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Authors

Kohn, Donald B
Kuo, Caroline Y

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New Frontiers in the Therapy of Primary Immunodeficiency: From Gene Addition to Gene Editing

Donald B. Kohn[1,2,3,4] and **Caroline Y. Kuo**[1]

[1]Department of Pediatrics, David Geffen School of Medicine, University of California, Los Angeles

[2]Department of Microbiology, Immunology & Molecular Genetics, David Geffen School of Medicine, University of California, Los Angeles

[3]Eli & Edythe Broad Center of Stem Cell Research & Regenerative Medicine, David Geffen School of Medicine, University of California, Los Angeles

[4]Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, University of California, Los Angeles

Abstract

The most severe Primary Immune Deficiencies (PID) have been successfully treated with allogeneic hematopoietic stem cell transplantation for more than four decades. However, such transplants have the best outcomes when there is a well matched donor available, as immune complications such as graft versus host disease are higher without a matched sibling donor. Gene therapy has been developed as a method to perform autologous transplants of a patient's own stem cells that are genetically corrected. Through an iterative bench-to-bedside-and-back process, methods to efficiently add new copies of the relevant gene to hematopoietic stem cells have led to safe and effective treatments for several PID, including forms of Severe Combined Immune Deficiency, Wiskott-Aldrich Syndrome, and Chronic Granulomatous Disease. New methods for gene editing may allow additional PID to be treated by gene therapy, as they will allow the endogenous gene to be repaired and expressed under its native regulatory elements, essential for genes involved in cell processes of signaling, activation and proliferation. Gene therapy is providing exciting new treatment options for patients with PID and advances are sure to continue.

Keywords

Hematopoietic stem cell transplantation; Gammaretroviral vector; Lentiviral vector; Gene Editing; Site-specific endonuclease; Zinc Finger Nuclease; CRISPR/Cas9

Gene therapy has developed from an attractive, but unrealized concept to a first licensed medicine over the past few decades. Much of the work on gene therapy for primary immune deficiency diseases (PID) has been based around the application of hematopoietic stem cell transplantation (HSCT) as a potentially lifelong, curative therapy. Since HSCT using bone marrow transplant (BMT) was first successfully performed for PID patients with Severe

Corresponding author: Donald B. Kohn, M.D., 3165 Terasaki Life Science Bldg., 610 Charles E. Young Drive East, Los Angeles CA 90095, P- 310-794-1964, F- 310-206-0356, dkohn1@mednet.ucla.edu.

Combined Immune Deficiency (SCID) in the late 1960's[1], the power of replacing a patient's HSC bearing a disease causing mutation that disables the immune system with HSC from a healthy donor has advanced greatly. HSCT has been successfully applied to many of the most severe PID, including SCID, Wiskott-Aldrich Syndrome (WAS), Chronic Granulomatous Disease (CGD), X-linked hyper IgM (X-HIM), Leukocyte Adhesion Deficiency (LAD) and others. The success of HSCT has relied on identification of a well-matched stem cell donor, ideally a human leukocyte antigen (HLA)-matched sibling or family member donor. For SCID, such matched sources of HSC may provide a graft that is accepted without conditioning to eliminate the patient's endogenous stem cells or immune system. It was learned that for patients with less severe cellular-mediated immune defects (e.g. WAS, CGD), it was necessary to apply some regimen of cytoreductive conditioning to "make space" for donor HSC to engraft and often immune suppressive drugs to prevent residual immunity from rejecting the donor's HSC.[2] Increased pre-treatment conditioning increases risks of allogeneic HSCT due to direct toxic effects of the conditioning agents (often cytotoxic chemotherapy drugs), immune suppression in patients with pre-existing immune defects, and potential burdens of chronic infections.

Because the majority of PID patients will not have an eligible HLA matched family donor, techniques were developed to use HSC from less well-matched donors, either unrelated donors identified through increasingly large registries or from half-matched parental donors. These mismatched transplants carry higher risks of either graft rejection or the converse, graft versus host disease, in which donor T cells attack the patient's body. Therefore, manipulation of the donor graft, such as T cell depletion, or more potent immune suppression may be needed peritransplant, with attendant morbidity. As experience with these more challenging transplants has increased, the outcomes have steadily improved.

Nevertheless, alternative approaches that may be safer with the same prospect of benefit are needed. The key concept of gene therapy for PID has been to provide an autologous HSCT option, using the patient's own HSC that are corrected *ex vivo* by either adding a normal copy of the responsible disease-related gene or, in the near future, correcting the endogenous defective gene in situ in the HSC (Figure 1). The key technical challenge has been to effectively and non-toxically introduce the normal gene into the stem cells, without causing them to lose their stem cell capacity for life-long multi-lineage blood cell production. Over 2-3 decades, such techniques have been developed and used in clinical trials for a growing number of diseases (Table 1).

Technical advances that are enabling effective gene therapy of PID

The successes in gene therapy have been due to three sets of advances: better vectors, better HSC processing methods and better conditioning regimens. In the mid-1980's murine retroviruses were developed that could transfer a foreign gene into mammalian cells (Figure 2).[3] Several studies demonstrated their ability to introduce their gene into murine HSC, which could be transplanted and give rise to blood cells of both myeloid and lymphoid lineages containing and expressing the new gene.[4,5] Initial attempts to use these vectors with human HSC were much less effective, but improvements in vector production methods to yield higher vector titers and changes in the viral envelope protein used to coat the virus and

target it to cellular receptors led to higher levels of gene transfer to human HSC. Simultaneously, new hematopoietic growth factors were identified that could stimulate activation and proliferation of human HSC which greatly increased their ability to be take up the vectors.[6] Additionally, it was learned that having an extracellular matrix protein, such as fibronectin or recombinant fragments thereof, could increase the uptake of virus by the stem cells and also better preserve their activity during the 2-4 days of *ex vivo* culture.[7] Finally, the use of relatively low doses of the myelotoxic drug busulfan was shown to greatly enhance re-engraftment of the ex vivo-modified HSC by creating space in the bone marrow niche.[8,9] Bringing these advances together in the late 1990's, several investigators were able to provide significant benefit to patients with ADA-SCID and XSCID using gene therapy with patient autologous bone marrow HSC.[8–19] Similar studies for WAS and XCGD followed and also led to restoration of the defective immune cell function.[20–23]

However, severe complications occurred in many of these patients, with leukemia developing a few years after gene therapy.[24–29] The retroviral vectors that were used randomly inserted into the chromosomal DNA of the target stem cells, and the potent enhancer elements they carried to drive expression of the therapeutic gene could trans-activate expression of nearby cellular proto-oncogenes (*LMO2, MECOM*). This eventually led to clonal outgrowth of transformed cells. While most of the patients were successfully treated for the leukemia, this was an unacceptable level of toxicity.

Fortunately, the remedy was already in hand. A new generation of gene delivery vectors, derived from the lentivirus class of retroviruses, had been developed that had markedly better safety profiles. Lentiviral vectors (LV) could be produced in a way in which the enhancer elements "self-inactivated" (so-called "SIN" vectors) so they had minimal, if any, ability to turn on cellular genes near their integration sites (Figure 2).[30,31] Pre-clinical studies in murine cells showed SIN LV to have essentially no detectable transforming activity, whereas that of murine gammaretroviral vectors was readily demonstrable.[32–36] Thus, most trials are currently using SIN LV and ongoing trials for ADA-SCID, XSCID, WAS and XCGD have shown effective immune reconstitution and no vector related complications to date, with more than 50 patients treated.[37–40] Additionally, one study used a SIN gammaretroviral vector to treat XSCID with no complications of clonal expansions and good immune reconstitution in the majority of patients.[41]

One exception to the complications with murine gammaretroviral vectors has been in the setting of ADA SCID. More than 40 ADA SCID patients have been safely treated with no leukemia-like complications using gammaretroviral vectors since the landmark studies from The San Raffaele Telethon Institute for Gene Therapy (TIGET), Milan, Italy began in the late 1990's, followed by studies at University College London/Great Ormond Street Hospital and at the University of California, Los Angeles.[9,11,13,14] The work from TIGET led to the approval by the European Medicines Agency of their gammaretroviral vector gene therapy for ADA SCID, marketed by GSK as Strimvelis. It is only the second gene therapy product to receive licensure in the European Union (with none in the US or Canada to date). Why the gammaretroviral vectors have not had clinical safety problems in contrast to similar vectors for other PID remains unknown. Both the TIGET and UCLA groups have demonstrated

vector integrations near some of the same genes implicated in leukoproliferation in other diseases, but there have been no clinical manifestations thus far.[42]

It should be emphasized that in all of these gene therapy trials, there has been no need to use immune suppressive drugs (e.g. cyclophosphamide, fludarabine, ATG, cyclosporine, corticosteroids) and there have been no problems with graft rejection or graft versus host disease. The amounts of pre-transplant conditioning used for the non-SCID disorders (WAS and CGD) were higher (8-12 mg/kg busulfan) than used for the ADA SCID (4 mg/kg) and XSCID (0-8 mg/kg), because of the perceived need for greater cytoablation for higher engraftment of the corrected stem cells to correct all blood cell lineages and not just lymphocytes as is needed for the SCID patients. While the dosages of these potent drugs are significant and have some risk, the results to date have been gratifyingly good, with only the expected marrow suppression and some mucositis and no severe transplant-related complications.

Future approaches and prospects for gene therapy of PID

One of the drawbacks of gene therapy is that each disease genotype entails a separate drug development project to develop the vector for that specific gene, perform pre-clinical activity and toxicity studies, etc. In contrast, in allogeneic HSCT, the donor cells have all of the corrective genes and it is more of a “one size fits all” approach. Nevertheless, there are ongoing projects at many centers to develop gene therapy for additional PIDs. Other forms of SCID, such as Artemis, Rag1 and Rag2 deficiency, autosomal recessive forms of CGD (p47, p67) and other PID (LAD, HLH) are under pre-clinical study with the goal of advancing to early phase clinical trials. Thus, these gene therapy approaches of gene addition will compete with the always improving methods of allogeneic HSCT to provide the best treatments for many of these diseases.

However, for other PID, gene addition therapies are not currently applicable. CVID, perhaps the most prevalent PID, may likely be caused by one of several genes, only a few of which are currently recognized. Each will again entail a separate development process and the efficacy and safety will need to be high enough to outweigh the results obtained currently with best medical therapy. Gain-of-function mutations, such as in *STAT1* and *STAT3*, where the mutant allele may override proper expression of a new normal copy of the gene, poses additional challenges to traditional forms of gene therapy.

Additionally, genes that underlie some PID may not be amenable to the approach of gene addition using a viral vector. Genes that are involved in cell activation, intracellular signaling and other processes likely require precise temporal and physiologic control of their expression. For example, after the CD40 ligand gene was identified as a cause of X-HIM, studies were done to add that gene to bone marrow HSC from a gene knock-out mouse model.[43] While initial immune restoration was achieved with class switching of B cells, many of the mice developed lymphoproliferative complications from constitutive, unregulated expression of CD40 ligand. Other genes involved in PID that may similarly require precise regulation are *BTK*, involved in X-linked agammaglobulinemia, *FoxP3*, causative of Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome

(IPEX), as well as some of the B cell growth factors or their receptors that may be involved in some cases of CVID (e.g. *TACI, BAFF-R*). Efforts to derive vectors that display the precise gene regulation pattern of the endogenous gene have often been unsuccessful, as it may be difficult to include in the relative small genome size of a vector all of the DNA regulatory sequences that may surround and regulate these genes.

Gene editing to correct the genes causing PID

Once again, the answer is close at hand. New methods have been developed in the past few years for gene correction. If a disease-related gene could be repaired in its native chromosomal site, it should retain the normal expression pattern and restore the needed gene function. A real revolution of scientific advances has developed methods for gene editing that can either disrupt and turn-off a troublesome gene (e.g. dominant *STAT2*), repair a gene mutation (e.g. in *BTK*) or insert a new copy of the gene directly into the defective gene itself to use the gene expression regulatory elements to control the expression of the inserted gene (e.g. CD154). The critical component of this revolution in gene editing has been the recognition that such gene editing events can be greatly enhanced by introducing a precisely located break in the chromosomal DNA near the site of intended gene editing.[44] A succession of designer site-specific endonucleases have been developed, including homing endonucleases, zinc finger nucleases (ZFN), TALENs and more importantly CRISPR/Cas9. Each of these systems can be engineered to recognize and introduce a DNA break at essentially one site out of the entire genome. If the DNA break is left to be resolved by the cell, it is often reannealed through a process called non-homologous end-joining (NHEJ) that often removes or adds a few base pairs at the break site and lead to disruption of the gene's reading frame and gene silencing. However, if the cell is provided at the same time with copies of the target sequence as single stranded or double stranded DNA carrying the corrective DNA sequence, it may use the introduced information to repair the break by Homologous Directed Repair (HDR), "knocking-in" the corrective sequence and repairing the pathogenic mutation (Figure 3). This process of HDR may even be used to introduce new gene cassettes at a precise target site, such as a full length normal copy of the defective gene just downstream from the gene promoter sequences to maintain the gene's normal expression patterns.

These methods for gene editing have entered the clinic, with the first study using ZFN to disrupt the HIV CCR5 co-receptor in peripheral blood T cells from patients infected by HIV, rendering the cells relatively resistant to HIV infection.[45] Other studies nearing clinical trials will seek to disrupt the *CCR5* gene in HSC of HIV infected patients or disrupt the gene encoding a protein (*Bcl11a*) that represses expression of fetal globin to turn on the fetal hemoglobin as a way to treat beta-thalassemia and Sickle Cell Disease.

Gene correction in HSC for PID has been demonstrated in pre-clinical studies for XSCID, ADA SCID, XCGD, X-HIM and others.[46–48] The methods are reaching levels of efficacy and non-toxicity to HSC to lead to clinical efficacy (Figure 4). The gene editing approaches may allow a much wider range of PID to be treated by gene therapy and autologous transplant, including XLA, XHIM, IPEX, CVID and others.

In addition to all of the cell based PID discussed above, there are other PID that are due to defects in blood proteins, such as Hereditary Angioedema (HAE). A recent report described studies in a mouse model of HAE where a different viral vector – adeno-associated virus (AAV) – was used to introduced a normal *SERPING1* gene encoding the C1 inhibitor into cells of the liver that could then serve as a factory to produce the protein and release it into the bloodstream.[49] This approach of a protein factory has also been shown to be effective for ADA SCID. In the mouse model, a LV carrying a normal human ADA gene was injected intravenously, targeted the liver, and served as a source of ADA sufficient to sustain survival and correct immunity. Similar approaches are under active study for non-PID protein disorders such as hemophilia, anti-trypsin inhibitor deficiency and others.

Remaining challenges for gene therapy for PID

Of course, there are challenges that remain to be fully overcome. Production of the gene addition vectors or gene editing reagents has been at relatively small scale for pilot studies and need to be advanced to pharmaceutical production methods at sufficient scale to treat many patients. Both gene addition with SIN LV as well as gene editing with the various endonucleases may still have some risks of genotoxicity if they disrupt important genes by either insertion or off-target cutting.

The conditioning regimens used to date have been based on chemotherapy regimens. While these have not shown significant toxicity in the short-term, they may have some late effects, even at the reduced dosages used. Once again, progress has been made in this essential area, by the development of methods to “make space” for transplanted gene corrected HSC using monoclonal antibodies to proteins on HSC, such as ckit or CD45.[50–52] If effective, these new conditioning agents could greatly increase safety and allow applications of gene therapy to the somewhat milder PID such as XLA.

While current approaches of either gene addition or gene editing are done *ex vivo* with harvested bone marrow or mobilized stem cells, it may be advantageous, when possible, to deliver the needed gene or gene editing reagents *in vivo* and repair the HSC while remaining in their niches. And eventually, the use of pluripotent stem cells, such as induced pluripotent stem cells (iPSC), may allow autologous cell grafts to be produced, gene corrected, expanded and used for transplant with a much more abundant source of stem cells providing better immune reconstitution.[53]

It is an exciting time for science and a good time for patients as these new treatments using gene therapy may provide safer and more effective approaches that avoid the complications of allogeneic HSCT. Of course, it will take several years of comparative analyses to determine which approaches are best, but the existence of these various treatments for diseases that were once often fatal is indeed gratifying.

Abbreviations

AAV	Adeno-associated virus
BMT	Bone Marrow Transplant

CGD	Chronic Granulomatous Disease
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CVID	Common Variable Immune Deficiency
HAE	Hereditary angioedema
HLA	Human Leukocyte Antigen
HDR	Homology-directed repair
HLH	Hereditary lymphohistiocytosis
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplantation
IDLV	Integration-Defective Lentiviral Vector
IPEX	Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome
iPSC	Induced pluripotent stem cell
LAD	Leukocyte Adhesion Deficiency
LV	Lentiviral Vector
NHEJ	Non-homologous end-joining
PID	Primary Immune Deficiency
SCID	Severe Combined Immune Deficiency
SIN	Self-inactivating
TALEN	Transcription activator-like effector nucleases
TIGET	The San Raffaele Telethon Institute for Gene Therapy
WAS	Wiskott-Aldrich Syndrome
XLA	X-linked agammaglobulinemia
X-HIM	X-linked Hyper-IgM Syndrome
ZFN	Zinc Finger Nuclease

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Key Concepts

- Severe PID can be treated by transplantation of hematopoietic stem cells from a healthy matched donor, but risks of immunologic complications exist and are heightened when the transplant is from an unrelated donor or haplo-identical donor.
- Gene therapy for PID allows an autologous transplant in which the patient's own hematopoietic stem cells are corrected and transplanted, essentially eliminating the immunologic complications.
- Hematopoietic stem cells can be corrected by addition of a normal copy of the relevant gene using an integrating retroviral or lentiviral vector.
- Initial studies with retroviral vectors that showed clinical efficacy, but some had leukoproliferative complications from the vectors (this has not been seen for ADA SCID).
- Current trials using safer lentiviral vectors lacking strong enhancers have shown efficacy for ADA SCID, XSCID, WAS and CGD with no vector-related complications.
- Recently, methods have been developed for gene editing that could allow the disease-causing mutation to be corrected *in situ* within a patient's hematopoietic stem cells. This approach may be of particular value for disorders where the relevant PID-causing gene requires precise regulation of expression, such as CD40 ligand for X-HIM, BTK for XLA and FoxP3 for IPEX.

Therapeutic Implications

Gene therapy provides a new therapeutic option for treatment of patients with severe PID that respond to allogeneic hematopoietic stem cell transplantation.

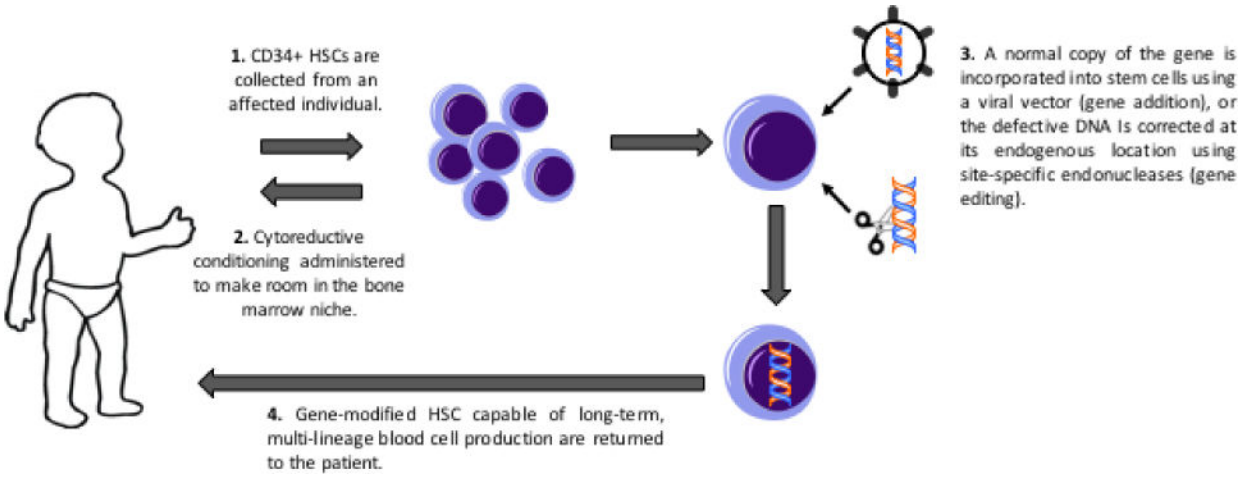

Figure 1.
Clinical schema of gene therapy for PID.

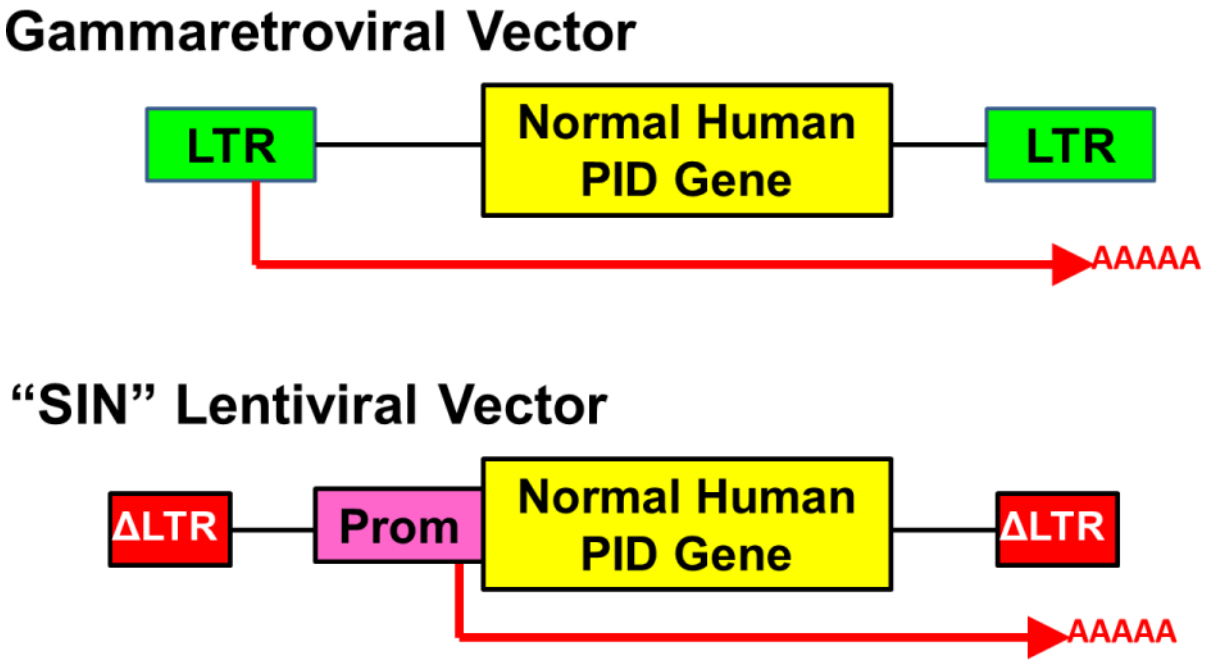

Figure 2. Gene delivery vectors used for gene therapy of PID

Upper: Gammaretroviral vectors have viral long terminal repeats (LTR) at each end with viral enhancers and promoters that drive transcription and termination/polyadenylation of a normal copy of the relevant human gene involved in the inherited PID. The messenger RNA produced from the vector is shown as a red arrow including the polyA tail. **Lower:** Self-inactivating ("SIN") lentiviral vector has LTR with the enhancers deleted (Δ) and an internal promoter (Prom) to drive transcription of the human PID gene. In current clinical trials, lentiviral vectors being used for ADA SCID and XSCID are using the human Elongation Factor Alpha-1 gene short promoter (EFS) to drive the ADA and IL2Rg genes, respectively, the WASP gene endogenous promoter to drive a WASP gene, and a chimeric myeloid promoter to drive the gp91phox gene for XCGD.

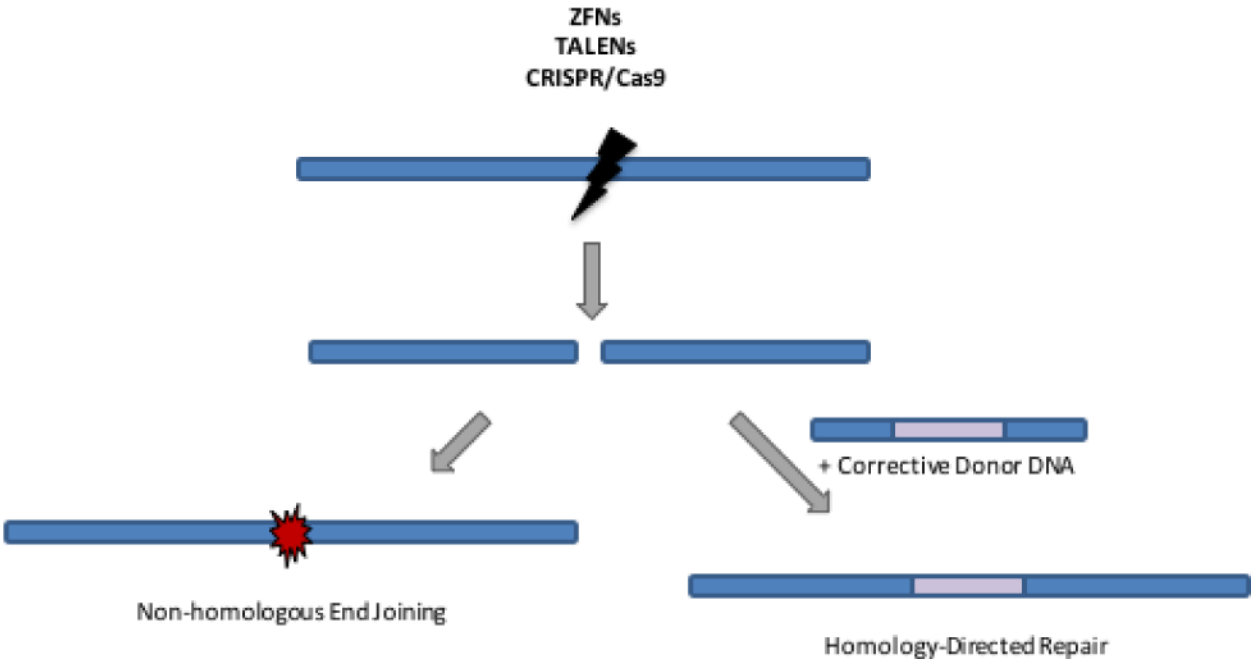

Figure 3. Gene editing using site-specific endonucleases

Site-specific endonucleases (ZFNs, TALENs, or CRISPR/Cas9) can be used to create targeted double stranded DNA breaks. These DNA breaks can be repaired by non-homologous end joining, which introduces small insertions and deletions and can be used for gene knockout strategies, as is employed in the CCR5 gene therapy trials for HIV. If a corrective donor DNA sequence is also provided to the cell, it can be used for homology-directed repair of the double-stranded DNA break, incorporating the therapeutic gene sequence and maintaining its expression under its endogenous regulatory elements.

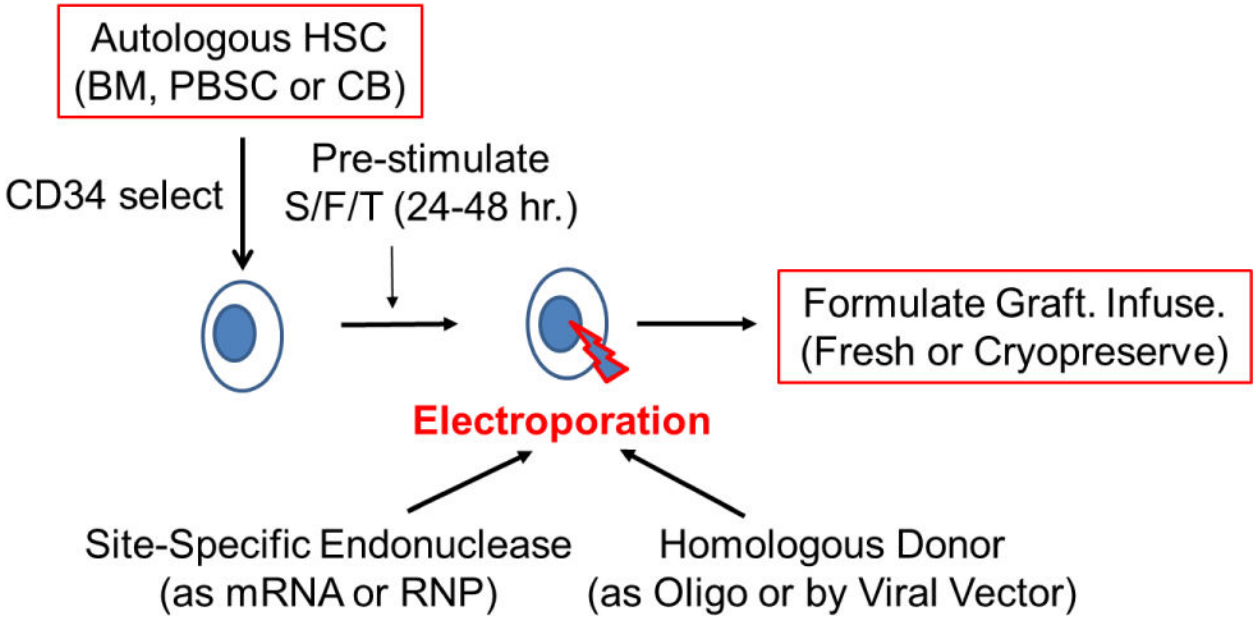

Figure 4. Site-specific Gene Editing of Autologous Hematopoietic Stem Cells for Gene Therapy of PID

Autologous hematopoietic stem cells (HSC) can be obtained from bone marrow (BM), mobilized peripheral blood stem cells (PBSC) or umbilical cord blood (CB). Usually the HSC are enriched using CD34 immunoselection. The HSC are pre-stimulated to improve gene modification by culture for 24-48 hours with a combination of recombinant hematopoietic growth factors, such as ckit ligand/flt-3 ligand and thrombopoietin (S/F/T). The cells are then treated with electroporation with the site-specific endonuclease (zinc finger nuclease, homing endonuclease, TALEN or CRISPR), to introduce a double stranded break at the target gene, delivered as either *in vitro* transcribed messenger RNA (mRNA) or pre-formed ribonucleoprotein (RNP) containing the Cas9 protein and a short-guide RNA for CRISPR-mediated processes. The homologous donor containing the corrective sequences may be provided either as an oligonucleotide (Oligo) that is co-electroporated with the endonuclease or via a viral vector (e.g. adeno-associated virus {AAV} or integrase defective lentivirus {IDLV}). Following electroporation, the gene-modified HSC are formulated for intravenous administration and may either be infused into the patient fresh or after cryopreservation and thawing.

Table 1

Diseases treated by gene therapy using hematopoietic stem cells in clinical trials.

Primary Immune Deficiencies
Adenosine deaminase (ADA)-deficient SCID
X-linked SCID (XSCID)
Wiskott-Aldrich Syndrome (WAS)
X-linked Chronic Granulomatous Disease (XCGD)
Leukocyte Adhesion Deficiency (LAD)
Lysosomal Storage and Metabolic Disorders
X-linked Adrenoleukodystrophy (XALD)
Metachromatic Leukodystrophy (MLD)
Hemoglobinopathies
Beta-thalassemia
Sickle Cell Disease
Stem Cell Defects
Fanconi’s anemia