Chapter #: Gene Therapy in Cellular Immunodeficiencies

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Abstract: (~150 words).
The treatment of cellular primary immunodeficiencies has benefitted from significant advances in the field of allogeneic stem cell transplantation (alloHSCT). However, whilst this therapy is curative for many PIDs, the procedure requires a suitably matched donor and carries significant risks of morbidity and mortality from complications such as graft-versus-host disease (GVHD). Autologous gene therapy (GT) approaches using stem cells isolated from patients and modified *ex vivo* using viral vectors or gene editing techniques, have the potential to offer curative therapy for PID without the immunological complications of alloHSCT. GT for PID has been developed over the last 30 years and whilst several setbacks have been encountered along the way, there is now a licensed GT product for ADA-SCID. and Promising results from phase I/II clinical trials have demonstrated that GT may offer clinical efficacy comparable to alloHSCT in several other PIDs. Developments in the field are broadening the application of GT and we expect that this therapeutic modality may become standard of care for the management of several PIDs in the near future. This chapter explores the development of this exciting technology over the last 30 years and outlines the application of GT to cellular primary immunodeficiencies.

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Chapter Text:

Introduction

Allogeneic hematopoietic stem cell transplantation (alloHSCT) has for many years been the only curative treatment option for primary immunodeficiencies (PIDs)(1). The procedure has been performed for PIDs for over 50 years, and advances in this field have led to survival rates of over 95% in paediatric severe combined immune deficiency (SCID) cohorts(2). The development of HLA-haploidentical stem cell transplantation using posttransplant cyclophosphamide (PTCy) or graft manipulation with α/β T-cell and B-cell depletion has reduced problems with donor availability and survival outcomes are now comparable to HLA-matched HSCT(3-5). However, alloHSCT still carries a risk of graft-versus-host disease (GVHD) and graft failure, which can result in significant morbidity and mortality. AlloHSCT carries higher risks in older cohorts with non-SCID PIDs, thus limiting the application of the procedure for some patients(6, 7).

Autologous gene therapy (GT) completely removes the risk of GVHD and the requirement for a suitably matched donor(8, 9). Autologous procedures are less toxic than allogeneic approaches, as the lack of immunogenicity permits engraftment of HSCs with reduced intensity conditioning and removes the need for immune suppression as GVHD prophylaxis. Current autologous GT approaches using lentiviral vectors have demonstrated clinical benefits at least equivalent to alloHSCT, without the associated immunological complications(10-12). This exciting emerging field has already shown impressive, long term results for a number of conditions with the potential to offer curative therapies for many more PIDs and supplant alloHSCT as the standard of care for these rare but devastating disorders.

Ex-vivo GT involves harvesting hematopoietic stem cells (HSCs) from a patient, either by direct aspiration from the bone marrow, or from mobilised peripheral blood using GCSF and plerixafor to allow collection of large numbers of CD34+ HSCs [Fig. 1]. Harvested HSCs are cultured *ex vivo* in conditions that favour expansion of cells with long-term repopulating potential(9, 13, 14). Gene transfer or gene editing is performed under sterile conditions and necessary quality checks are performed to ensure that the genetically modified product is safe to be re-infused to the patient, termed GMP (Good Manufacturing Practice) compliant. Prior to re-infusion, the patient receives conditioning with chemo-immunotherapy to deplete HSCs and permit engraftment of the genetically modified stem cells. Varying degrees of conditioning can be used to optimise engraftment but minimise side effects in different PIDs. For example, myeloreductive conditioning in SCID compared to more intense conditioning in GT trials for chronic granulomatous disease (CGD)(12, 15).

The genetically modified cells are re-infused to the patient and engraft in the bone marrow. After engraftment, the genetically modified HSCs have the potential to self-renew for the lifetime of the recipient and as they differentiate, give rise to gene-corrected progeny across all immuno-hematopoietic lineages(9, 16).

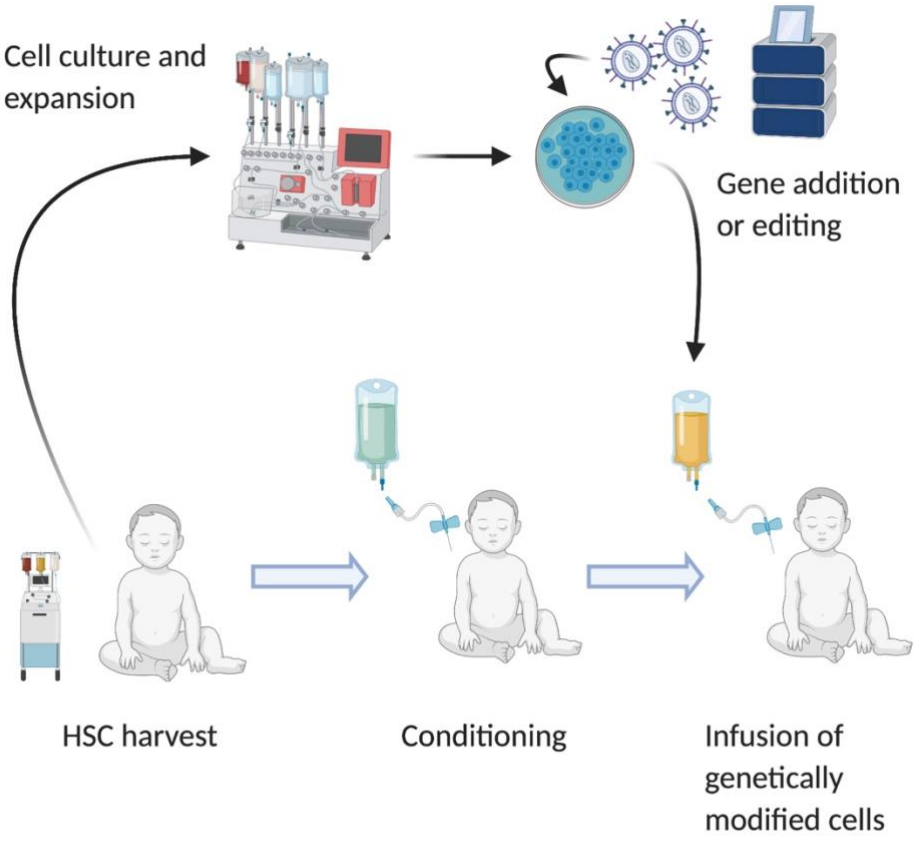

Figure 1: Overview of autologous gene therapy (GT) procedure

Retroviruses and lentiviruses were identified as candidate vectors for the transduction of HSCs due to their ability to efficiently integrate their DNA into host cells. HSCs are an ideal cellular target because they are long-lived, can be accessed with relative ease for *ex vivo* manipulation, and are multipotent, so genetic correction will be passed on to all progeny across different cell lineages(16, 17). Prior to a detailed discussion of GT in cellular primary immunodeficiencies, it is worth evaluating which genetic disorders are most suitable for a GT approach.

Most importantly, the nature of the genetic defect needs to be considered. PIDs are excellent candidates for GT due to the clear link between defined monogenic defects and a clinical phenotype of immune dysfunction and dysregulation. PIDs result from monogenic germline mutations that result in reduced or absent protein expression (loss-of-function, LOF), or increased or overactive protein expression (gain-of-function, GOF). Mutations can be heterozygous (haploinsufficiency or dominant negative) or homozygous (18). The mutational landscape and variability in both genetic penetrance and clinical phenotype are important. It is easier to target and then validate the efficacy of GT for diseases caused by single mutations that have a clear clinical phenotype, as opposed to diseases with heterogeneous mutational landscapes and variable clinical phenotype. Another important factor relates to the normal expression and function of the encoded protein. For example, it is helpful to know whether supra-physiological levels of ubiquitously expressed proteins are potential harmful. It is

logical therefore that a disease caused by homozygous LOF mutations resulting in reduced or absent expression of a ubiquitously expressed protein would be the least complicated disorder to attempt a GT approach. Indeed, adenine deaminase deficient SCID (ADA-SCID), fits this description and it is therefore, not surprising that ADA-SCID was the first genetic disorders for which *ex vivo* GT was attempted and for which GT approaches are now at the most advanced stage of clinical development.

As GT has advanced, the application of the technology to more complex disorders has been attempted and pre-clinical proof-of-concept studies are leading on to clinical trials. Where normal protein expression is tightly regulated and/or lineage restricted correction of expression by GT should, where possible replicate this. Gene editing will prove to be particularly useful in these disorders where correction in situ allows physiological gene regulation from all native regulatory elements. Finally, the effect of transgene expression on the HSCs and/or their progeny is important. Where the transgene confers a significant survival advantage, the corrected cells are more likely to proliferate *in vivo*.

This is a rapidly changing field and developments are broadening the application of GT to an increasing number of PIDs. Developments in gene editing techniques have the potential to overcome some of the limitations of viral gene addition strategies(9). This chapter will describe the history of GT and some of the early problems that were encountered with the technique. The early applications of GT will be described before outlining the application of GT to different PIDs. The limitations of gene addition will be considered, before explaining how gene editing may overcome some of these issues. Finally, gene editing techniques will be explained and their application to PID GT outlined.

History of gene therapy (GT)

The pathway to the successful application of *ex-vivo* HSC GT to PIDs has not been straightforward. The concept of using viral vectors to insert a functional copy of the defective gene directly into the cellular chromosomal DNA was met with initial excitement. The first PID for which GT was attempted was adenine deaminase deficient severe combined immunodeficiency (ADA-SCID), a multi-system, life-threatening disorder resulting from the absence of an essential enzyme, adenosine deaminase. Although a multi-system disorder it is the immune deficiency which is fatal and only this feature which can be corrected by both HSCT and GT(19). The first clinical trials in humans, published in 1995, used gammaretroviral (γRV) vectors to introduce the ADA gene initially into T lymphocytes and then into HSCs(15, 20-22). Most patients received low intensity conditioning chemotherapy prior to administration of gene-corrected HSCs. The results of these early trials showed great promise with evidence of metabolic correction and immune reconstitution. Importantly, the gene-corrected cells persisted in the patients treated, demonstrating that HSC GT could offer a cure for ADA-SCID.

Encouraged by the results in the ADA-SCID trials, researchers began working on GT approaches for X-linked SCID (X-SCID), which results from mutations in the common cytokine receptor gamma chain (IL-2 receptor gene (*IL2RG*)). Again, early results using γRV vectors were encouraging with successful immune reconstitution and persistence of gene-corrected

cells. However, optimism turned to concern when five of the twenty patients treated developed leukaemia(23, 24). This was caused by integration of the vector close to the known proto-oncogenes, and subsequent leukemic transformation. Although this was clearly of great concern, these early trials demonstrated the potential that HSC GT had for treating patients with X-SCID with one comparative study demonstrating that gene-corrected autologous HSCs offered improved immune reconstitution compared to haploidentical alloHSCT(25) Leukemogenesis was also observed in an early clinical trial using γRV vector-based therapy for Wiskott Aldrich syndrome (WAS). Whilst nine of the ten patients treated demonstrated correction of WAS protein (WASP) expression, seven patients developed leukaemia due to vector integration close to proto-oncogenes. In this trial additional chromosomal translocations were also observed(26, 27). A trial in a third PID, chronic granulomatous disease (CGD) using γRV vectors encountered a similar problem. Insertional activation of ectopic viral integration site 1 (*EVI1*), lead to oligoclonal hematopoiesis and myelodysplasia with monosomy 7 in patients treated(28). Further investigation demonstrated that the transgene was being silenced by methylation of promoter elements in the long-terminal repeats whereas the enhancer elements were unaffected, resulting in mutagenesis(29, 30). Interestingly, insertional mutagenesis was not observed in any of the patients treated with γRV vectors for ADA-SCID, despite similar vector integration patterns for reasons which remain unclear(15, 31).

Although it was shown that leukemogenesis requires the acquisition of several secondary genetic lesions in addition to the initial event resulting from vector insertion, there was significant concern about the ongoing safety of GT using γRV platforms(24). This prompted the development of alternative vectors with the aim of improving safety. Typically, γRV vectors use strong viral promoters in the long terminal repeat (LTR) sequences to drive transgene expression. Self-inactivating (SIN)-γRV-vectors were developed by mutating the LTR sequences and inserting a less powerful mammalian promoter(32, 33). Furthermore, a new gene transfer platform was developed based on the human immunodeficiency virus (HIV), a lentivirus (LV) which has an integration pattern associated with a lower risk of oncogene activating insertions(34-36). The LV gene transfer vectors utilise the basic platform of HIV enabling insertion of genetic material into chromatin. Additional significant alterations removing their pathogenic potential and utilising other viral envelopes for packaging, have been made, thus enabling a range of cell types to be targeted(37, 38). LV vectors were not only shown to have a more neutral insertion profile compared to γRVs but were also more efficient at gene transfer(34). Induction of cell cycling by cytokine stimulation is required for retrovirus integration(39). This pre-stimulation step prolongs the *ex vivo* manipulation, and has been shown to reduce the long-term repopulating potential of the manipulated HSCs and impair their engraftment(40, 41). Lentiviruses in contrast are able to transduce proliferating and non-proliferating cells, reducing the amount of ex vivo manipulation required and preserving HSC function(42, 43).

Since the development of SIN-γRV and SIN-LV vectors, no insertional oncogenesis events have been reported thus, although a theoretical risk exists, these platforms appear to be safe. To date, 29 phase I/II HSC GT clinical trials have been completed, are in follow-up or are recruiting for the most common PIDs (SCID, WAS and CGD)(44). Indeed, a SIN-γRV vector for ADA-SCID was the first HSC GT product to receive marketing approval in 2016(45). *Strimvelis*, a γRV vector containing the adenine deaminase (ADA) coding DNA (cDNA), is now available in

Europe for patients with ADA-SCID and is currently licensed for use in patients who lack a suitable sibling donor for alloHSCT(31, 46). The results of these trials and the application of HSC GT to specific PIDs will be discussed later in this chapter.

Just as the number of PIDs for which HSC GT can be applied expands as the field develops, there has been also been a change in the demographics of patients who may benefit from HSC GT procedures. Trials of GT for different forms of SCID exclusively treated young children, as survival after infancy is not possible without definitive treatment. As HSC GT approaches were developed for non-SCID PIDs the possibility of treating adolescents and adult patients arose. There are many patients with PIDs such as WAS and CGD who survive to adulthood without an alloHSCT procedure, due to earlier mild disease phenotype, better supportive care, lack of a suitable donor for alloHSCT or the presence of co-morbidities which increase the risks of an allogeneic procedure(6, 7). There were concerns that older patients may not retain their ability to recover a full T-cell repertoire using an autologous GT approach. The first report of HSC GT in adults was published in 2017 in a 30-year-old patient with WAS for whom a HLA-matched donor was not available and a haploidenticial alloHSCT was deemed too high risk due to pre-existing disease-related comorbidities(47). Fears regarding differing reconstitution patterns in older patients appeared to be unfounded and this first patient underwent rapid engraftment and expansion of a polyclonal pool of gene-corrected T cells and had sustained gene marking in myeloid and B cell lineages(47). Adult patients have since been recruited to HSC GT trials including the recent, international, multi-centre, phase I trial of lentivirus-based gene therapy for X-linked CGD. This trial recruited nine patients but six of these were aged over 18 years old and the oldest was 27. Following GT, all patients had successful engraftment of gene-modified HSCs and seven of those treated remained free of new infections at last follow-up(12). Indeed, with advances in alloHSCT and haploidentical approaches reducing problems with donor availability it may be that older patients, who have a higher risk of GVHD post alloHSCT, will stand to benefit most from autologous GT procedures(48).

The pathway to successful GT for cellular PIDs has been far from straight forward. However, the early tragic adverse events have driven a huge number of scientific advances in vector development and gene delivery. Optimism has returned to the field, as several clinical trials of GT approaches have demonstrated promising results in an increasing number of cellular PIDs.

Current status of gene therapies in PID

Severe combined immune deficiencies, SCID

ADA-SCID

HSC GT approaches for the commonest genetic causes of SCID were some of the first to be developed, and consequently are at the most advanced stage of clinical development. As previously stated, ADA-SCID was the first application of HSC GT. The disease is an ideal candidate for an autologous approach due to the relatively small size of the *ADA* cDNA (1.5kb), simplifying cloning into a viral vector. Ubiquitous expression of ADA makes it safer to use the strong viral promoters in the γRV LTRs to drive transcription of the gene(49).

Untreated, ADA-SCID is fatal in infancy and prior to GT, treatment options were either chronic enzyme-replacement therapy or alloHSCT. Due to the inability of a patient with ADA-SCID to reject sibling HSCs, alloHSCT can be performed in the absence of conditioning. In later GT clinical trials for ADA-SCID, prompt and secured engraftment of gene-corrected HSCs following low-dose busulfan conditioning (≤25% myeloablative dose) was observed(50, 51). The finding that improved multi-lineage engraftment could be achieved with the addition of reduced intensity conditioning (RIC) has resulted in the adoption of conditioning for most autologous GT applications, even for underlying diseases with profound T cell lymphopenia(45). As previously mentioned, a γRV-vector GT product, *Strimvelis* is licensed in Europe for ADA-SCID and is available for patients who lack a HLA-matched donor for alloHSCT(46). This is a fresh cell product meaning that patients must travel to Milan (the only treatment centre) for the procedure. This fresh cell product model was used in many clinical trials but the advent of cryopreserved products will improve logistical accessibility, although probably not economic accessibility.

A SIN-LV approach for ADA-SCID has also been developed and efficacy has been further improved by codon optimisation of the ADA-cDNA, expressed under the control of the elongation factor 1α short (EFS) promoter. Promising preclinical studies of this product have led on to clinical trials that utilise preconditioning with a single dose of busulfan (4-5mg/kg) or more recently targeted AUC, prior to infusion of gene-modified HSCs(22, 52). To date, over 30 patients have been treated with lentiviral GT in the United States and the United Kingdom with 100% survival and no complications associated with vector insertion. Commercialisation of a LV GT product for ADA SCID is expected soon(44, 53). Longer-term follow up is required to assess extent and durability of immune reconstitution.

X-linked common gamma chain SCID

For X-SCID, initial trials with γRV vectors were complicated by insertional mutagenesis. Subsequently, a trial of a SIN-γRV vector transduced HSCs in X-SCID delivered without pre-conditioning demonstrated similar efficacy in terms of immune reconstitution compared to the earlier γRV vector trials but with less clustering of insertion sites around proto-oncogenes(32). AlloHSCT can be performed in X-SCID without any conditioning, however, as B-cell development is preserved this may result in mixed chimerism in non-T cell lineages and persistence of recipient HSCs. The experience of autologous HSC GT in X-SCID has been similar and gene-corrected T cells have been shown to consistently develop in the absence of conditioning, as gene-correction results in functional T cell survival and proliferation conferring a competitive advantage. However, as no gene-correction is seen in the myeloid compartment and B-cells, patients remained immunoglobulin dependent(32). Humoral immunity can be restored by HSC GT using low-dose busulfan conditioning prior to infusion of gene-marked stem cells(54). At the time of writing, there are several clinical trials for X-SCID using LV-vectors and low-dose busulfan conditioning(44). Preliminary results of a dual-centre phase I/II trial recently reported were extremely encouraging. After a median follow up of 16.4 months, the eight patients treated demonstrated normal T-cell and NK-cell numbers and sustained gene marking across all lineages. The patients demonstrated normal IgM levels and antibody responses(54). Whilst longer term follow up will be required to assess the durability of the immune reconstitution, lentiviral GT for X-SCID appears very promising.

RAG1 and Artemis SCID

Due to the severity of the clinical phenotype, SCID caused by mutations in recombination genes are obvious candidates for HSC GT. Mutations in the *Artemis* gene and in recombinase-activating gene 1 and 2, *RAG1* and *RAG2* result in V(D)J recombination defects that cause severe impairment of T cell and immunoglobulin receptor rearrangement. Clinically this result in profound immune dysregulation(55). A varied spectrum of combined immunodeficiency results from *RAG1* defects with the clinical phenotype resulting from hypomorphic mutations being particularly variable(56).

In addition to the absence of T and B cells, *Artemis* mutations result in cellular radiosensitivity and a predisposition to malignancy(57). *Artemis* SCID is difficult to treat with alloHSCT, as the conditioning therapy is poorly tolerated due to the underlying sensitivity to ionising radiation and alkylating chemotherapy(58). A HSC GT approach using SIN-LV vectors with transgene expression driven by the phosphoglycerate kinase (PGK) promoter, has been shown in murine models to result in restoration of T- and B- cell repertoires(57). More recently, a SIN-LV vector incorporating the *Artemis* cDNA under the influence of the endogenous *Artemis* promoter has been shown to restore T and B cell function *in vivo* following adoptive transfer of transduced murine stem cells(59). A humanised SIN-LV vector based on this has been shown to correct the radiosensitivity of fibroblasts isolated from Artemis-SCID patients. Restoration of T and B cell development after transduction of mobilised CD34+ cells isolated from an Artemis SCID patient has also been demonstrated(58). This vector has now entered phase I clinical trials in the United Sates (NCT03538899).

HSC GT approaches for *RAG1*-deficiency have previously used γRV vectors which restored T and B cell function but resulted in lymphoproliferation(60). A SIN-LV based approach incorporating codon-optimised *RAG1* cDNA has demonstrated adequate immune reconstitution, but only at high vector copy numbers in a backbone unsuitable for large-scale production(61). Generation of low RAG1 expression levels in cells transduced with this vector with a lower copy number, resulted in incomplete thymic reconstitution and the development of an Omenn-like syndrome with autoreactive T-cells(62). Whilst the development of GT for *RAG1* deficiency has been challenging, at the time of writing a SIN-LV based approach has recently been reported. In the development of this vector, different promoters were tested and an MND (myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer binding site substituted promoter) driven vector was found to result in restoration of *RAG1* deficiency at lower vector copy numbers. Using this vector, B and T cell reconstitution was observed in mice with adequate *RAG1* expression(63). This approach is expected to enter phase I clinical trials in the next 12 months.

Non-SCID PIDs

Chronic granulomatous disease, CGD

Mutations in genes coding for the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex are responsible for CGD, a multisystem PID characterised by hyperinflammation and severe, recurrent bacterial and fungal infections. The most common, X-linked variant of the disease is caused by mutations in *CYBB* which encodes the gp91phox subunit of NADPH-oxidase. The three autosomal recessive forms of CGD are rarer and

cumulatively account for a third of CGD cases(64). AlloHSCT has been shown to be curative in CGD across all age groups(65, 66).

As previously mentioned, HSC GT was attempted for CGD some 15 years ago with Notabk but initial results were disappointing. The first trials in 1995 and 1997 for AR-CGD and X-CGD respectively, used murine Moloney retroviral vectors to transduce HSCs, which were then administered without conditioning(67, 68). Contrary to GT in SCID, the genetically modified HSCs did not have a significant survival advantage over uncorrected HSCs and thus very low levels of engraftment and persistence were observed(68). Following the observation in ADA-SCID GT that low-dose busulfan conditioning aided engraftment, subsequent CGD trials have used pre-conditioning prior to infusion of gene-modified HSCs(50). In 2005, a German group used a γRV vector derived from a murine spleen focus-forming virus which was expected to offer improved but not specific gene transfer to the myeloid lineage(69). Whilst improved gene-marking in myeloid cells was observed initially, unfortunately this trial was complicated by potent enhancer element-driven clonal expansion following gene insertion in the *EVI1/MDS1* gene complex and the development of a monosomy 7 derived MDS(28, 70). A later trial in 2006 using the same γRV vector used in the original 1997 trial but with busulfan conditioning achieved improved, but still poor long term correction of neutrophils (1.1% at six months, as measured by flow cytometric analysis of dihydrorhodamine oxidation)(70).

In order to mitigate the risk of insertional mutagenesis whilst improving gene marking in myeloid cells, a SIN-LV vector has been designed for X-CGD which encodes the codon optimised human *CYBB* cDNA driven by a novel chimeric myeloid specific promoter(71, 72). This vector entered multicentre clinical trials in the United States and Europe and the initial results of nine patients have recently been reported. At six months, 16-46% of neutrophils were oxidase-positive in treated patients with stable vector copy numbers(12). This trial included six patients aged >18 years at time of entry to the study, providing evidence that GT is safe and effective in older patients with CGD. A similar SIN-LV based strategy for AR-CGD is also in development(64). Many older patients with CGD have significant co-morbidities as a result of many years of infections and antimicrobial therapy and/or refractory inflammation. AlloHSCT for adults with CGD is safe and effective, but the TRM is higher than for children, particularly if only unrelated donors are available(6, 73, 74). These promising results offer hope of a curative therapy for older CGD patients who may otherwise have limited treatment options. Longer follow up and expanded studies are needed to assess clinical efficacy of HSC GT against alloHSCT in lower risk patients.

Wiskott-Aldrich Syndrome (WAS)

WAS is an X-linked PID characterised by recurrent infections, thrombocytopenia and eczema and frequently complicated by autoimmunity and lymphoid malignancy. It results from mutations in the WAS gene which encodes WASP. WASP regulates the polymerisation of actin and is critical for immunological synapse formation, cell migration and cytotoxicity(75). The first GT trial for WAS used a γRV vector. WASP expression was driven by a strong viral promoter but this resulted in insertional mutagenesis due to gene insertion close to proto-oncogenes and the majority of patients treated developed acute leukaemia or myelodysplasia(26, 27).

As previously noted, SIN-LV vectors have a more neutral insertion pattern compared to γRV vectors. A SIN-LV vector was developed that used a fragment of the endogenous *WAS* gene promoter(76). This approach entered clinical trials in Europe and the United States in 2010 with a reduced-intensity conditioning regimen consisting of busulfan and fludarabine. Over twenty patients have now been treated and results are encouraging. The GT procedure resulted in good immune reconstitution. Engraftment of gene marked cells was maintained with several patients now having over five years of follow-up. WASP expression was increased across all lineages and a significant clinical improvement was noted with seven of eight patients in one cohort ceasing their immunoglobulin replacement therapy(77). Platelet count recovery was variable, but many patients have become independent of platelet transfusions(10, 77, 78). As previously mentioned, this trial included the first demonstration that HSC GT could be safely performed in adults with PID when a 30-year-old patient was successfully treated. This adult patient had significant disease-related co-morbidities and no HLA-matched donor available. Following GT, they were able to discontinue immunosuppression for autoimmune complications and stop immunoglobulin replacement therapy(47). No genotoxicity has been observed with prolonged follow up and this approach appears to be a very promising and safe alternative to alloHSCT for WAS.

Leukocyte Adhesion Defect Type 1 (LAD-1)

LAD-1 results from defects in the *ITGB2* gene which encodes the CD18 integrin subunit expressed at the plasma membrane. Reduced membrane expression of CD18 results in impaired neutrophil migration and manifests clinically as severe, recurrent bacterial infections. Untreated, few patients survive past infancy and alloHSCT remains the only curative therapy. GT approaches with γRV vectors failed to result in sustained gene marking(79). Successful pre-clinical work using lentiviral vectors in murine and canine models however, has led on to human clinical trials in Europe and the United States (NCT03825783, NCT03812263) using busulfan conditioning (80, 81).

Other-non-SCID PIDs

Gene therapy in rarer monogenic non-SCID PIDs is more challenging as gene-modified HSCs have less of a survival advantage compared to the non-modified stem cells, and the clinical phenotype may be variable. However, these challenges are increasingly being overcome as evidenced by the progress in CGD and WAS described above and the application of GT to other PIDs looks increasingly promising(8). Whilst many GT approaches for other non-SCID PIDs are in the preclinical stage of development, in several diseases, phase I clinical trials have begun or are expected to start in the near future.

RAG2 deficiency

Correction of *RAG2* deficiency has been demonstrated using *RAG2* knock-out mice and a γRV vector, although a selective advantage of transduced lymphoid cells was noted, raising concerns of insertional mutagenesis(82). A SIN-LV vector containing codon-optimised *RAG2* driven by the ubiquitous chromatin opening element (UCOE), has been shown to correct *RAG2* deficiency in a murine model without any potentially oncogenic events in 28 treated mice(83). A clinical trial is being planned.

Hypomorphic mutations in recombination activating genes result in the distinct phenotype, Omenn syndrome (as seen when low level *RAG1* activity was generated in the early *RAG1* SIN-LV preclinical study)(62). More recently, a SIN-LV vector encoding *RAG2*, corrected the immunodeficiency and autoimmunity in a murine model of Omenn syndrome (*RAG2*$^{R229Q/R229Q}$). This preclinical work demonstrates that HSC GT may be effective in an autoinflammatory environment where the risks of alloHSCT may be higher(84). Absence
Absence

IPEX Syndrome

Regulatory T cells (T_{reg}) require the FoxP3 transcription factor for normal development and mutations in FoxP3 result in the devasting PID, immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. IPEX syndrome is characterised by severe autoimmunity with enteropathy, type 1 diabetes and eczema(85). AlloHSCT is the only curative therapy however, overall survival from this procedure in IPEX syndrome is similar to patients who received chronic immunosuppression and was frequently complicated by immune-mediated complications(85). It has been shown in murine adoptive transfer experiments, that *ex-vivo* correction using a LV vector encoding *FoxP3* and the EF1α promoter can generate functional T_{reg}-like cells from conventional $CD4^+$ T cells(86). However, it is possible that a T-cell based strategy may not generate adequate numbers of T_{reg}-like cells that persist long-term(87). Modified HSCs with their self-renewing properties may circumvent the potential problems of a T-cell transfer approach. A California-based group recently demonstrated lineage-specific FoxP3 expression and abrogation of autoimmunity following adoptive transfer of LV-transduced HSCs in a mouse model of IPEX syndrome. They found that ubiquitous FoxP3 expression was detrimental to HSC proliferation and differentiation, with marked defects in peripheral mature cells when a LV vector containing the FoxP3 cDNA used the MNDU3 promoter. An alternative LV vector was designed incorporating the endogenous FoxP3 promoter and including some of the FoxP3 regulatory regions. HSCs transduced with this alternative LV vector were able to confer lineage-specific gene expression in the progeny of the transduced HSCs(88). GT approaches for IPEX syndrome have not entered clinical trials at the time of writing although this is expected in the near future. The development of this preclinical proof-of-concept GT approach for IPEX syndrome highlights how the choice of promoter can influence the success of the strategy.

Familial hemophagocytic lymphohistiocytosis (FHL)

In PIDs where the defect is confined to lymphoid cells, there is the potential for employing a strategy where gene-modified T cells alone are transferred as opposed to HSCs. The rapidly advancing field of adoptive cellular therapies has demonstrated that genetically modified autologous T cells can persist and proliferate *in vivo*(89, 90). A T-cell approach is particularly attractive when the transgene is not expressed at the progenitor level such as the perforin 1 (*PRF1*) gene. Mutations in *PRF1* account for the majority of cases of familial hemophagocytic lymphohistiocytosis (FHL)(91). In a murine model of FHL (*Prf* $^{-/-}$), $CD8^+$ T cells transduced with a γRV vector incorporating the Perforin cDNA, successfully engrafted and demonstrated restored cytotoxic function(91). Clinical trials are needed in order to assess whether T cell GT could be an effective long-term treatment or whether this should be used as a bridging therapy to more definitive treatment such as alloHSCT or HSC GT. Indeed, the potential of HSC GT for perforin defects has also been demonstrated in a pre-clinical setting. LV vectors

expressing the human perforin gene under the influence of a phosphoglycerate kinase (PGK) promoter resulted in perforin expression in mature T and natural killer (NK) cells following gene transfer to murine progenitor cells and adoptive transfer experiments(92). Perforin GT has not entered clinical trials at the time of writing although trials are in the planning stages. Gene therapy approaches are also being developed for the other major cause of FHL, Munc 13-4 defects(92).

X-linked lymphoproliferative disease 1 (XLP1)

X-linked lymphoproliferative disease 1 (XLP1), is another PID for which a T-cell GT strategy may be effective. Mutations in the *SH2D1A* gene result in defects in the SLAM-associated protein (SAP), an intracellular adaptor protein, important for normal T-cell and NK-mediated cytotoxicity(93). AlloHSCT remains the only curative therapy for XLP1 and outcomes are influenced by the presence or absence of active disease at the time of transplantation(94). As patients with active disease at transplantation have inferior outcomes, GT may be particularly beneficial for this group. Promising pre-clinical work has demonstrated that T cells transduced with an LV vector incorporating *SAP* cDNA were able to engraft in *SAP*-deficient mice. T-cells from patients affected by XLP1 demonstrated improved cytotoxicity following transduction with this LV vector(95). These results suggest that autologous T-cell GT may present an alternative therapeutic option for patients with XLP1 and a clinical trial is being planned.

Gene Editing

Whilst SIN-γRV and SIN-LV HSC GT approaches are either 'in clinic' or in the advanced stages of development for a number of disorders, many challenges to this approach remain. Whilst there have been no instances of insertional mutagenesis with SIN vectors, a theoretical risk of genotoxicity from semi-random integration of the transgene after gene transfer remains(8, 9). SIN-γRV and SIN-LV HSC GT approaches have been proven to be highly successful in some PIDs such as ADA-SCID where ubiquitous protein expression is needed, and overexpression of the protein does not have adverse effects(50). However, even in diseases where LV-mediated gene addition has been successful such as WAS and variants of CGD, the extent of gene transfer and engraftment of the genetically modified HSCs can vary between patients and trials(10). For other PIDs, gene addition strategies are unlikely to be successful.

Diseases resulting from defects in genes which require stringent control of expression such as those involved in cell activation or intracellular signalling may be less likely to benefit from traditional gene addition and indeed such strategies may carry significant risks. PIDs which fit this description include X-linked agammaglobulinaemia (XLA) caused by mutations in Bruton Tyrosine Kinase (*BTK*) and X-linked hyper IgM syndrome, caused by mutations in CD40 ligand(96). In recent years, gene editing technologies have made it possible to repair genetic defects, insert additional genetic material or remove deleterious sequences with relative ease. For the GT field this technology offers great potential as for the first time, a gene can be repaired or altered in its native site retaining endogenous regulatory elements(96, 97).

Gene editing has been made possible by the development of designer DNA endonucleases that can introduce a double-stranded DNA break at a specific target sequence. Transcription activator-like effector nuclease (TALENs), zinc-finger nucleases and meganucleases can all

perform a similar function, however in 2012 the development of the CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats associated with Cas9 endonuclease) RNA-based system has spurred the development of gene editing strategies(98). The CRISPR-Cas9 system enables the same high specificity of sequence targeting of the other endonuclease systems yet is much easier to use due to the guidance of the Cas9 endonuclease to its target site being governed by Watson-Crick base pairing(97, 99). Left to its own devices, DNA preferentially repairs through error-prone non-homologous end-joining (NHEJ). However in 1994 it was demonstrated that the introduction of a 'donor' DNA template could result in homology-directed repair (HDR) [Fig. 2](100). The 'donor' DNA template can be single- or double-stranded DNA with homology arms which extend either side of double-strand DNA break. Adeno-associated virus serotype 6 (AAV6) vectors have been proven to be a highly-efficient platform to introduce a 'donor' template to T-lymphocytes and HSCs and high levels of HDR have been observed using a combination of CRISPR-Cas9 (or TALENS) and AAV6 vectors(101-103). AAV6 vectors have been developed without an integrative capacity by removing *rep* and *cap* from the viral genome(104). A gene cassette up to 4.8 kilobases long can be cloned into the AAV6 genome between the inverted terminal repeats (ITRs) at either end of the single-stranded DNA (ssDNA) genome. When cells are transduced with an AAV6 vector at the same time as a double-strand DNA break is made, the AAV6 vector enters the cell nucleus and provides the repair template that enables HDR to occur. The AAV6 DNA is lost when the cell divides as the episomal DNA is not replicated in the process of cell division(102, 105-107). The genetic edit which resulted from the induced HDR however is passed on to the progeny of the cell resulting in permanent modification of the genome.

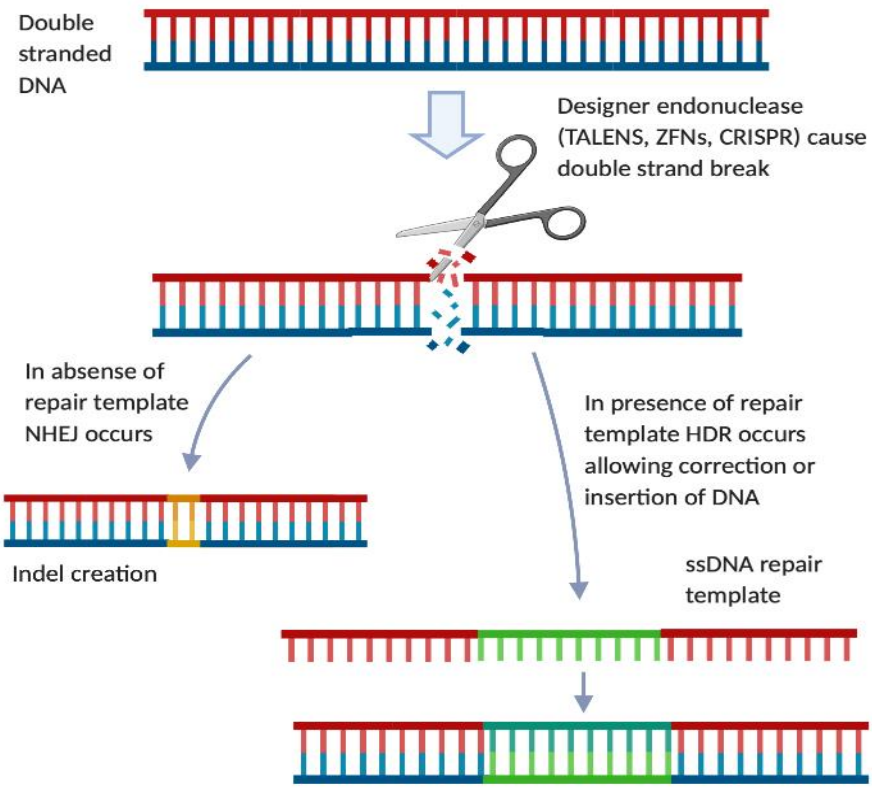

Figure 2. Schematic representation of DNA repair mechanisms utilised in gene editing

Gene editing techniques have entered clinical trials in humans. The first study modified the CCR5 co-receptor for HIV using ZFNs in CD4+ T cells(108). At the time of writing, there are 17 clinical trials of gene editing based therapeutic interventions worldwide listed on international clinical trials registries. These include gene editing of the CCR5 co-receptor in HSCs, gene editing of immune checkpoints such as PD1 in T cells to treat malignancies, and gene editing for the treatment of the hemoglobinopathies; sickle cell anaemia and β-thalassaemia major. Whilst there are no gene editing based strategies for PID in clinical trials at the present time, several promising pre-clinical studies have been conducted. Proof-of-concept has been demonstrated for many PIDs including ADA-SCID, X-SCID, X-CGD, IPEX syndrome, WAS and CD40ligand deficiency(101, 103, 109-112).

There are several different gene editing approaches that can be utilised depending on the mutational landscape of the disease being treated. The first, *direct repair of the disease-causing mutation*, is appropriate when a single mutation is responsible for the majority of clinical cases. An example of this in PIDs is the direct repair of the *CYBB* 676 locus in $CD34^+$ cells for the treatment of X-CGD. This proof-of-concept study confirmed repair in >20% of HSCs, which was sufficient to restore NADPH oxidase function in myeloid cells(111) The second gene editing approach is to *insert a cDNA cassette into the endogenous locus of a particular gene*. Examples in PIDs are SCIDX1, hyper IgM syndrome, X-linked agammaglobulinaemia (XLA) and WAS (101, 103, 113, 114). The preclinical studies in hyper IgM syndrome and XLA are particularly noteworthy as these are two PIDs for which a gene addition strategy is unlikely to be successful due to concerns about regulation of the transgene. Expression of CD40L (in hyper IgM syndrome) has been achieved with gene addition strategies but unregulated expression resulted in lymphoproliferation(115). Similarly gene addition strategies have been successful in preclinical studies for XLA, but as BTK confers a significant selective advantage to the expressing cells, gene editing is likely to be a safer option as the endogenous regulatory machinery can remain intact(116, 117). Similarly, heterozygous mutations in immune regulatory proteins such as CTLA4 or where GOF mutations result in PID such as mutations in APDS1, gene editing may be able to achieve physiological correction in a way that wouldn't be possible using viral gene addition.

Whilst gene editing offers many exciting therapeutic possibilities, safety concerns have yet to be fully addressed. Double-strand breaks are a potential source of genomic instability which may increase risk of oncogenic mutations and translocations(118). Off-target gene edits are also a concern. Careful gRNA design and *in silico* simulations are a starting point for reducing this risk(97). Improvements in editing specificity have been made to help mitigate off-target editing, for example, by reducing the exposure of DNA to the nuclease by delivering Cas9-gRNA as a ribonucleoprotein complex(119). Several assays also exist to assess genome wide off-target edits in preclinical validation experiments(120, 121). Whilst genome wide screening is useful for detecting off target effects of gene editing, the clinical relevance of any mutations is difficult to predict(122, 123). Whilst *in vivo* safety has been demonstrated using adoptive transfer experiments in murine models, they may not predict results in humans. Careful analysis of the gene editing therapeutics currently in phase I clinical trials will be required to ascertain any potential toxic effects in humans.

Future developments, challenges and conclusions

The exciting field of gene therapy has a bright future. A new class of autologous curative therapies will hopefully be available for patients with a variety of monogenic PIDs in the near future. Gene editing has the potential to offer autologous HSC based therapies for a wider spectrum of PIDs and refine existing GT approaches. However, despite the exciting developments, several challenges need to be overcome before HSC GT becomes the standard of care for the management of PID.

The advantages of GT over alloHSCT need to be assessed against contemporary transplant practice which itself has benefitted from major advances. Advances in haploidentical transplantation have reduced issues with donor availability, whilst *ex vivo* graft manipulation, improved GVHD prophylaxis and better prophylaxis and treatment of infections have all reduced the morbidity and mortality associated with allogeneic transplant(124). The benefits of GT need to be assessed against the increased costs compared to alloHSCT in order to make this a feasible option for healthcare providers and the patients that use them. Whilst there are increasing numbers of patients with long-term follow up post-GT, longer term toxicities can only be assessed with time and as larger numbers of patients are treated. The genotoxic effects of gene editing in humans is currently unknown. Clinical trials of gene edited products are underway, thus initial safety analyses will hopefully reveal in the near future whether this novel group of therapeutics is truly feasible and safe in humans. Longer term follow-up will be needed to assess the persistence of gene-edited cells and the durability of any beneficial effects.

Conditioning regimens for GT (and alloHSCT) currently use chemo, radio and/or serotherapy. Whilst reduced intensity regimens have reduced overall toxicity without compromising engraftment, adverse late effects as a result of conditioning therapy do occur. The ability to avoid these agents would be a significant therapeutic advance and likely broaden the appeal and application of GT procedures. Antibody-based conditioning agents that deplete HSCs are being developed. An approach using a hematopoietic-cell-specific immunotoxin, saporin (SAP) conjugated to an antibody targeting CD45 can result in >90% engraftment of donor cells after a single dose in an in vivo model using immunocompetent mice(125). Another approach targets c-kit (CD117), a dimeric transmembrane receptor tyrosine kinase expressed by HSCs. It has been shown that anti-mouse c-kit monoclonal antibodies can deplete HSCs and permit engraftment of exogenous HSCs(126-128). CD45 is present on lymphocytes in addition to HSCs so using a combined CD45-c-kit targeted approach would result in lymphodepletion in a similar way to alkylating agents which is undesirable. In order to circumvent this, c-kit (CD117) antibody drug conjugates have been developed e.g. streptavidin-saporin-anti-CD117 and similarly to the CD45 targeting approaches this agent leads to >99% depletion of host-HSCs without causing clinically significant side effects(127, 129). Phase I trials of non-genotoxic conditioning agents are now in progress in the context of alloHSCT for SCID, and early results are promising with evidence of sustained engraftment of multipotent HSCs, however, it remains to be seen if these agents will permit stem cell engraftment in a non-SCID setting(130). Should these new agents prove to be successful in the non-SCID *adult* setting, the ability to perform autologous GT procedures without alkylating agents or irradiation will increase the advantages and applicability of GT strategies whilst reducing the risks further.

Historically, GT procedures involved infusion of a fresh product meaning that patients had to travel to a site where the *ex vivo* manipulation, conditioning, infusion and recovery had to take place. The ability to deliver a cryopreserved product manufactured centrally will improve the availability of GT and may reduce the cost of the therapy (131). Limited capacity to manufacture cellular therapy and GT products is an issue as the technology is more widely adopted. However, as the results of clinical trials in gene and cell therapy have demonstrated the utility of these products for the treatment of a variety of disorders, interest and development of manufacturing capabilities has increased. Large scale, serum-free, good manufacturing practice (GMP) compliant automated systems are now available for virus manufacture. These platforms reduce the use of animal-derived products and the handling required thus, lowering the risk of contamination (132, 133). The development of culture media which maintains HSC potency as well as the availability of transduction enhancers which improve the efficiency of gene transfer will also help lower the cost of manufacture as less virus per product will be required (134-136). Together these developments will help lower the cost and improve the availability of GT products.

In conclusion, whilst significant challenges remain, there is undoubtedly the potential that GT may become the standard of care for many PIDs in the not-to-distant future. Sustained international collaborative efforts between scientists, clinicians and industry will be required in order to make these treatments available to patients affected by these rare but devastating diseases. We look forward to the advances in the years to come and hope that even as you read this chapter, we are a few steps closer to making GT an effective therapy for patients affected by cellular PIDs.

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