Gene Editing in Human Haematopoietic Stem Cells for the Treatment of Primary Immunodeficiencies

Sameer Bahal[1], Klesti Karaxhuku[1] and Giorgia Santilli [1,2]

1 Molecular and Cellular Immunology section, UCL Great Ormond Street Institute of Child Health, University College London, London, United Kingdom.

2 NIHR Great Ormond Street Hospital Biomedical Research Centre, London, UK.

Corresponding Authors:

Giorgia Santilli (email@: g.santilli@ucl.ac.uk)

Sameer Bahal (email@: sameer.bahal.19@ucl.ac.uk)

Abstract

In recent years, gene editing technologies have revolutionised precision medicine and human trials of this technology have been reported in cell-based cancer therapies and other genetic disorders. The same techniques, have the potential to reverse mutations in monogenic Primary Immunodeficiencies (PIDs) and transplantation of edited haematopoietic stem cells may provide a functional cure of these diseases. In this review we discuss the methods of gene editing being explored and describe progress made so far with several PIDs. We also detail the remaining challenges, how to confidently detect off target effects and chromosomal abnormalities in a timely manner, how to obtain long term benefits and how to achieve physiological levels of expression of the therapeutic gene. With advances in gene editing, we envisage a robust clinical translation of this technology in the coming decade.

Key Points

- The recent development of gene editing technologies can be applied to haematopoietic stem cells of patients with Primary Immunodeficiencies to create potential curative cell therapies.
- Although great progress has been made, vector associated genotoxicity and off-target effects remain a concern.

Introduction

Primary Immunodeficiencies (PIDs) have a reported prevalence of between 1 in 1,000 and 1 in 100,000 people[1]. Patients suffer from recurrent and severe infections which can be

accompanied by autoimmunity and increased predisposition to malignancy. More than 400 genetic causes have been identified in increasing proportions of patients as a result of advancing gene sequencing technologies, with genes involved in the development and function of the immune system most commonly implicated. Despite this, management regimes have remained similar over the last few decades. Firstly, PID patients are given infection prevention advice and infections are treated with longer courses of antibiotics than standard. Depending on the individual condition and its severity patients can be provided with prophylactic antibiotics and in the case of antibody deficiency, Immunoglobulin Replacement Therapy (IRT) can be initiated[2]. Despite these treatments, breakthrough infections occur with varying frequency and severity. In addition, non-infectious complications such as autoimmune disease, chronic lung disease and malignancy are challenging to ameliorate.

Allogeneic Haematopoietic Stem Cells Transplantation (HSCT) is the only curative option for many PIDs. Although HSCT can restore immune function; especially when performed in early childhood, the overall mortality rate is roughly 10%[3] if not higher when a matched family or unrelated donor is unavailable. This is mainly due to immunosuppression related infections and graft vs host disease.

In the past 30 years, ex vivo correction of autologous Haematopoietic Stem Cells (HSCs) followed by transplantation of these cells (conventional gene therapy) has entered clinical practice for the treatment of a number of PIDs including severe combined immunodeficiency (SCID), Wiskott Aldrich Syndrome (WAS) and Chronic Granulomatous disease(CGD),[4]. This process obviates the risk of graft versus host disease. Gamma-retroviral and lentiviral vectors are often used to deliver the donor DNA containing the correct gene (known as the therapeutic cassette) into target cells and genes are integrated into host DNA at a semi-random location; but this poses a risk of insertional mutagenesis[5].

In the last two decades, the discovery of DNA endonucleases which create breaks in a specific DNA locus have paved the way for precision medicine by gene editing[6]. Scientists

have now a suite of programmable endonucleases to choose from including Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and, most recently, the RNA-guided CRISPR-Cas9 (clustered, regularly interspersed palindromic repeats-CRISPR-associated protein 9) system.

ZFNs were the first nucleases to be employed by scientists[7]. They were developed after the discovery of zinc fingers which serve multiple roles in procaryotic organisms including regulation of gene transcription. A Zinc finger is a zinc ion-regulated protein motif, which binds to a specific 3 base pair sequence. The zinc finger domain is conjugated to the FokI endonuclease that works as heterodimer (Figure 1A). Two separate ZFNs targeting adjacent areas of DNA allow pairing of the Fok1 domains and activation of the enzyme leading to the creation of a DNA double strand break (DSB). While ZFNs are effective at creating DBSs in the genome, they are difficult to design and are limited by the range of Zinc Fingers available.

Another programmable nuclease that has been widely used in the laboratory are derived from Transcription Activator-Like Effector (TALE) proteins from Xanthomonas bacteria[8]. Each subunit binds to a single nucleotide and a sequence of these are again conjugated to a FokI endonuclease allowing pairs of TALENs to make DSBs (Figure 1B). While these endonucleases are more customisable than ZFNs, they take long to design and manufacture, limiting their utility in research laboratories.

Recently, Jennifer Doudna and Emmanuelle Charpentier developed a new endonuclease that has emerged from investigations on the adaptive immunity of bacteria. Clustered regularly interspaced short palindromic repeats (CRISPR) of DNA had been described in bacteria since 2002[9]. These lay adjacent to sequences that had close homology to viral DNA[10]. Later it was found that CRISPR associated (Cas) proteins would complex with these sequences and target viral DNA with direct homology to them[11]. One Cas protein; Cas9, was then repurposed to make targeted cuts in the genome once complexed with a guide RNA (gRNA). This resulted in a highly efficient and easily customisable method to

target specific areas of the genome (Figure 1C). Each DNA target sequence needs to be adjacent to specific sequence known as a Protospacer Adjacent Motif (PAM). Although *S. pyogenes* Cas9 (SpCas9) is the most commonly used, other engineered Cas9 homologs have been identified recognising alternate PAM sequences, hence dramatically expanding the target choice.

Subsequently, the advent of base editors (BEs) unveiled new potential treatment strategies for several hematological disorders caused by point mutations. These are created by fusing a cytidine or adenine deaminase to an inactive Cas9 or nickase (single strand cleaver) Cas9 (Figure 1D and E),[12].

The most recent gene editing tool is prime editing (Figure 1F) which utilizes an extended guide RNA (pegRNA) that mediates the DNA targeting and, at the same time, serves as an RNA template for a reverse transcriptase fused to the Cas9 nickase[13]. This system amends not only point mutations but also small insertions and deletions, and a recent optimization (grand editing) can mediate the substitution of larger DNA fragments (~400bp) without the need for DSBs[14].

The gene editing method used depends on the individual PID. For example, in gain-of-function mutations it may be appropriate to knock out a gene whereas in a loss-of-function mutation, site directed repair may be employed.

Gene editing strategies

Non-homologous end joining repair to address gain-of-function (GoF) mutations

Repair of the DNA break mostly happens via non-homologous end joining (NHEJ), an error-prone process that creates small insertions and deletions (INDELs). Scientists have successfully employed the NHEJ pathway to knockdown genes such as CCR5 for HIV prevention[15] or to modulate gene expression by disruption of chromatin repressors, as in the case of beta-thalassemia[16]. Cas9 mediated disruption of specific GoF mutations for Activated Phosphoinositide 3-kinase δ syndrome(APDS) [17] can be potentially applied for the treatment of this PID (Figure 2A). Gene knock outs can also be obtained with base editors introducing a stop codon.

A limitation of this technique is that it is not always possible to precisely target the mutated allele and therefore a compensating gene addition procedure may need to be employed. Diseases where the GoF mutations affect the expression of a protein with a redundant function make an exception to this rule. An example is the neutrophil disorder Severe Congenital Neutropenia (SCN) which is most often caused by autosomal dominant (AD) mutations in *ELANE* encoding neutrophil elastase[18]. CRISPR/Cas9 ribonucleoprotein (RNP) based ELANE knock out in HSCs from SCN patients have indeed proved the efficacy of this approach[19]. More recently a clinical trial for transthyretin amyloidosis has disclosed the full potential of the Cas9 “hit and run” approach to knock out the mutated misfolded transthyretin in the liver[20].

Challenges with gene knockout include maintaining a high enough efficiency to result in disease resolution.

Homology directed repair to achieve targeted gene editing or insertion

When the mutation involves a single base change, base editors or homology directed repair (HDR) with short oligos can be used. Unlike the NHEJ pathway, which occurs throughout the cell cycle, HDR occurs only during the S and G2 phases, making it less efficient. By harnessing the cellular homology recombination machinery together with adequate

concentrations of repair template DNA, scientists can integrate a chosen DNA sequence in a precise locus. Targeted integration achieves correction of single mutations or insertion of entire open reading frames near endogenous regulatory sequences to rescue physiological gene expression.

Site specific repair: Allelic Exchange

This method can be used for AD mutations. A DSB is made specific to a disease-causing mutation. The host repair machinery repairs the break using the wildtype gene as a template, correcting the mutation (Figure 2B). Potential PID applications for this approach include APDS and the AD version of Hyper Immunoglobulin E Syndrome which is caused by *STAT3* mutations.

Site specific repair: oligonucleotide-mediated site directed repair

This method can be used for a wide large of mutations (apart from large deletions) but must be customised to each mutation loci (Figure 2C). Edited cells will have target gene sequences homologous to the wildtype version and hence this technique will be preferable in diseases where highly regulated gene expression in required; such as X-Linked Hyper Immunoglobulin M Syndrome[21].

Insertion of therapeutic Transgene or Exon at endogenous gene location using vectors such as viruses.

For this approach a DSB is created generally in an early exon or intron of a specific gene. Subsequently, cells are generally treated with a virus or lipid nanoparticles containing the cDNA encoding the therapeutic transgene flanked by homology areas identical to the areas adjacent to the DSB. Cells use this DNA as a template for repairing the mutation and

therefore introduce the transgene into the endogenous gene location via HDR (Figure 2D). Integrase-deficient lentiviruses (IDLV), and Adeno Associated Vector 6 (AAV6) with an improved tropism for blood cells are often used[22].

Codon optimised or divergent sequences are used to ensure that the transgene does not contain the target sequence for the gene editing machinery and is therefore not excised. Additionally, a divergent sequence facilitates the design of a unique “In” primer for an In-Out Polymerase Chain Reaction (PCR) often used to evaluate correction frequency. In this assay the “In” Primer anneals to a region specific to the donor construct and the “Out” Primer anneals a genomic sequence adjacent to the integration site. Thus, amplification can only result from sites with successful integration of the donor cassette.

This represents a one size fits all solution with the same virus, donor template and gRNA being used for almost any mutation. Importantly insertion of the coding sequence next to its endogenous regulatory elements often provides physiological levels of expression of the therapeutic gene. However, loss of intronic regulatory elements [23,24] can alter protein expression although more recently studies have mitigated this with the inclusion of short intronic regulatory sequences within the donor template[25]. In addition, scientists have reported variable levels of HDR frequency in HSCs from different donors. Finally, cell toxicity and growth arrest are common post gene editing, mainly due to AAV6 related cell death[26] and the DNA damage response following the DSB. The resulting fall in HDR rates post transplantation limits translation into clinical practice[27].

Towards clinical application of gene editing strategies: a step-by-step guide

In general, studies contain the following steps:

1. Identify a target in the genome

2. In vitro gene editing

3. Determine the efficiency of the process

4. Identify off target effects

5. Proof of concept: in vitro studies

6. Proof of concept: transplantation studies in mouse models

Identifying a target in the genome

For each genetically defined PID studied, an integration site for the therapeutic gene is chosen. This is usually an early exon/intron in the gene of interest or a “safe-harbour” site which will not alter endogenous gene expression[28] . Researchers make use of online libraries such as Zinc Finger Targeter[29], E-TALEN[30] and E-CRISP[31]for ZFN, TALEN and CRISPR/Cas9 editing respectively. These libraries rank targets in order of their off-target potential, with the most unique sequences favoured.

In vitro gene editing

Researchers initially optimise their gene editing strategy in an appropriate cell line. Electroporation is commonly used to deliver gene editing tools into target cells and it must be determined which platform settings and reagent concentrations achieve the correct balance of high transduction efficiency and low cell toxicity.

Subsequently, editing on primary cells such as HSCs or T cells is performed. Ideally, optimisation on healthy donor cells takes place prior to patient cells. T cells are easily acquired from peripheral blood donation with CD3 based cell selection. HSCs are now usually harvested from mobilised peripheral blood and this source has mostly replaced bone marrow over the last few decades[32]. Peripheral bood stem cell donors are first administered a short course of Granulocyte-Colony Stimulating Factor (G-CSF), sometimes in combination with the CXCR4 antagonist Plerixafor[33]. These cause HSC mobilisation into

peripheral blood and cells can then be collected via apheresis and isolated using the CD34 marker for positive selection.

Determining the efficiency of the process

The efficacy of a gene editing approach depends, in the first instance, on the frequency of DSBs made by the gene editing machinery. Endonucleases are generally provided as mRNA or RNP complexes (in the case of CRISPR/Cas9) through nucleofection so that they are in the cells for a short time, minimising off target risks. Sequencing based methods such as “Tracking of Indels by Decomposition” (TIDE) Analyses[34] or “Inference of CRISPR Edits” (ICE),[35] are commonly used to determine the “cutting efficiency”. After nucleofection, genomic DNA is extracted and DNA spanning the target region is amplified in a PCR reaction. The PCR product sequence is analysed using online software which compare the wildtype and edited sequences to determine efficiency. An alternative method utilisesT7 Endonuclease 1 (T7E1) which recognizes and cleaves structural deformities in non-perfectly matched DNA[36]. The PCR product DNA spanning the cut site is denatured and slowly re-annealed. Heteroduplexes form when DNA of different lengths (created by NHEJ) combine. T7E1 then cleaves these DNA strands, resulting in smaller fragments. The DNA products are run on a gel and the intensity of the bands corresponding to the cut fragments are compared to non-cleaved, DNA. The relative sizes indicate the cut efficiency. This is a low cost and relatively simple assay but lacks the accuracy of sequencing-based methods[37].

The “editing efficiency” is the efficiency of HDR and implicitly takes into account the cutting efficiency. Droplet Digital PCR with In and Out primers is often used when it is not possible to promptly verify the gene expression by FACS analysis This technique is highly sensitive and can detect editing rates of 0.1%[38]

Identify off target effects

Limitations in the specificity of the gene editing machinery result in off target effects which are a major concern as potential disruption of a gene or regulatory elements could lead to malignant transformation [39]. Therefore several approaches have been developed to identify these unintended genomic changes.

In silico approaches such as Cas-OFFinder[40] use a genetic database to identify predicted off target effects by identifying sequences with close homology to the gRNA. These areas can be amplified using PCR and analysed using Next Generation Sequencing (NGS) to identify the frequency of indels. These off-target predictions are often an underestimation of the real frequencies of undesirable cuts (biased identification), as, for example, they do not consider the presence of genetic variants. More sophisticated methods such as Guide-Seq and Circle Seq enable the unbiased identification of off-targets in in vitro cultures.

GUIDE-seq(Genome-wide, unbiased identification of DSBs enabled by sequencing), relies on exposing a 34 base pair double-stranded oligodeoxynucleotide (dsODN) flanked by phosphothiorate linkages to cells being edited[41]. The dsODN is integrated into DSBs and integration points are then identified using dsODN specific primers. Sequencing adapters allow for unidirectional amplification of the dsODN and the adjacent host DNA. These DNA fragments are sequenced and compared to a reference genome using bioinformatic software to determine the location of the off target effects. This method allows for the identification of off target sites not predicted by the standard in silico methods.

Circle-Seq also relies on Next Generation Sequencing (NGS). Initially, genomic DNA is turned into a circular molecule by intramolecular ligation, and the remaining linear DNA is degraded using exonucleases. The molecules containing an endonuclease recognition site will break, and the ends of the DNA will be freed to allow adapter and ligation molecules to attach. The resulting molecules are amplified using PCR and undergo paired-end

sequencing[42]. The resulting data reveal off target sites. Circle-Seq has a detection frequency lower than 0.1%, and background reads can be omitted, improving sensitivity. Moreover, the technique can be performed by widely implemented next generation sequencers (NGS), making it easily accessible to researchers. Lastly, off-target sites resulting from sequence variations such as single-nucleotide polymorphisms (SNPs), which may not necessarily be included in reference genomes can be studied. Hence, a personalised off-target effect profile is created[42,43]. Limitations include the large amount of DNA required for analyses and the presence of cellular factors altering nuclease on-target activity[43].

Other methods, such as Cast-seq (Chromosomal aberration analysis by single targeted LM-PCR sequencing) not only are able to identify off targets but also translocations and other chromosomal aberrations[44]. Cast-seq uses Linker Mediated (LM) PCR. On-target activity can cause homology mediated translocations (HMTs), which are conciliated by a homologous recombination (HR) mechanism. These translocations will appear between the on-target sites, and the regions that have a high percentage of homology with the on-target site. CAST-seq can be utilised to identify those regions. Firstly, genomic DNA is fragmented to roughly a 350bp size and linker molecules are attached for three consecutive PCR steps. In the first step, three primers are used, the first (bait) binding to the on-target region of DNA, the second (pray) binds to the linker sequence and the last, the decoy primer would recognise the target sequence and prevent on target amplification. Exclusively, during a loss of decoy recognition site due to translocations or alterations at the on-target region, the next two PCR steps would lead to amplification of these sites. Although this method detects rearrangements with high specificity, it can miss off-target effects repaired by NHEJ, for which a translocation does not occur. Furthermore, creating the primers can be difficult, for instance, when repeated GC content is not persistent. Lastly, as with CIRCLE-seq, large quantities of DNA are required and the cellular factors causing these alterations are not identified[44].

If significant off target effects are detected several options can be employed. Firstly, a different target can be chosen with a more favourable predicted off-target profile. Secondly, the target specificity can be increased in several ways such as using paired Cas9 nickases (which enable a 40 base pair rather than a 20 base pair specificity), or using high-fidelity versions of Cas9[45–47]. We would need to take into consideration that off target effects are only the tip of the iceberg as genotoxicity can be a consequence of AAV random integration and chromotripsis[48].

Proof of concept: in vitro studies

After gene editing, in vitro functional assays must be carried out to evaluate the fitness of edited cells and in some cases, specialised culture techniques are required to differentiate edited stem cells into the leukocytes affected by the PID. One example is the use of a B cell differentiation culture involving sequential exposure of cells to Flt3-L, IL-6, IL-7 and ICAM1 to test a potential gene editing strategy in X-Linked Agammagloulinaemia (XLA)[49]. The types and numbers of assays used will depend on the PID being studied. Additionally, confirmation of restored protein expression is performed by western blotting or FACS analysis. In vitro colony forming unit (CFU) assays are also used to explore the potential of edited cells to differentiate into erythroid and myeloid lineages as a first screening of cell fitness[50].

Proof of concept: transplantation studies in mouse models

Subsequent work includes testing edited cells in mouse models. The editing machinery needs to be adapted to murine genomes when a mouse model of a specific disease is used. More often for PID, human HSCs are transplanted into an immunodeficient mouse model,

with the NOD/scid/γcnull (NSG) mouse being a frequent choice due to their ability to engraft human HSCs[51]. Immune reconstitution is assessed over 3-6 months, with interval venesection determining human cell engraftment, frequency of editing and where appropriate for the specific PIDs, immunoglobulin levels and vaccine responses. At termination, human HSCs from the bone marrow of primary transplanted animals may subsequently be selected and transplanted into a second set of mice (secondary transplantation)[52] to test the ability of gene edited stem cells to self-renew.

Animal experiments have highlighted a limitation of the gene editing approach for HSCs. While gene addition using retroviral vectors rarely impacts on cell engraftment, scientists using endonuclease-mediated gene targeting approaches are struggling to find good levels of corrected cells in mice regardless of the levels of correction achieved in the product. This is probably due to the combined effects of p53 activation resulting from the DSB and AAV6 transduction delivering the donor template[27]. It appears that the repopulating/homing ability of HSCs is indeed compromised, and often high frequencies of HDR translate into poor engraftment. A solution is to opt for p53 direct inhibition using short hairpin RNAs possibly in combination with inhibitors of 53BP1, an early regulator of the DSB repair pathway that promotes NHEJ over HDR. Gene editing with p53 inhibition results in better cell viability and better engraftment of corrected cells in immunodeficient mice while i53 (the inhibitor of 53BP1) can increase HDR[53].

Progress in gene editing for PIDs

Gene editing studies have been reported in increasing numbers of PIDs in the last 20 years. Table 1 shows some examples of PIDs for which gene editing strategies have been reported.

Disease	Reference	Method	Editing Machinery	Donor Vector	Cells Edited	Animal Model
SCID-X1	Urnov et al. [54]	Site directed Repair using HDR	ZFN	Plasmid	K562 cells and Human CD4+ Cells	N/A
	Lombardo et al.[55]	Insertion of therapeutic transgene with HDR	ZFN	IDLV	K-562 cells, EBV transformed B cells, Jurkat cells, HUES-3, HUES-1 and cord blood derived HSCs	N/A
	Schiroli et al.[56]	Insertion of therapeutic transgene with HDR	CRIPR/Cas9 and ZFNs	IDLV and AAV6	SCID X1 mouse HSCs	SCID-X1 mice
	Pavel-Dinu et al.[57]	Insertion of therapeutic transgene with HDR	CRIPR/Cas9	AAV6	Patient HSCs	NSG
JAK3-SCID	Chang et al. [58]	Site directed Repair using HDR	CRIPR/Cas9	Plasmid	Patient iPSC	N/A
X-Linked Hyper Immunoglobulin M Syndrome	Hubbad et al.[59]	Insertion of therapeutic transgene with HDR	TALEN	rAAV	Primary human T Cells	NSG
	Kuo et al.[60]	Insertion of therapeutic transgene with HDR	TALEN and CRISPR/Cas9	IDLV and AAV6	K562 and Jurkat cell line. Healthy donor CD4 T Cells and HSCs	NSG
Wiskott–Aldrich syndrome	Laskowski et al. [61]	Insertion of therapeutic transgene with HDR	ZFN	Plasmid	Patients generated iPSC	N/A

	Gutierrez-Guerrero et al. [62]	Insertion of therapeutic transgene with HDR	CRISPR/Cas 9 and ZFN	Plasmid	K562 cells	N/A
	Rai et al.[63]	Insertion of therapeutic transgene with HDR	CRISPR/Cas 9	AAV6	Patient HSCs	NSG
Chronic granulomatous disease (All Forms)	Merling et al. [64]	Insertion of therapeutic transgene with HDR	ZFN	Plasmid	Patient generated iPSC lines	N/A
X-Linked Chronic granulomatous disease (X-CGD)	Ravin et al. [65]	Insertion of therapeutic transgene with HDR	ZFN	AAV6	Healthy donor HSCs	NSG
	Ravin et al. [66]	Site directed repair	CRISPR/Cas 9	ssODN	Healthy donor and patient HSCs	NSG
	Sweeney et al.[67]	Site directed repair of Exon 5 and insertion of therapeutic transgene with HDR	CRISPR/Cas 9 and TALEN	Plasmid	$Gp91^{phox}$ deficient iPSC lines	N/A
$P47^{phox}$ deficient Chronic granulomatous disease (p47-CGD)	Merling et al.[68]	Site directed repair	ZFN	rAAV2	$P47^{phox}$ deficient iPSC lines and patient HSCs	N/A
	Klatt et al.[69]	Insertion of therapeutic transgene with HDR	CRISPR/Cas 9	Plasmid	$P47^{phox}$ deficient iPSC lines	N/A
Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX)	Goodwin et al. [70]	Insertion of therapeutic transgene with HDR	CRISPR/Cas 9	rAAV	Healthy donor HSCs, CD4+ cells, MT-2 T_{reg} cell line, Patient T cells	NSG-SGM3

X-Linked Agammaglobulinaemia (XLA)	Gray et al. [25]	Insertion of therapeutic transgene with HDR	CRISPR/Cas9	AAV6	BTK-deficient K562 and Ramos cell lines	N/A
X-linked immunodeficiency with magnesium defect, EBV infection, and neoplasia (XMEN)	Brault et al. [71]	Insertion of therapeutic transgene with HDR	CRISPR/Cas9	AAV6	Patient HSCs and T cells	NSGS

Table 1 Studies of gene editing in PIDs

SCID-X1 X-Linked Severe Combined Immunodeficiency, *HDR* Homology Directed Repair, *ZFN* Zinc Finger Nucleases, *N/A* Not Applicable, *IDLV* Integrase Deficient Lentivirus, *EBV* Epstein-Barr Virus, *HSCs* Haematopoietic Stem Cells, *CRISPR/Cas9* Clustered, regularly interspersed palindromic repeats/CRISPR-associated protein 9, *NSG* NOD/scid/γc null, *AAV* Adeno-Associated Virus, *JAK3* Janus kinase 3, *iPSC* Induced Pluripotent Stem Cells, *TALEN* Transcription Activator-Like Effector nucleases, *rAAV* recombinant adeno-associated virus, *ssODN* single-stranded oligodeoxynucleotide, *BTK* Bruton's Tyrosine Kinase, NSGS NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}TgS

Severe Combined Immunodeficiency

Severe Combined Immunodeficiency (SCID) is a rare, and usually fatal immunodeficiency presenting with severe infections and failure to thrive within the first few months of life. T cell numbers are low or absent and B and NK cells can be reduced depending on the particular subtype[72]. Urnov et al. explored the use of ZFNs in editing a T cell line to correct a mutation in exon 5 of the *IL2RG* gene; that causes X-Linked SCID (SCID-X1)[55]. They

achieved correction in 2.4% of asynchronously growing erythroleukemia K562 cells, and HDR rates of 5.3% in primary CD4+ T cells. These low efficiencies likely resulted from suboptimal vector choice. This was addressed by Lombardo et al. who used a IDLV to deliver ZFN DNA as well as a *IL2RG* donor construct to cells[55]. Efficiencies of 6% were achieved in Epstein-Barr Virus (EBV) transformed B cells and 0.11% in CD34+ cord blood derived HSCs as suggested by the expression of the Green Fluorescent Protein(GFP) reporter. Interestingly, the researchers targeted gene insertion into the *CCR5* gene as well as the *IL2RG* gene and reported efficiencies of up to 50% in cell line editing.

Subsequently Schiroli et al. trialled a gene correction approach in a SCID-X1 mouse model[27]. Firstly, they determined the lowest fraction of healthy HSCs required to rescue T cell development and confirmed that healthy HSCs had a strong survival advantage compared to SCID-X1 HSCs. They developed a gene editing strategy involving insertion of a therapeutic transgene and when transplanting edited SCID-X1 mouse HSCs back into these mice, promising levels of HDR and T cell rescue was observed.
More recently, Pavel-Dinu et al. targeted integration of the *IL2RG* cDNA into the endogenous start codon using a CRISPR/Cas9 and AAV6 based strategy, This led to 20% targeted integration frequencies in long term HSCs[57]. Transplantation of edited HSCs from multiple patients into an NSG mouse model revealed restoration of T cell development.

Less common SCID causing genes have also been studied and in 2015 Chang et al. described gene editing cells containing homozygous *JAK3* mutations which cause a T-B+NK- SCID phenotype[58]. Patient induced pluripotent stem cells (iPSCs) were transfected with a plasmid encoding Cas9 and a gRNA and well as plasmids containing a repair template for the disease-causing mutation in exon 14. Restoration of JAK 3 expression was demonstrated using RT-PCR to detect *JAK3* mRNA and western blot for the JAK 3 protein. Whole genome sequencing demonstrated no off-target effects. To determine if gene edited stem cells could differentiate into T cells, they were cultured with a OP9 stromal cells. Flow

cytometry for CD7, CD16, CD56, CD4, CD8 revealed that gene corrected cells were able to differentiate into T and NK cells at numbers comparable to wildtype cells.

X-Linked Hyper Immunoglobulin M Syndrome

Hyper Immunoglobulin M syndrome refers to a group of PIDs affecting class switch recombination and somatic hypermutation. Mutations in the gene encoding CD40 Ligand (CD40L); *CD40LG,* cause the commonest form which is X-Linked (XHIM)[73]. During the immune response, CD40L expressed on T Cells binds to surface CD40 on B Cells to induce class switching from IgM to IgG IgA and IgE[74]. Patients suffer from a combined immunodeficiency characterised by bacterial and fungal infections[75]. A previous attempt at *CD40LG* gene therapy in a mouse model resulted in abnormal lymphoid proliferation, lymphoma and death[21]. This emphasised that CD40L expression is highly regulated and in any further attempts a transgene should be under the control of the endogenous regulatory environment. Subsequently, Hubbard et al. showed that a gene editing approach could restore regulated CD40L expression in T Cells[59]. A TALEN pair was designed to target the 5’ untranslated region (5’UTR) in exon 1 of the *CD40LG* gene. Electroporation of the TALEN mRNA had an efficiency of 88%. PCR amplification of 10 predicted off-target sites detected no off-target activity. HDR of up to 46.7% was achieved in healthy donor CD4+ cells as determined by the expression of the GFP reporter. When editing T cells from 3 XHIM patients, HDR rates varied between 5.3% and 31.7%. The editing resulted in CD40L expression and CD40 binding comparable to healthy donor T cells. The ability of edited cells to achieve class switching in cocultured B cells after TLR9, CpG oligodeoxynucleotide and IL-2 stimulation was explored. As predicted, editing XHIM T Cells rescued their ability to induce class switching (as determined my measuring IgG expression on B cells). Finally, survival of edited cells was confirmed after adoptive cell transfer to a NSG mouse model with

GFP positive cells measured after 5 weeks. This study confirmed the potential use of gene corrected T cells as a bridging therapy to HSCT. The potential of HSC editing to produce a long-term cure was not explored in this study but was later addressed by Kuo et al. who targeted the 5'UTR of the CD40LG gene with either TALEN pairs or Cas9/gRNA[60]. IDLV and AAV6 vectors were trialled with each containing a codon divergent CD40L cassette. Roughly 15% HDR was achieved using both the TALEN + AAV6 and the Cas9 +AAV6 combinations in XHIM T cells. When editing HSCs, HDR rates of 13.2% and 20.8% were achieved with the TALEN + AAV6 and the Cas9 +AAV6 combinations respectively. Restored physiological function of edited cells was confirmed by CD40L expression. Transplanting edited HSCs into sublethally irradiated NSG mice revealed engraftment levels and immune reconstitution comparable to unmodified cells. This study showed that gene editing can lead to physiological transgene expression in a gene where aberrant expression can lead to significant complications.

Wiskott–Aldrich syndrome

Wiskott–Aldrich syndrome (WAS) is an X-Linked PID caused by *WAS* gene mutations. WAS protein (WASp) regulates the actin cytoskeleton and is essential for immune synapse formation[76]. Therefore, its deficiency leads to a complex immunodeficiency typified by microthrombocytopenia, eczema, recurrent infections, autoimmunity and malignancy[77]. The first exploration of gene editing in WAS was described in 2016 by Laskowski et al [61]. Patient derived iPSCs were generated and edited using a cWAS-GFP construct targeting a ZFN-mediated DSB in Intron 1. T cell differentiation and NK cell function were restored in selected edited cells. A second study in 2018 compared various methods of creating a DSB in the *WAS* gene. ZFN and CRISPR/Cas9 machinery were delivered into K562 cells using either plasmids or IDLVs. HDR rates of between 0.3 and 6% were achieved when using the GFP reporter delivered via a plasmid[62].

In 2020, Rai et al. described a gene editing approach with a Cas9 mediated DSB created in exon 1 of the *WAS* gene followed by insertion of codon-optimised *WAS* cDNA via an AAV6

vector[63]. Up to 90% cutting efficiency was achieved in HSCs and HDR rates of up to 69% were described when using the GFP reporter gene. CFU assays confirmed the ability of edited cells to differentiate into myeloid and erythroid lineages. Edited WAS patient T cells showed restored ability to proliferate in response to TCR/CD3 stimulation. The researchers had access to WAS patient HSCs and were able to confirm restoration of physiological expression of WASp cell function post editing. Finally, edited patient HSCs led to immune reconstitution following primary and secondary transplantation in an NSG mouse model. This promising study is now in preclinical development leading to a phase I study.

Chronic granulomatous disease

Chronic granulomatous disease (CGD) results from mutations in one of the 5 subunits of NADPH Oxidase, a complex which produces reactive oxygen species in phagocytes which kill bacteria. Patients are prone to bacterial and fungal infections as well as inflammatory complications[78]. The X-linked form of this disease (X-CGD) is the most common and is caused by mutations in the *CYBB* gene encoding the core subunit $gp91^{phox}$. A group led by Harry Malech in Bethesda, developed a “one size fits all’ approach for all 5 subunits using gene editing. The strategy was to mediate, through ZFN, the insertion of a cassette containing either the $gp91^{phox}$, $p47^{phox}$, $p22^{phox}$, $p67^{phox}$ or $p40^{phox}$ coding sequences into the Adeno-Associated Vectors integration site 1 (AAVS1) locus in chromosome 19, known to be a safe harbour for targeted insertion of transgenes [64]. When trialled in HSCs from one X-CGD patient, this strategy resulted in 15% correction of cells that were able to engraft immunodeficient NSG mice[65]. This approach, although viable, relied on exogenous regulatory sequences to drive gene expression and seems to nullify one important advantage of targeted gene insertion: the positioning of genes next to their own regulatory sequence to guarantee physiological expression levels. The same group later showed higher editing rates of >20% when attempting site directed repair of HSCs from a X-CGD patient with a C676T substitution in exon 7 of the *CYBB* gene[66]. Restored antibacterial activity was confirmed by culturing edited cells with *G. bethesdensis*, a known CGD pathogen. Gene

corrected cells were transplanted into an NSG mouse model and were detected after 20 weeks. Although the sustained immune reconstitution was promising, this method was only translatable to the 6% of CGD patients with this mutation[79]. In another study published in 2017, Sweeny et al. published a gene editing strategy for X-CGD [67] that showed that retention of intronic sequences was required to restore expression and hence illustrated the importance that introns possess in regulating expression.

Another approach involving site directed repair was used by Merling et al. in an autosomal recessive form of chronic granulomatous disease due to deficiency in $p47^{phox}$. More than 94% of p47 deficient CGD patients bear a dinucleotide deletion at the start of exon 2 [68]. This mutation has probably arisen by a homologous recombination event between the *NCF1* gene and its two pseudogenes, *NCF1B* and *NCF1C*. A ZFN approach targeting the start of exon 2, and a rAAV2 delivering the donor template for that specific mutation could not only rescue the normal sequence/activity of the gene but can also resurrect the pseudogenes. Indeed, the exon 2 repair also restored function in a patient with an exon 8 mutation suggesting that the rescue of the $p47^{phox}$ expression was probably due to the resurrection of *NCF1B* and *NCF1C*.

On the other hand, the presence of pseudogenes can complicate gene editing as their similarity to the target gene leads to unwanted DSBs. Klatt et al. addressed this concern in p47-CGD by inserting a codon optimised p47 transgene into a CRISPR/Cas9 -mediated DSB in the AAVS1 safe harbour locus[69]. A donor plasmid was used, and restoration of phagocyte function was achieved.

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked

Gene editing in PIDs with primarily autoimmune manifestations have also been studied. One such disease, Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) is

caused by mutations in FOXP3[80], a transcription factor essential for the development of regulatory T-cells. Disease manifestations include autoimmune enteropathy (usually presenting with diarrhoea), Type 1 Diabetes Melitus and Autoimmune Thyroid Disease[81].

Goodwin et al. designed an approach that inserted codon divergent FOXP3 cDNA adjacent to the endogenous gene's start codon[70]. Their donor construct included the reporter gene truncated nerve growth factor receptor (tNGFR), which is a benign surface marker used to determine editing efficiency and for positively selecting edited cells. GUIDE-seq identified four off target effects, none of which were in coding regions. Edited patient T regulatory cells showed increased suppressive activity in coculture with T effector cells when compared to non-edited patient cells. Finally, engraftment of edited healthy donor HSCs into the NSG-SGM3 mouse model[70] revealed lineage development comparable to WT cells after 3 months. A further step would be to edit patients HSCs to confirm restoration of T_{reg} development and function, however, it is often challenging to perform peripheral blood stem cell harvesting in patients who have complex medical conditions which may preclude this.

X-Linked Agammaglobulinaemia

X-Linked Agammaglobulinaemia (XLA) is caused by mutations in the gene encoding Bruton's Tyrosine Kinase (BTK), an essential signal transducer for B cell development[82–84]. Hence, the majority of patients have an absolute B cell deficiency with absent immunoglobulins[85]. The disease burden is characterised by recurrent and severe bacterial infections and is ameliorated by IRT. XLA is an ideal disease to trial gene editing in for several reasons. Firstly, as it is X-linked and therefore just a single mutant allele requires correction. Secondly, corrected cells have a selective advantage over patient cells and therefore even a modest number of corrected cells could reconstitute the humoral Immune system as shown previously in a BTK knockout mouse model.[86] . Finally, due to the introduction of IRT there is an established cohort of patients with stable disease who could donate stem cells for research and be potential recruits in a Phase 1 trial. Gray et al. developed a strategy in 2021 involving the AAV6 mediated delivery of the codon optimised

BTK gene into a CRISPR/Cas 9-mediated DSB in exon 2[25]. Initial low BTK expression led to the researchers adding a Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) sequence and truncated portions of Intron 18. This resulted in BTK expression comparable to WT levels but integration rates of just 10% were observed. Two off-target effects were identified in coding regions but these were virtually eliminated by the substitution of WT Cas9 with high-fidelity versions[45–47] .HSCs suffered high toxicity levels after editing most likely as a result of the AAV6 virus used. This hurdle needs to be overcome before future patient trials as the edited cells will need to proliferate at normal rates throughout the life of the patient to result in a persistent cure.

X-linked immunodeficiency with magnesium defect, Epstein-Barr virus (EBV) infection, and neoplasia

Mutations in the magnesium transporter 1 (*MAGT1*) gene cause a recently discovered syndrome named “X-linked immunodeficiency with magnesium defect, Epstein-Barr virus (EBV) infection, and neoplasia,” (XMEN)[87]. Brault et al designed a gene editing strategy involving electroporation of Cas9 mRNA and gRNA targeting an area adjacent to the *MAGT1* start codon, followed by AAV6 mediated insertion of *MAGT1* cDNA [71]. Engraftment of edited cells was increased to over 60% by suppressing AAV-associated DNA damage with i53, an inhibitor of p53-binding protein 1 and human genetic suppressor element. In addition, NK group 2 member D (NKG2D) expression and function (which is lost in XMEN patients) was restored.

Conclusion

PIDs represent a group of diseases relatively amenable to gene editing strategies for two reasons. Firstly, targets cells (usually HSCs) are easily harvested via G-CSF mobilisation

and apheresis facilitating ex-vivo editing. Secondly, edited cells will locate their target niche and engraft after simple intravenous injection allowing straightforward replacement of the immune compartment. These autologous cells also obviate the risk of graft vs host disease seen after allogenic HSCT.

Recent progress over the last decade has accelerated due to the advent of CRISPR/Cas9 editing methods. Several challenges remain prior to clinical trials. The potential for off-target effects are concerning considering previous malignant transformation from gene therapy products in SCID and WAS[5,88]. Although our ability to predict and mitigate this risk improves, the significant genetic variation between individuals mean that gene editing will always have an inherent risk of mutagenesis.

In addition, the longevity of edited cells can be suboptimal, especially when AAV vectors are employed. We have already discussed methods to reduce p53 mediated HSC apoptosis following editing [27] ,although the inclusion of extra mediators during editing requires further clinical evaluation.

We also need to consider that the homologous recombination machinery works when the cell enters the S/G2 phase of the cell cycle. Culture conditions that promote cell proliferation often induce HSC differentiation and/or modify the expression of cell surface molecules relevant for BM homing. For example, it has been recently shown that the expression of CXCR4 decreases after gene editing cultures and this could partly explain the poor homing of corrected cells if they are not provided with an engraftment advantage in the form of CXCR4 mRNA[89].

Therefore, the use of transient upregulation or expression of cytokines (through mRNA delivery) could increase homing and preserve the long-term repopulating ability of corrected cells.

As these hurdles are overcome, it is likely Phase I human trails will begin in the next decade and careful trial design is imperative. Conditioning regimes must be considered especially where edited cells have no clear selective advantage, such as in CGD. Conversely,

conditioning may not be required in XLA where only edited cells could cause humoral reconstitution. Additionally, regulation surrounding cell-based therapies needs to be developed. Several countries have created regulatory frameworks independently[90], however, promoting convergence will be essential to simply and expedite human trials; many of which are likely to be multinational.

As such, efforts to produce cell-based gene editing products and design trials must progress prudently as this technology enters clinical practice.

Declarations

Funding and/or Conflicts of interests/Competing interests.

Conflicts of Interest: Sameer Bahal, Klesti Karaxhuku and Giorgia Santilli declare that they have no conflicts of interest.

Author Contributions

SB, KK wrote the manuscript.GS wrote and edited the manuscript.

Funding

No funding was required for this article.

References

1. Abolhassani H, Azizi G, Sharifi L, Yazdani R, Mohsenzadegan M, Delavari S, et al. Global systematic review of primary immunodeficiency registries. Expert Review of Clinical Immunology. Taylor & Francis; 2020;16:717–32.

2. Shehata N, Palda V, Bowen T, Haddad E, Issekutz TB, Mazer B, et al. The use of immunoglobulin therapy for patients with primary immune deficiency: an evidence-based practice guideline. Transfus Med Rev. 2010;24 Suppl 1:S28-50.

3. Hahn T, McCarthy PL, Hassebroek A, Bredeson C, Gajewski JL, Hale GA, et al. Significant improvement in survival after allogeneic hematopoietic cell transplantation during a period of significantly increased use, older recipient age, and use of unrelated donors. J Clin Oncol. 2013;31:2437–49.

4. Touzot F, Hacein-Bey-Abina S, Fischer A, Cavazzana M. Gene therapy for inherited immunodeficiency. Expert Opin Biol Ther. 2014;14:789–98.

5. Baum C. Insertional mutagenesis in gene therapy and stem cell biology. Curr Opin Hematol. 2007;14:337–42.

6. Doudna JA. The promise and challenge of therapeutic genome editing. Nature. Nature Publishing Group; 2020;578:229–36.

7. Kim YG, Cha J, Chandrasegaran S. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proceedings of the National Academy of Sciences of the United States of America. National Academy of Sciences; 1996;93:1156.

8. Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, et al. Breaking the code of DNA binding specificity of TAL-type III effectors. Science. 2009;326:1509–12.

9. Jansen R, Embden JDA van, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol. 2002;43:1565–75.

10. Mojica FJM, Díez-Villaseñor C, García-Martínez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol. 2005;60:174–82.

11. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, et al. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature. Nature Publishing Group; 2011;471:602–7.

12. Porto EM, Komor AC, Slaymaker IM, Yeo GW. Base editing: advances and therapeutic opportunities. Nat Rev Drug Discov. Nature Publishing Group; 2020;19:839–59.

13. Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature. Nature Publishing Group; 2019;576:149–57.

14. Wang J, He Z, Wang G, Zhang R, Duan J, Gao P, et al. Efficient targeted insertion of large DNA fragments without DNA donors. Nat Methods. Nature Publishing Group; 2022;19:331–40.

15. Xu L, Yang H, Gao Y, Chen Z, Xie L, Liu Y, et al. CRISPR/Cas9-Mediated CCR5 Ablation in Human Hematopoietic Stem/Progenitor Cells Confers HIV-1 Resistance In Vivo. Molecular Therapy. Elsevier; 2017;25:1782–9.

16. Frangoul H, Altshuler D, Cappellini MD, Chen Y-S, Domm J, Eustace BK, et al. CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. N Engl J Med. 2021;384:252–60.

17. Florea AED, Braylan RC, Schafernak KT, Williams KW, Daub J, Goyal RK, et al. Abnormal B-cell maturation in the bone marrow of patients with germline mutations in PIK3CD. Journal of Allergy and Clinical Immunology. Elsevier; 2017;139:1032-1035.e6.

18. Horwitz MS, Corey SJ, Grimes HL, Tidwell T. ELANE Mutations in Cyclic and Severe Congenital Neutropenia—Genetics and Pathophysiology. Hematol Oncol Clin North Am. 2013;27:19–41.

19. Nasri M, Ritter M, Mir P, Dannenmann B, Aghaallaei N, Amend D, et al. CRISPR/Cas9-mediated ELANE knockout enables neutrophilic maturation of primary hematopoietic stem and progenitor cells and induced pluripotent stem cells of severe congenital neutropenia patients. Haematologica. 2020;105:598–609.

20. Gillmore JD, Gane E, Taubel J, Kao J, Fontana M, Maitland ML, et al. CRISPR-Cas9 In Vivo Gene Editing for Transthyretin Amyloidosis. New England Journal of Medicine. Massachusetts Medical Society; 2021;385:493–502.

21. Sacco MG, Ungari M, Catò EM, Villa A, Strina D, Notarangelo LD, et al. Lymphoid abnormalities in CD40 ligand transgenic mice suggest the need for tight regulation in gene therapy approaches to hyper immunoglobulin M (IgM) syndrome. Cancer Gene Ther. 2000;7:1299–306.

22. Bulcha JT, Wang Y, Ma H, Tai PWL, Gao G. Viral vector platforms within the gene therapy landscape. Sig Transduct Target Ther. Nature Publishing Group; 2021;6:1–24.

23. Rigo F, Martinson HG. Functional coupling of last-intron splicing and 3'-end processing to transcription in vitro: the poly(A) signal couples to splicing before committing to cleavage. Mol Cell Biol. 2008;28:849–62.

24. Nesic D, Cheng J, Maquat LE. Sequences within the last intron function in RNA 3'-end formation in cultured cells. Mol Cell Biol. 1993;13:3359–69.

25. Gray DH, Villegas I, Long J, Santos J, Keir A, Abele A, et al. Optimizing Integration and Expression of Transgenic Bruton's Tyrosine Kinase for CRISPR-Cas9-Mediated Gene Editing of X-Linked Agammaglobulinemia. CRISPR J. 2021;4:191–206.

26. Rapti K, Stillitano F, Karakikes I, Nonnenmacher M, Weber T, Hulot J-S, et al. Effectiveness of gene delivery systems for pluripotent and differentiated cells. Molecular Therapy - Methods & Clinical Development. 2015;2:14067.

27. Schiroli G, Conti A, Ferrari S, Della Volpe L, Jacob A, Albano L, et al. Precise Gene Editing Preserves Hematopoietic Stem Cell Function following Transient p53-Mediated DNA Damage Response. Cell Stem Cell. 2019;24:551-565.e8.

28. Sadelain M, Papapetrou EP, Bushman FD. Safe harbours for the integration of new DNA in the human genome. Nat Rev Cancer. 2011;12:51–8.

29. Sander JD, Zaback P, Joung JK, Voytas DF, Dobbs D. Zinc Finger Targeter (ZiFiT): an engineered zinc finger/target site design tool. Nucleic Acids Res. 2007;35:W599–605.

30. Heigwer F, Kerr G, Walther N, Glaeser K, Pelz O, Breinig M, et al. E-TALEN: a web tool to design TALENs for genome engineering. Nucleic Acids Res. 2013;41:e190.

31. Heigwer F, Kerr G, Boutros M. E-CRISP: fast CRISPR target site identification. Nat Methods. 2014;11:122–3.

32. Körbling M, Freireich EJ. Twenty-five years of peripheral blood stem cell transplantation. Blood. 2011;117:6411–6.

33. Dreger P, Haferlach T, Eckstein V, Jacobs S, Suttorp M, Löffler H, et al. G-CSF-mobilized peripheral blood progenitor cells for allogeneic transplantation: safety, kinetics of mobilization, and composition of the graft. Br J Haematol. 1994;87:609–13.

34. Etard C, Joshi S, Stegmaier J, Mikut R, Strähle U. Tracking of Indels by DEcomposition is a Simple and Effective Method to Assess Efficiency of Guide RNAs in Zebrafish. Zebrafish. 2017;14:586–8.

35. Conant D, Hsiau T, Rossi N, Oki J, Maures T, Waite K, et al. Inference of CRISPR Edits from Sanger Trace Data. CRISPR J. 2022;5:123–30.

36. Mashal RD, Koontz J, Sklar J. Detection of mutations by cleavage of DNA heteroduplexes with bacteriophage resolvases. Nat Genet. 1995;9:177–83.

37. Sentmanat MF, Peters ST, Florian CP, Connelly JP, Pruett-Miller SM. A Survey of Validation Strategies for CRISPR-Cas9 Editing. Sci Rep. Nature Publishing Group; 2018;8:888.

38. Peng C, Zheng M, Ding L, Chen X, Wang X, Feng X, et al. Accurate Detection and Evaluation of the Gene-Editing Frequency in Plants Using Droplet Digital PCR. Frontiers in Plant Science [Internet]. 2020 [cited 2022 Aug 27];11. Available from: https://www.frontiersin.org/articles/10.3389/fpls.2020.610790

39. Cheng Y, Tsai SQ. Illuminating the genome-wide activity of genome editors for safe and effective therapeutics. Genome Biology. 2018;19:226.

40. Bae S, Park J, Kim J-S. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics. 2014;30:1473–5.

41. Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nat Biotechnol. Nature Publishing Group; 2015;33:187–97.

42. Tsai SQ, Nguyen NT, Malagon-Lopez J, Topkar VV, Aryee MJ, Joung JK. CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR–Cas9 nuclease off-targets. Nat Methods. Nature Publishing Group; 2017;14:607–14.

43. Lazzarotto CR, Nguyen NT, Tang X, Malagon-Lopez J, Guo JA, Aryee MJ, et al. Defining CRISPR–Cas9 genome-wide nuclease activities with CIRCLE-seq. Nat Protoc. Nature Publishing Group; 2018;13:2615–42.

44. Turchiano G, Andrieux G, Klermund J, Blattner G, Pennucci V, el Gaz M, et al. Quantitative evaluation of chromosomal rearrangements in gene-edited human stem cells by CAST-Seq. Cell Stem Cell. 2021;28:1136-1147.e5.

45. Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F. Rationally engineered Cas9 nucleases with improved specificity. Science. 2016;351:84–8.

46. Vakulskas CA, Dever DP, Rettig GR, Turk R, Jacobi AM, Collingwood MA, et al. A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. Nat Med. Nature Publishing Group; 2018;24:1216–24.

47. Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, et al. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature. 2016;529:490–5.

48. Leibowitz ML, Papathanasiou S, Doerfler PA, Blaine LJ, Sun L, Yao Y, et al. Chromothripsis as an on-target consequence of CRISPR–Cas9 genome editing. Nat Genet. Nature Publishing Group; 2021;53:895–905.

49. Kraus H, Kaiser S, Aumann K, Bönelt P, Salzer U, Vestweber D, et al. A feeder-free differentiation system identifies autonomously proliferating B cell precursors in human bone marrow. J Immunol. 2014;192:1044–54.

50. Wognum B, Yuan N, Lai B, Miller CL. Colony forming cell assays for human hematopoietic progenitor cells. Methods Mol Biol. 2013;946:267–83.

51. Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol. 2005;174:6477–89.

52. Ramkumar C, Gerstein RM, Zhang H. Serial transplantation of bone marrow to test self-renewal capacity of hematopoietic stem cells in vivo. Methods Mol Biol. 2013;976:17–24.

53. Sweeney CL, Pavel-Dinu M, Choi U, Brault J, Liu T, Koontz S, et al. Correction of X-CGD patient HSPCs by targeted CYBB cDNA insertion using CRISPR/Cas9 with 53BP1 inhibition for enhanced homology-directed repair. Gene Ther. 2021;28:373–90.

54. Urnov FD, Miller JC, Lee Y-L, Beausejour CM, Rock JM, Augustus S, et al. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature. 2005;435:646–51.

55. Lombardo A, Genovese P, Beausejour CM, Colleoni S, Lee Y-L, Kim KA, et al. Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. Nat Biotechnol. 2007;25:1298–306.

56. Schiroli G, Ferrari S, Conway A, Jacob A, Capo V, Albano L, et al. Preclinical modeling highlights the therapeutic potential of hematopoietic stem cell gene editing for correction of SCID-X1. Sci Transl Med. 2017;9:eaan0820.

57. Pavel-Dinu M, Wiebking V, Dejene BT, Srifa W, Mantri S, Nicolas CE, et al. Gene correction for SCID-X1 in long-term hematopoietic stem cells. Nat Commun. 2019;10:1634.

58. Chang C-W, Lai Y-S, Westin E, Khodadadi-Jamayran A, Pawlik KM, Lamb LS, et al. Modeling Human Severe Combined Immunodeficiency and Correction by CRISPR/Cas9-Enhanced Gene Targeting. Cell Rep. 2015;12:1668–77.

59. Hubbard N, Hagin D, Sommer K, Song Y, Khan I, Clough C, et al. Targeted gene editing restores regulated CD40L function in X-linked hyper-IgM syndrome. Blood. 2016;127:2513–22.

60. Kuo CY, Long JD, Campo-Fernandez B, de Oliveira S, Cooper AR, Romero Z, et al. Site-Specific Gene Editing of Human Hematopoietic Stem Cells for X-Linked Hyper-IgM Syndrome. Cell Rep. 2018;23:2606–16.

61. Laskowski TJ, Van Caeneghem Y, Pourebrahim R, Ma C, Ni Z, Garate Z, et al. Gene Correction of iPSCs from a Wiskott-Aldrich Syndrome Patient Normalizes the Lymphoid Developmental and Functional Defects. Stem Cell Reports. Elsevier; 2016;7:139–48.

62. Gutierrez-Guerrero A, Sanchez-Hernandez S, Galvani G, Pinedo-Gomez J, Martin-Guerra R, Sanchez-Gilabert A, et al. Comparison of Zinc Finger Nucleases Versus CRISPR-Specific Nucleases for Genome Editing of the Wiskott-Aldrich Syndrome Locus. Hum Gene Ther. 2018;29:366–80.

63. Rai R, Romito M, Rivers E, Turchiano G, Blattner G, Vetharoy W, et al. Targeted gene correction of human hematopoietic stem cells for the treatment of Wiskott - Aldrich Syndrome. Nat Commun. 2020;11:4034.

64. Merling RK, Sweeney CL, Chu J, Bodansky A, Choi U, Priel DL, et al. An AAVS1-targeted minigene platform for correction of iPSCs from all five types of chronic granulomatous disease. Mol Ther. 2015;23:147–57.

65. De Ravin SS, Reik A, Liu P-Q, Li L, Wu X, Su L, et al. Targeted gene addition in human CD34(+) hematopoietic cells for correction of X-linked chronic granulomatous disease. Nat Biotechnol. 2016;34:424–9.

66. De Ravin SS, Li L, Wu X, Choi U, Allen C, Koontz S, et al. CRISPR-Cas9 gene repair of hematopoietic stem cells from patients with X-linked chronic granulomatous disease. Sci Transl Med. 2017;9:eaah3480.

67. Sweeney CL, Zou J, Choi U, Merling RK, Liu A, Bodansky A, et al. Targeted Repair of CYBB in X-CGD iPSCs Requires Retention of Intronic Sequences for Expression and Functional Correction. Mol Ther. 2017;25:321–30.

68. Merling RK, Kuhns DB, Sweeney CL, Wu X, Burkett S, Chu J, et al. Gene-edited pseudogene resurrection corrects p47phox-deficient chronic granulomatous disease. Blood Adv. 2016;1:270–8.

69. Klatt D, Cheng E, Hoffmann D, Santilli G, Thrasher AJ, Brendel C, et al. Differential Transgene Silencing of Myeloid-Specific Promoters in the AAVS1 Safe Harbor Locus of Induced Pluripotent Stem Cell-Derived Myeloid Cells. Hum Gene Ther. 2020;31:199–210.

70. Goodwin M, Lee E, Lakshmanan U, Shipp S, Froessl L, Barzaghi F, et al. CRISPR-based gene editing enables FOXP3 gene repair in IPEX patient cells. Sci Adv. 2020;6:eaaz0571.

71. Brault J, Liu T, Bello E, Liu S, Sweeney CL, Meis RJ, et al. CRISPR-targeted MAGT1 insertion restores XMEN patient hematopoietic stem cells and lymphocytes. Blood. 2021;138:2768–80.

72. Fischer A, Notarangelo LD, Neven B, Cavazzana M, Puck JM. Severe combined immunodeficiencies and related disorders. Nat Rev Dis Primers. Nature Publishing Group; 2015;1:1–18.

73. Yazdani R, Fekrvand S, Shahkarami S, Azizi G, Moazzami B, Abolhassani H, et al. The hyper IgM syndromes: Epidemiology, pathogenesis, clinical manifestations, diagnosis and management. Clin Immunol. 2019;198:19–30.

74. Noelle RJ. CD40 and its ligand in host defense. Immunity. 1996;4:415–9.

75. Winkelstein JA, Marino MC, Ochs H, Fuleihan R, Scholl PR, Geha R, et al. The X-linked hyper-IgM syndrome: clinical and immunologic features of 79 patients. Medicine (Baltimore). 2003;82:373–84.

76. Calvez R, Lafouresse F, Meester JD, Galy A, Valitutti S, Dupré L. The Wiskott-Aldrich syndrome protein permits assembly of a focused immunological synapse enabling sustained T-cell receptor signaling. Haematologica. 2011;96:1415–23.

77. Suri D, Rikhi R, Jindal AK, Rawat A, Sudhakar M, Vignesh P, et al. Wiskott Aldrich Syndrome: A Multi-Institutional Experience From India. Frontiers in Immunology [Internet]. 2021 [cited 2022 Apr 3];12. Available from: https://www.frontiersin.org/article/10.3389/fimmu.2021.627651

78. Arnold DE, Heimall JR. A Review of Chronic Granulomatous Disease. Adv Ther. 2017;34:2543–57.

79. Kuhns DB, Alvord WG, Heller T, Feld JJ, Pike KM, Marciano BE, et al. Residual NADPH oxidase and survival in chronic granulomatous disease. N Engl J Med. 2010;363:2600–10.

80. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet. Nature Publishing Group; 2001;27:20–1.

81. Park JH, Lee KH, Jeon B, Ochs HD, Lee JS, Gee HY, et al. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome: A systematic review. Autoimmunity Reviews. 2020;19:102526.

82. Tsukada S, Saffran DC, Rawlings DJ, Parolini O, Allen RC, Klisak I, et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. 1993. J Immunol. 2012;188:2936–47.

83. Wang Q, Pechersky Y, Sagawa S, Pan AC, Shaw DE. Structural mechanism for Bruton's tyrosine kinase activation at the cell membrane. Proc Natl Acad Sci U S A. 2019;116:9390–9.

84. Vetrie D, Vorechovský I, Sideras P, Holland J, Davies A, Flinter F, et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. Nature. 1993;361:226–33.

85. El-Sayed ZA, Abramova I, Aldave JC, Al-Herz W, Bezrodnik L, Boukari R, et al. X-linked agammaglobulinemia (XLA): Phenotype, diagnosis, and therapeutic challenges around the world. World Allergy Organ J. 2019;12:100018.

86. Rohrer J, Conley ME. Correction of X-linked immunodeficient mice by competitive reconstitution with limiting numbers of normal bone marrow cells. Blood. 1999;94:3358–65.

87. Li F-Y, Chaigne-Delalande B, Su H, Uzel G, Matthews H, Lenardo MJ. XMEN disease: a new primary immunodeficiency affecting Mg2+ regulation of immunity against Epstein-Barr virus. Blood. 2014;123:2148–52.

88. Braun CJ, Witzel M, Paruzynski A, Boztug K, Kalle C von, Schmidt M, et al. Gene therapy for Wiskott-Aldrich Syndrome—Long-term reconstitution and clinical benefits, but increased risk for leukemogenesis. Rare Diseases. Taylor & Francis; 2014;2:e947749.

89. Omer-Javed A, Pedrazzani G, Albano L, Ghaus S, Latroche C, Manzi M, et al. Mobilization-based chemotherapy-free engraftment of gene-edited human hematopoietic stem cells. Cell. 2022;185:2248-2264.e21.

90. Drago D, Foss-Campbell B, Wonnacott K, Barrett D, Ndu A. Global regulatory progress in delivering on the promise of gene therapies for unmet medical needs. Molecular Therapy - Methods & Clinical Development. 2021;21:524–9.

Figures

Figure 1

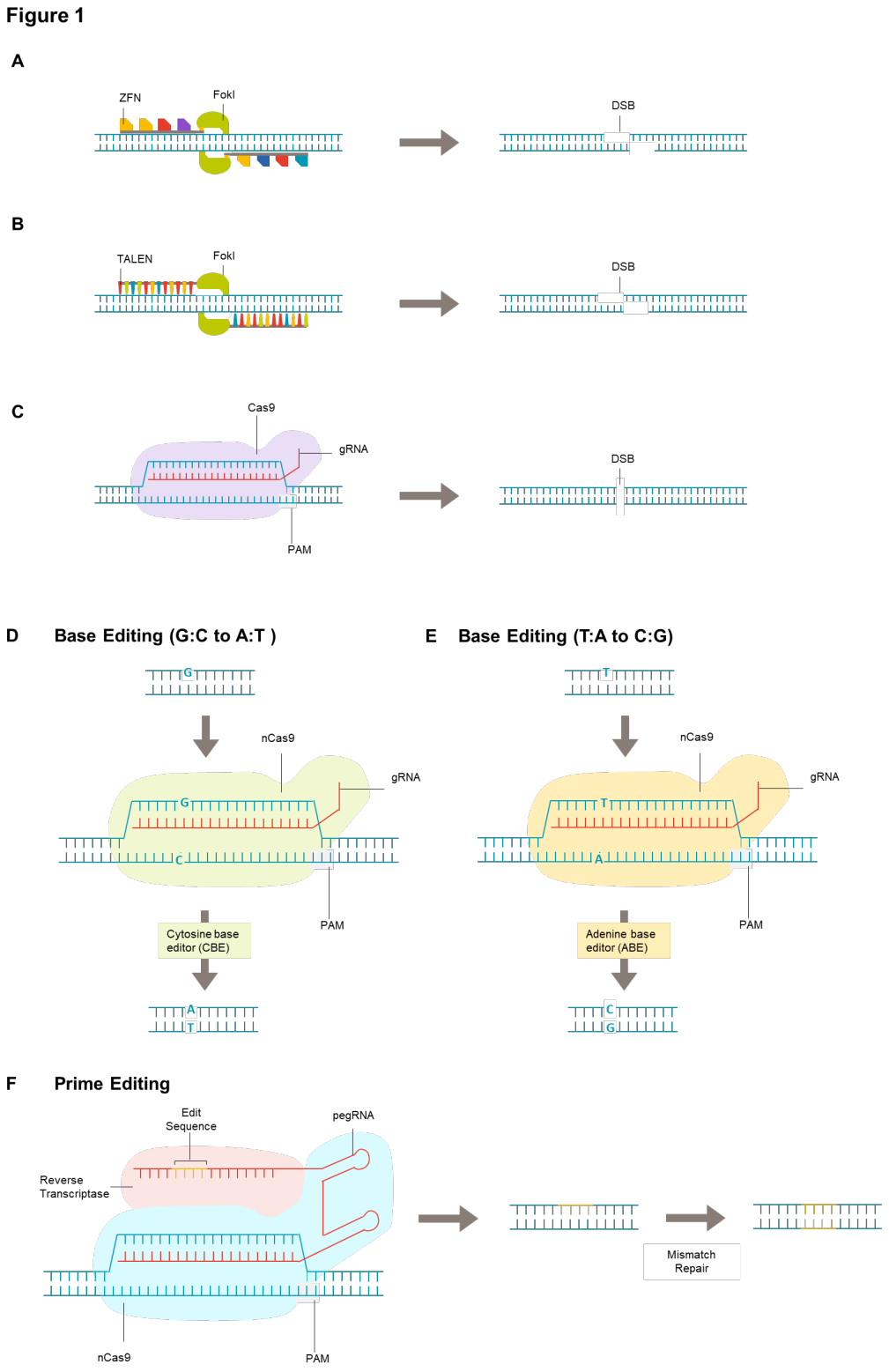

Figure 2

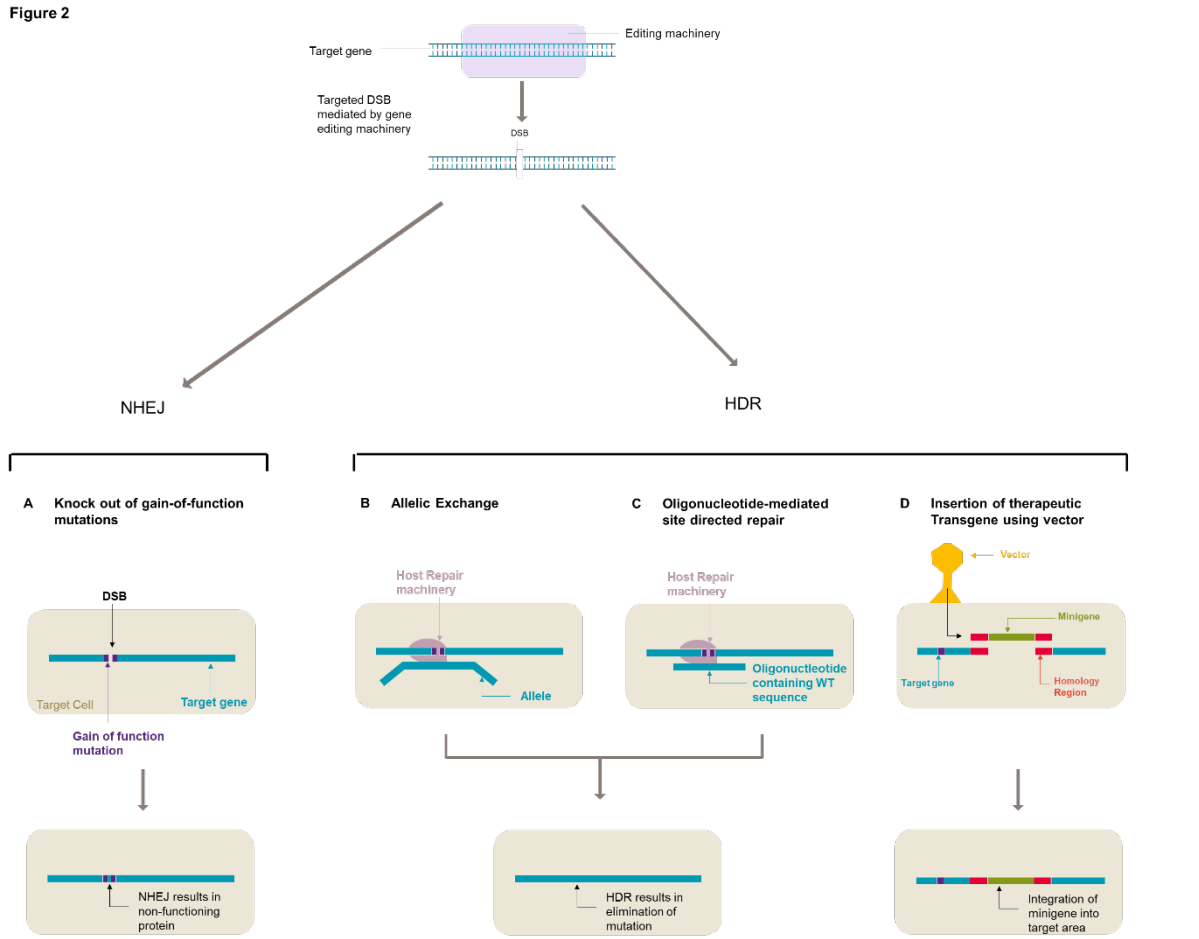
Figure 2
Target gene
Editing machinery
Targeted DSB mediated by gene editing machinery
DSB
NHEJ
HDR
A Knock out of gain-of-function mutations
DSB
Target Cell
Target gene
Gain of function mutation
NHEJ results in non-functioning protein
B Allelic Exchange
Host Repair machinery
Allele
C Oligonucleotide-mediated site directed repair
Host Repair machinery
Oligonuctleotide containing WT sequence
HDR results in elimination of mutation
D Insertion of therapeutic Transgene using vector
Vector
Minigene
Target gene
Homology Region
Integration of minigene into target area
B

Figure Legends

Figure 1

Editing machinery used to make target specific DSBs in target cell genome. This can be delivered in either RNP or plasmid format. **A.** ZFN editing depends on a FokI nickase coupled to several ZFNs each with a 3 base pair specificity. Paired constructs bind either side of the target creating the desired DSB. **B**. In TALEN based editing, TALEs with single base pair specificities are instead coupled to FokI. Again, a paired approach on either side of the target produces the DSB. **C.** The CRISPR/Cas9 method is easily programmable by designing a gRNA specific to the target area. Firstly, Cas9 and gRNA are complexed together. This assembly locates the identical sequence to the gRNA in the host genome and the Cas9 nuclease activity results in a DSB. Base editors comprise altered Cas9 (such as nCas) bound to cytidine or adenine deaminase. They facilitate single base pair substitutions of either G:C to A:T **(D)** or T:A to C:G **(E)**. Their editing window normally falls between 4 and 7 base pairs from the 5' end of the gRNA. **F.** In prime editing an altered Cas9 molecule attached to reverse transcriptase is complexed with an extended gRNA known as a pegRNA which contains a targeting gRNA sequence as well as a correction template. After the Cas9 locates the area in the genome complimentary to the pegRNA, a nick is made by the Cas9 component. The reverse transcriptase transcribes the correction template into one strand while the cell's mismatch repair mechanism completes the edit in the complementary strand.

DSB double stranded breaks, *RNP* ribonucleoprotein, *ZFN* zinc finger nuclease, *TALEN* transcription activator-like effector nucleases, *CRISPR/Cas9* clustered, regularly interspersed palindromic repeats/CRISPR-associated protein 9, *gRNA* guide RNA, *PAM* protospacer adjacent motif, *nCas9* nickase Cas9, *CBE* cytosine base editor, *ABE* adenine base editor. *pegRNA* prime editing guide RNA

Figure 2

Gene editing strategies employed for PIDs**.** Editing machinery (either using ZFNs, TALENs or the CRISPR/Cas9 system) creates a targeted DSB that can be repaired by Non-homologous end joining-NHEJ (A) or by Homology Directed Repair-HDR (B-D). **A.** When the DSB targets GoF mutations, the resulting indels from NHEJ disrupt gene expression. **B.** Allelic exchange also involves a targeted DSB of the mutation. However, the host repair machinery uses the wildtype allele as a correction template; eliminating the mutation. **C.** Oligonucleotide based editing is similar to allelic exchange, however, a synthetic oligonucleotide that is co-transfected with the editing machinery acts as the correction template. **D.** In the "one size fits all" strategy a targeted DSB is made at a safe harbour or a predetermined location in the target gene. A vector (such as an AAV or lipid nanoparticles) is used to insert a donor construct usually containing a codon-optimised minigene flanked by homology regions adjacent to the cut. The cellular repair mechanism then inserts the construct via HDR.

PID Primary Immunodeficiency, *ZFN* Zinc Finger Nucleases, *TALEN* Transcription Activator-Like Effector nucleases, *CRISPR/Cas9* Clustered, regularly interspersed palindromic repeats/CRISPR-associated protein 9, *DSB* Double Stranded Breaks, *GoF* Gain of function, *NHEJ* Non-homologous end Joining, HDR Homology Directed Repair, *AAV* Adeno-Associated Virus, *gRNA* guide RNA, *WT* wildtype