematopoietic 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 that 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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