Safer Vectors for Gene Therapy of Primary Immunodeficiencies

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Abstract: Primary immunodeficiencies (PID) are caused by mutations in genes that impair the development or activity of the immune system. Although bone marrow transplants achieve long time restoration in up to 90% of treated patients, morbidity and mortality are still high for some PID and adequate donors are not always available. Gene Therapy (GT) was envisioned as an alternative treatment for PID by inserting the correct gene into the patient’s haematopoietic stem cells (HSCs). Up to date, GT for PID has succeeded in 40 of 44 patients treated in four clinical trials. However, five children enrolled in the SCID-X1 clinical trial developed leukaemia-like disease produced by aberrant expression of oncogenes. This phenomenon resulted fatal in one patient and represented a severe setback for gene therapy. Since then, vector development has been a priority in the GT field, by refining existing Murine Laeukemia virus (MLV)-based vectors or by developing new ones. This review summarizes existing methodologies for PID GT highlighting the importance of animal models in the PID GT success and focusing on new gene transfer vectors to achieve safe, efficient and stable gene modification.

Keywords: Gene therapy, primary immunodeficiencies, gammaretroviral vectors, lentiviral vectors, animal models, safety, site-directed integration, gene correction, efficiency.

INTRODUCTION

Primary immunodeficiencies (PID) are caused by mutations in genes that affect the development or activity of the immune system [1, 2]. PID includes B- and T-cell defects, phagocytic disorders, and complement deficiencies with the common feature of frequent life-threatening infections. Management of patients with PID relies in intravenous injection of immunoglobulins, bone marrow transplantation (BMT) and antibiotics. Adequate donor selection based on high resolution tissue typing and age allows good survival rates in most PID after BMT [3]. Still, therapeutic alternatives to BMT are needed due to life threatening complications derived from BMT, the risks involved in transplantation and the lack of suitable donors for nearly 50% of patients [3, 4]. These secondary effects included graft versus host disease (GVHD), autoimmune diseases, recurrent infections and/or nutritional support. Gene therapy (GT) was envisioned as an alternative treatment for PID by stable correction of the mutated gene on the patient’s haematopoietic stem cells (HSCs). In order to achieve therapeutic benefit gene transfer vectors must overcome two significant hurdles: poor efficiency, since HSCs are very restrictive to gene modification; and genotoxicity, since the stable gene modification can alter the HSCs’ expression pattern.

Retroviral vectors derived from gammaretrovirus (MLV) have been the selected vectors for PID GT studies and the only one used in clinical trials so far. They are highly efficient, integrative, easy to manipulate, poorly immunogenic and they derive from a non-pathogenic virus. These properties have made MLV-based vectors the only alternative for PID GT for years. However HSCs are quiescent or very slowly dividing cells and MLV-based vectors require active cell division for transduction [5]. Therefore HSCs transduction protocols using MLV-based vectors require cytokine "pre-stimulation" to induce cell proliferation [6], a process that can modify the intrinsic characteristics of the haematopoietic precursors [7]. Another drawback for MLV-based vectors is the high level of transgene silencing via chromatin remodeling and/or methylation [7, 8].

The first successful gene therapy clinical trial used MLV-derived vectors expressing common cytokine-receptor gamma chain (γc) cDNA in HSCs from X-linked severe combined immunodeficiency (SCID-X1) patients [9]. So far, 40 out of 44 PID patients treated with GT have been successfully treated in three clinical trials[10-14]. However, in five children, GT treatment resulted in clonal T-cell proliferation (leukemia-like disease) [12] (Ref. [12B]: Howe SJ, Mansour, MR, Schwarzwaelder, K, et al). Two main conclusions can be drawn from these clinical trials: 1- Gene therapy is a valid alternative strategy for the treatment of PID and 2- Safer and more efficient vectors are needed to improve the outcome of the procedure.

This review has been written to allow a general view of the PID GT problematic, focusing on gene transfer vectors. In addition we have tried to explain the different requirements that gene transfer vector must have based on the PID to be treated. A more detailed update of PID GT clinical data...
and strategies to improve clinical outcome have been revised elsewhere [15].

CURRENT ACHIEVEMENTS OF GENE THERAPY FOR PRIMARY IMMUNODEFICIENCIES

X-Linked Severe Combined Immunodeficiency (SCID-X1)

SCID-X1 syndrome is caused by mutations in the interleukin-2 receptor gamma chain gene (named \(\gamma_c\), IL2RG, or CD132), resulting in disruption of development of T lymphocytes and natural-killer cells, consequently B-lymphocyte function is also intrinsically compromised [16]. SCID-X1 has two characteristics that made this disease an easy target for GT; \(I^c\)-the mutated gene \(\gamma_c\) is ubiquitously expressed in haematopoietic cells, minimizing vector requirement and \(ii\)-the patient’s cells expressing the transgene have a growth advantage over non-expressing cells [17, 18], allowing immune reconstitution with low number of gene-corrected cells. In addition mice model of SCID-X1 [19, 20] have allowed comprehensive preclinical studies of GT strategies. \(\gamma_c\) deficient mice have abnormal T cell development [21, 22], severe reductions in B and NK cells and gut-associated intraepithelial lymphocytes, mimicking many characteristics of patients with SCID-X1.

The successful correction of \(\gamma_c\)-deficient patient’s cells by gene therapy [23, 24] prompted French scientists to assess the potential of GT for the treatment of this disease [9], achieving the first unequivocal success of gene therapy in the two patients treated. The authors transduced bone-marrow isolated CD34 + cells \(ex vivo\) with a MLV-based vector expressing the \(\gamma_c\) cDNA driven by the MLV Long Terminal Repeat (LTR). They harvested HSCs from SCID-X1 patients by positive selection (CD34+) and following pre-activation with SCF, polyethylene glycol-megakaryocyte differentiation factor (PG-MDF), IL-3 and Flt3-L (Fig. 1c; SCID (F)), they were transduced daily for three days. Gene-modified CD34 + cells were infused into the patients without chemotherapy. The continuation of this work enrolled a total of 10 children between 1999 and 2002 into a SCID-X1 GT clinical trial [10, 25]. A second GT trial for the SCID-X1 was initiated also in the United Kingdom by Prof. Adrian Thrasher [11, 26] with a slightly different transduction protocol (Table 1 and Fig. 1c, SCID (E)). To date, 17 out of 20 enrolled SCID-X1 patients have been successfully treated in both clinical trial.

However, 4 patients in the French trial and 1 in the English trial developed leukaemia 3-6 years after treatment. This leukaemia-like disease was a result of vector-mediated up-regulation of host cellular oncogenes (i.e. LMO2) [11, 27]. Several studies in cell lines and animal models have demonstrated that MLV-derived vectors integration favour transcriptionally active genes, CpG islands and transcription start sites (TSSs) [28-30]. In addition, some authors have also suggested that the \(\gamma_c\) gene might have contributed to the establishment or progression of the malignancies, although this is controversial [31-34]. Recently Thrasher and Co-workers have suggested that leukemogenesis was the result of insertional mutagenesis (involving activation of the LMO2 oncogene) combined with the acquisition of genetic abnormalities unrelated to vector insertion, such as the increase activity of NOTCH1 or the deletion of CDKN2A gene [11]. Finally, we must always consider the own patients genetic background to interpret these results. For instance, the poor NK activity of treated patients (even after GT treatment) could also help to cancer development [35]. These clinical trials were of major importance to verify the potential of GT as a real alternative for PID therapy and to envision the requirement of more efficient and safer vectors.

Adenosine Deaminase-Deficiency (ADA) Severe Combined Immunodeficiency (ADA-SCID)

The ADA-SCID immunodeficiency is an autosomal recessive disorder where purine metabolites accumulate in plasma, lymphoid tissues and red blood cells (RBCs) due to mutations in the adenosine-deaminase (ADA) gene [36]. ADA-SCID patients suffer from lymphopenia, absent cellular and humoral immunity, failure to thrive, and recurrent infections. Additionally, skeletal, hepatic, renal, lung, and neurologic abnormalities have been observed in some patients [37, 38]. Like for SCID-X1, bone marrow transplantation is the best therapeutic alternative, but an HLA identical donor is needed and it is associated with a high morbidity and mortality [39, 40]. On the other hand, enzyme replacement therapy with polyethylene-glycol-conjugated bovine ADA (PEG-ADA) generates adequate metabolites detoxification but not enough immune reconstitution [41]. As for SCID-X1, the availability of an animal model resembling the main characteristics of the human ADA-SCID syndrome was of crucial importance. The ADA-SCID mouse model [42, 43] develop severe T and B cell lymphopenia, a pronounced accumulation of 2’-deoxyadenosine and dATP in the thymus and spleen, inhibition of S-adenosylhomocysteine hydrolase and accumulation of adenosine in most tissues. They also exhibited severe pulmonary insufficiency, bone abnormalities, and kidney pathogenesis.

ADA deficiency has been successfully treated by GT using a similar approach that the one used for SCID-X1 (Fig. 1), but requiring mild bone-marrow chemoablation prior to gene therapy treatment [44]. Aiuti et al. showed immunological and metabolic reconstitution after transplantation of CD34+ HSCs transduced with ADA-expressing-MLV based vectors (Fig. 2). Again, the selective growth advantage of ADA expressing lymphocytes in an ADA-deficient patient played an important role in the success of this trial. In fact, GT benefits in ADA-SCID were only observed after PEG-ADA treatment disruption of GT-treated patients [45] demonstrating that PEG-ADA treatment abolished the selective advantage of genetically modified cells and hinder GT potential. Similar findings have recently reported by Gaspar et al. and again by Aiuti et al. [13]. Up to date, SCID-ADA immunodeficiency has been partially or fully corrected in 19 out of 20 patients in four different clinical trials [12, 13, 26, 44, 46, 47].

It is important to remark that no leukaemia-like disease have been observed in the ADA-SCID gene therapy trial. However, in the last clinical trial some non-life threatening adverse effects have been reported such as neutropenia (2 patients), treatment-related infections (2 patients), Epstein-Barr virus reactivations (1 patient) and autoimmune hepatitis (1 patient). Aiuti et al. [47] have made a detailed analysis of preferred retroviral integrations sites in MLV-ADA-
Fig. (1). Procedure for current Primary Immunodeficiencies Gene Therapy clinical trials. Haematopoietic stem cells (HSCs) are isolated from patients’ bone marrow (a, blue circles). Simultaneously, high amounts of MLV-based retroviral vectors expressing the correct version of the gene are produced (b) and use to modify the patients HSCs (c). All PID GT clinical trials (CT) used a similar transduction protocol using X-vivo medium supplemented with SCF, IL3 and FL3-L. In addition each CT added two additional components as indicated in the figure; SCID(F): SCID-X1 French CT; SCID(E): SCID-X1 English CT; ADA(I): ADA Italian CT; CGD(G): CGD German CT. After transduction, gene corrected cells (c, red circles) where expanded two-three times, characterize for expression of the transgene and infused into the patients (d). Gene modified HSCs migrate to the bone marrow allowing the development of a healthy immune system.

Fig. (2). Strategies to achieve safer and more efficient retroviral vectors. Main projects to improve PID GT efficiency and safety are directed toward the development of either new gammaretroviral-based vectors or lentiviral based vectors, using very similar strategies.
modified HSCs. They found insertion sites proximal to proto-oncogenes, genes controlling cell growth and self renewal, including LMO2. However, these integrations were not associated with clonal selection or expansion in vivo. The authors propose that the differences between SCID-X1 and SCID-ADA might be related with SCID-X1 genetic background or the role of the therapeutic transgene (ADA is a housekeeping enzyme whereas γc is a potential oncogene growth factor receptor).

X-Linked Chronic Granulomatous Disease (X-CGD)

Chronic granulomatous disease (CGD) is a rare PID disorder of phagocytic cells resulting in failure to kill a characteristic spectrum of bacteria and fungi. Patients with CGD have also defective degradation of inflammatory mediators leading to granuloma formation. The disease is caused by mutations that produce absence or malfunction of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits in phagocytic cells (reviewed in [48]). The X-linked form of this disorder (X-CGD) results from mutations in the X-linked gene for gp91phox, the larger subunit of flavocytochrome b 558. An unusual autosomal recessive form of CGD is caused by mutations in the gene encoding p22phox, the small subunit of flavocytochrome b 558. The remaining cases of autosomal recessive CGD are caused by mutations in either p47phox or p67phox, two soluble proteins which interact with flavocytochrome b 558 [49, 50]. The result of these mutations is the inability to generate superoxide by neutrophils, macrophages, and eosinophils. As a consequence, affected patients suffer from recurrent life-threatening, difficult to treat infections by bacterial and fungal species.

Different mice models for the different forms of CGD have been developed. The mice model for X-linked CGD has null allele for the gene encoding gp91phox. Affected male lack phagocyte superoxide production, manifest an increased susceptibility to infection with Staphylococcus aureus and Aspergillus fumigatus and have an altered inflammatory response in thioglycollate peritonitis [51]. In the autosomal form caused by mutations in gene encoding p47phox, leukocytes from p47phox−/− mice do not produce superoxide and therefore kill staphylococci ineffectively and develop lethal infections and granulomatous inflammation similar to those found in human CGD patients [52].

First preclinical studies to treat CGD used a murine stem cell virus vector (MSCV) for expression of human gp91phox to reconstitute NADPH oxidase activity in murine bone marrow cells [53]. Expression of gp91phox and respiratory burst activity was restored in granulocyte-monocyte progeny derived from X-CGD mice bone marrow cells. CGD HSC cells transduced with the gp91phox-therapeutic vector and transplanted into lethally irradiated syngeneic X-CGD mice engrafted into treated mice. Superoxide production was detected in 80% of peripheral blood neutrophils for up to 35 weeks after transplantation [54, 55]. Although neutrophil superoxide production was significantly lower than wild-type neutrophils, most of the mice with 50% to 80% NBT+ neutrophils did not develop lung disease after respiratory challenge with A fumigatus spores. However, protection against bacterial pathogens required greater numbers of oxidant-generating phagocytes compared to protection against A fumigatus [56]. In this sense Dr. Grez group in Germany, achieved higher transduction efficiencies and higher NADPH levels using bicistronic retroviral vectors expressing gp91-phox and a selectable transgene (deltaLNGFR) [57].

Several GT clinical trials for CGD have been conducted since 1997. However, initial studies using retroviral vector to express p47-phox into CD34+ cells, resulted in low and short-term engraftment of CGD-correted cells [58]. Ott, M et al. [14] completed a small clinical trial to correct X-linked CGD in two adults using retroviral vectors where gp91phox expression is driven by the friend mink cell spleen focus-forming virus LTR. Granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood CD34+ cells from two individuals with X-CGD were pre-stimulated for 36 hours with IL-3, SCF, Flt3-L and TPO and then transduced three times on consecutive days (Fig. 1c; CGD(G)). In addition, engraftment was enhanced by busulfan conditioning prior to infusion of gene-corrected cells. Dr Grez and co-workers observed a large number of functionally corrected phagocytes and reconstitution of microbial killing activity in both patients. However, they observed an unexpected expansion and chromosomal instability of gene-modified cells due to retroviral insertions in MDS1-EVI1, PRDM16 and SETBP1 genes. In addition, the therapeutic benefits last about two years due to CpG methylation at the viral LTR promoter that resulted in silencing gp91phox expression. The authors also demonstrated that CpG methylation abrogated promoter but not enhancer activity of the LTR and, therefore, the altered expression of MDS1-EVI1, PRDM16 and SETBP1 genes was still on, even in the absence of gp91phox expression. Unfortunately, one patient (P1) died from severe sepsis with multiorgan dysfunction 2.5 years after treatment [59]. Recently the same authors have extended a similar study in two children (5 and 8 years old) with impressive short term results showing recovery from severe pulmonary and spinal aspergilliosis. Treatment also achieved recovery from paraparesis of both legs in one of the children. However, transgene expression was again silencing due to CpG methylation 1-2 years post-treatment [60].

Wiskott-Aldrich Syndrome (WAS)

Wiskott–Aldrich syndrome (WAS) is a rare X-linked hereditary immunodeficiency characterized by platelet defects and variable immune dysfunction. The disease is the result of mutations in the WAS gene that cause either missing or diminished activity of the WAS protein (WASP). This missing activity of WASP translates into abnormal signalling and impaired cytoskeleton regulation in haematopoietic cells. The WAS protein is expressed exclusively in haematopoietic cells and reconstitution of microbial killing activity in both

WASP-deficient (WKO) mice, have some, however not all the clinical and immunologic characteristic of WAS pa-
patients [70, 71]. These mice are lymphopenic, only mildly thrombocytopenic with normal-sized platelets and develop chronic colitis. WKO mice have defects in T-cell receptor-induced proliferation and aberrant regulation of the actin cytoskeleton but have normal immunoglobulin levels and production of specific antibodies. As for all other immunodeficiencies, transduction of HSCs with MLV-based vectors coding for the corrected gene have been the standard for WAS GT strategies.

Correction of WAS deficiency has been demonstrated both in vitro [72, 73] and in vivo, where WAS-deficient-mice have been successfully treated by infusion of MLV-transduced-HSCs, restoring protein expression, correcting defects in cytoskeleton, cellular activation and inflammation [74, 75]. This prompted Dr Klein group (Hannover Medical School, Hannover, Germany) to initiate the first clinical trial to assess feasibility, toxicity, and potential therapeutic benefit of transplanting autologous WASP-reconstituted hematopoietic stem cells [76]. However, no data have been published so far of this clinical trial.

X-Linked Agammaglobulinemia (XLA)

X-linked agammaglobulinemia (XLA) or Bruton’s disease, is an X-linked immunodeficiency caused by the Bruton’s tyrosine kinase (btk) gene mutation, Btk is a member of Tec family of non-receptor kinases, expressed throughout B-lineage development, except in plasma cells. The principal developmental defect in XLA happens at the pre-B cell transition, with an increase in pro-B cell and strong reduction in cycling pre-B cells and all posterior stages [77-79]. Current treatment for XLA consists in regular intravenous immunoglobulin (Ig) injections that improve significantly patient’s life stile. However, there are still a high frequency of infections and chronic pulmonary diseases with fatal outcomes. Therefore the expression of the Btk transgene into HSCs could represent an alternative and definitive cure to XLA patients [80].

The development of mouse models for human XLA has been critical for GT progress. There are two main class of murine models for XLA: 1) The Xid and Btk-/- mice (a less severe form than XLA) that express Tec and have nearly-normal early B-lineage development but with splenic B-cell development compromised [81, 82]; 2) The Btk/Tec -/- mice, which exhibit a severe block in B-lineage development that is nearly identical to XLA [83]. These models try to simulate the different clinical severity found in XLA as a result of the specific mutations on Btk. Interestingly for GT success, in Btk/Tec -/- mice, Btk+ pre-B cells have growth selective advantage over Btk- cells. This selective advantage of Btk-expressing cells is not shown in Xid and Btk-/- mice models illustrating a possible problem to achieve efficient rescue in mild forms of XLA.

Yu et al. [84] used a MSCV-based retroviral vector to express Btk on HSCs and demonstrated that transduced cells had selective advantage over non-transduced. Mice engrafted with Btk-transduced-HSCs exhibited both primary and peripheral B-lineage development, recovery of peritoneal B cells, and correction of serum IgM and IgG(3) levels. MSCV-Btk GT also restored T-independent type II immune responses and B-cell antigen receptor (BCR) proliferative responses. In spite of this impressive results in animal models, XLA GT clinical trial approval will depend on a better risk–benefit ratio compared to standard intravenous Ig injection therapy. Risks of serious adverse effects, such as those observed in SCID-X1, associated with the use of ubiquitous gammaretroviral vector are not acceptable in the context of Bruton’s disease.

X-Linked Hyper IgM Syndrome (X-HIGM1)

XHIGM1 is an X-linked immunodeficiency characterized by failure of immunoglobulin isotype switching from IgM to IgG, IgA and IgE [85, 86]. This disease is caused by mutations in the gene encoding CD40-Ligand (CD40L, CD154, gp39 or TRAP), a type II membrane glycoprotein member of the TNF superfamily. The natural receptor for CD40L is CD40, a member of the TNF receptor superfamily that is expressed on B cell, macrophages, dendritic cells, endothelial cells and epithelial cells. The interaction between CD40L and CD40 plays a critical role in the immune system [87]. Binding of CD40L to CD40 induces B cells to undergo proliferation, immunoglobulin isotype switching and to escape apoptosis. In addition, CD40L-CD40 interaction induces T cell-mediated inflammatory responses (up-regulation of adhesion molecules, production of inflammatory cytokines and chemokines) as well as activation of macrophage effector functions.

CD40L gene mutations in X-HIGM1 patients are highly heterogeneous and include missense, nonsense, splice site mutations, insertions or deletions [88, 89]. In most instances, these mutations limit the interaction between the activated CD4+ T cells and B cells, responsible for the immunoglobulin isotype switching. Because CD40L is required for the functional maturation of T lymphocytes and macrophages, patients with X-HIGM1 also have a variable defect in T-lymphocyte and macrophage effector function. Patients with X-HIGM1 have increased susceptibility to infection with a wide variety of bacteria, viruses, fungi, and parasites. In addition, they have increased risk for developing autoimmune disorders and malignancies. At present, Ig replacement remains the mainstay of therapy by decreasing the frequency of life-threatening infections in X-HIGM1 patients. Another option for X-HIGM1 patients is bone marrow transplantation. However, there are still a significant number of patients that die as a consequence of recurrent infections or treatment complications [90]. Therefore, the development of safer and more efficient therapies is mandatory. One alternative is the transplantation of HSCs expressing functional CD40L.

Again, an animal model that mimics X-HIGM1 syndrome is required to assess the feasibility and safety of X-HIGM1 GT as an alternative treatment. In this context the CD40L-knockout mice [91] is the best model so far, mimicking some of the deficiencies found in human patients, although the immune defect is not as severe. CD40L knockout mice show deficiencies in humoral immunity and failed to generate secondary antigen-specific responses to immunization with a thymus-dependent antigen. However, contrary to X-HIGM1 human patients, the percentages of B and T cell subpopulations are normal and they produce antigen-specific antibody of all isotypes, except IgE, in response to a thymus-independent antigen.
Like in the other PIDs, first attempts to treat X-HIGM1 with GT used retroviral vectors. Brown et al. [92] developed a retroviral vector encoding de murine CD40L cDNA (mCD40L) driven through a constitutive promoter. Murine bone marrow cells were transduced and injected into CD40L-/- mice. The constitutive expression of mCD40L led to full or partial correction of both cellular and humoral immune defects, but more than half of treated mice developed uncontrolled thymic lymphoproliferations. The author proposed that constitutive expression of CD40L could produce abnormal proliferative responses in developing T lymphocytes, apparently through aberrant interaction between CD40L+ cells and thymocytes with an unusual phenotype: TCR+CD40.

Contrary to other PID, such as SCID-X1, SCID-ADA or WAS where the mutated genes are constitutively expressed in haematopoietic cells, in X-HIGM1, the mutated CD40L gene is tightly regulated and failure to regulate its expression properly could induce deleterious effects. An interesting work by Sacco et al., [92B] studied the effect of unregulated expression of CD40L by producing transgenic mice in which CD40L expression was expressed by different, non-physiological promoters. They found that widespread ectopic expression of CD40L was lethal while overexpression in mature T cells was tolerated, although one-third of the mice developed atypical lymphoid proliferations. Another hurdle for X-HIGM1 GT is the absence of selective advantage of CD40L-expressing cells, since mutations in CD40L do not affect directly the survival of T cells. The data accumulated to date clearly show that X-HIGM1 GT requires the development of strategies that achieve highly efficient and physiologically expression of CD40L.

Other Immunodeficiencies

JAK-3 immunodeficiency is an autosomal recessive form of SCID as result of mutations in the receptor tyrosine kinase gene JAK-3, expressed almost exclusively in haematopoietic cells. JAK-3 associates with the interleukin-2 receptor gamma chain common (γc) and is activated after ligand binding. The immune defects found in these patients are, therefore, very similar to those found in SCID-X1 (with the γc gene mutated). Murine models for JAK-3 deficiency have been treated by GT, demonstrating resistance to viral infection [93], reconstitution of deficient lymphocyte populations and restoration of cell- and humoral immunity [94]. However, overexpression of JAK-3 has been involved in cancer development [95] and GT vectors should therefore be developed to express JAK3 physiologically. RAG-1 and RAG-2 deficiencies have also been corrected in animal models [96, 97]. Although these genes are finely regulated, gene transfer vector constitutively expressed RAG1 and RAG2 without major problems for the treated animals. However, further studies would be required before clinical translation of these vectors.

NEW STRATEGIES TO IMPROVE GENE THERAPY OF PRIMARY IMMUNODEFICIENCIES

All the preclinical and clinical data reviewed previously pointed to the requirement of safer and more efficient vectors for PID GT. These studies have made clear that LTR-driven gammaretroviral vectors are not the ideal choice, since they can be silenced and/or can induce leukaemia-like disease. In this section we will describe the current alternatives that scientist use to overcome the main problems encountered by GT treatments (Figs. 2 and 3; Table 1).
Self-Inactivated Retroviral Vectors for SCID-X1, SCID-ADA and X-CGD

Most efforts to improve safety and efficiency for SCID GT have focussed in the development of new self-inactivated (SIN) gammaretroviral and lentiviral vectors (LV) (Fig. 2). One of the major drawbacks of first generation retroviral vectors has been the use of the viral LTR U3 region to drive the expression of the transgene. The U3 contains strong viral promoter and enhancer sequences that have been responsible for the activation of downstream host genes leading to transformation and leukemia-like diseases [98]. One of the ways to improve safety of retroviral vectors is to eliminate the U3 region from the 5' LTR and to use alternative promoters to drive the expression of the transgene (Fig. 2). Different authors have included deletions at the U3 region in retroviral [99, 100] and lentiviral vectors [101, 102]. Since the U3 region harbors the promoter and enhancer regions required for RNA transcription, these mutations not only abrogate potential mobilization of vector but also reduce genotoxicity due to promoter/enhancer interference [8]. In addition it also allow expressing the transgene from any promoter.

In this direction Prof. Thrasher’s group has recently evaluated the efficacy of SIN-gammaretroviral vectors driven by the human elongation factor-1alpha promoter for SCID-X1 GT [103] (Table 1). These vectors were capable of fully restore the lymphoid differentiation potential of γc-deficient lineage negative cells. The same group has also develop SIN-LV based in human immunodeficiency virus-1 (HIV-1) incorporating the ubiquitously acting chromatin opening element (UCOE) that achieves reproducible and stable γc gene expression [104]. LVs offers several advantages for PID GT over gammaretroviral vectors [105] such as the efficient transduction of HSCs without preactivation (that could lead to changes in HSC characteristics) [106] and the

### Table 1. Gene Therapy for Primary Immunodeficiencies

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<th>Disease</th>
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<th>Results</th>
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<th>New Therapeutic Vectors</th>
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<tbody>
<tr>
<td>SCID-X1</td>
<td>MLV-based vector driven by the LTRs</td>
<td>17 out of 20 treated patients cured [9, 10, 26]</td>
<td>Leukaemia development [11, 27]</td>
<td>Human promoter-driven SIN-gammaretroviral vector [103]</td>
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<td></td>
<td></td>
<td>Fully functional immune system</td>
<td></td>
<td>Enhancer-less- SIN-HIV1-based lentiviral vector [104]</td>
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<tr>
<td>ADA- SCID</td>
<td>MLV-based vector driven by the LTRs</td>
<td>19 out of 20 treated patients cured [12, 13, 26, 44, 47]</td>
<td>No life-threatening adverse effect observed.</td>
<td>SIN -HIV-based lentiviral vector driven by the PGK promoter [110]</td>
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<tr>
<td></td>
<td></td>
<td>Fully functional immune system</td>
<td>Neutropenia, Epstein-Barr virus reactivations, Autoimmune hepatitis</td>
<td>SIN -HIV-based lentiviral vector driven by the MND enhancer/promoter [111]</td>
</tr>
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<td>X-CGD</td>
<td>MLV-based vector driven by the SFFV LTR</td>
<td>Temporary recovery of 4 out 4 treated patients [14, 60]</td>
<td>Expansion and chromosomal instability of gene-modified cells.</td>
<td>SIN -HIV-1-based lentiviral vector [112]</td>
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<td></td>
<td></td>
<td>Recovery from severe pulmonary and spinal aspergiliosis</td>
<td>Gene silencing by CpG methylation</td>
<td>SIV-based lentiviral vector [113]</td>
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<tr>
<td>WAS</td>
<td>MLV-based vector driven by the LTRs</td>
<td>Correction of cytoskeleton defects, cellular activation and inflammation [74, 75]</td>
<td>No adverse effect observed.</td>
<td>Haematopoietic-specific SIN-HIV-1 Lentiviral vector [64, 66, 119]</td>
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<tr>
<td>XLA</td>
<td>Retroviral vectors based on MSCV</td>
<td>Primary and peripheral B-lineage development, correction of T-independent type II immune responses [84]</td>
<td>No adverse effect observed.</td>
<td>B-lineage specific SIN-HIV-1 based lentiviral vector[122-125]</td>
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<td>XHIGM-1</td>
<td>MLV-based vectors driven by constitutive promoter</td>
<td>Full or partial correction of both cellular and humoral (Ig isotype switching) immune defects [92]</td>
<td>Uncontrolled thymic limphoproliferations [92]</td>
<td>Transplicer[127]: Correction of CD40L RNA</td>
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<td>Human artificial chromosome harbouring a CD40L genomic fragment [129]</td>
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**Safer Vectors for Gene Therapy of Primary Immunodeficiencies**

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lower genotoxicity [107-109]. Therefore, UCOE SIN-LV appears as a very attractive tool for PID GT both, in term of efficiency and safety. However, formal demonstration that the UCOE element confer lower genotoxicity than other physiologic promoters needs further investigation.

Contrary to SCID-X1 clinical trials the use of standard LTR-driven gammaretroviral vectors in the two ADA-SCID GT clinical trials were a complete success, with no life threatening secondary side effects [13, 44]. However there is still the possibility that further extension of the trials could lead to the same problems found in SCID-X1 trial. In this sense Mortellaro et al. [110] have developed a SIN-lentiviral vector driving ADA by the PGK promoter. Bone marrow cells of ADA-/- mice were transduced and injected via the tail vein. ADA activity was normalized in lymphocytes and partially corrected in RBCs, resulting in full metabolic detoxification and prevention of severe pulmonary insufficiency. Here, pre-transplant irradiation was crucial for long-term survival of ADA-/-mice, since animals given transplants of ADA-/- BM cells without irradiation died 2 weeks after transplantation due to poor engraftment.

In a different approach, Carbonaro et al. [111], used a SIN-lentiviral vector driving ADA expression through the MND enhancer/promoter (that yields high level of expression in haematopoietic cells) to treat neonatal ADA-/- mice in vivo (by direct intravenous injection of LVs). In addition to prolonged survival, the mice had significant improvement of their immunologic systems, with increased numbers of T and B cells and reconstitution of mitogen-induced T-cell proliferative responses. However, they observed an absence of the selective survival of the gene-corrected T cells, derived from the lack of HSC transduction by systemically administered vectors and therefore this strategy can not be as effective as ex vivo transduction of HSCs in clinical settings.

The case for CGD is different from SCID-X1 and SCID-ADA clinical trials due to the absence of selective advantage of gp91phox-expressing cells which facilitates the progressive loss of transgene expression due to promoter methylation. However, vector development followed a very similar line; Dr. Malech’s group has developed SIN lentiviral (HIV-1-based) vector encoding gp91phox under constitutive promoters and compared their efficiency to that of LTR-driven gammaretroviral vector expressing the same transgene [112]. They showed that LV(gp91phox), but not the gammaretroviral-based vectors, achieved significant correction of human X-CGD neutrophils in the NOD/SCID xenotransplant model. However, the author did not study transgene expression stability, an important point in this immunodeficiency as demonstrated in Dr. Grez’ clinical trial. Another important factor to evaluate is the possible deleterious effects of ectopic and/or deregulated expression of the gp91phox transgene using this LV.

The same group has also developed LVs based in simian immunodeficiency virus (SIV) rather than in HIV-1 [113]. This SIV-based vector system has most current retroviral safety features such as SIN design and absence of accessory protein genes [114]. In addition, the viral particles were enveloped by a modified version of the feline endogenous virus envelope protein (RD114/TR) [115] which confers augmented transduction of human HSCs. They found sustained gp91phox expression and correction of oxidase function in human X-CGD myeloid cells arising from the human xenograft in the marrow of NOD/SCID mice. Using this system, the author obtained in vivo expression of gp91phox that was 20% of normal cells. This expression levels can result in significant restoration of the oxidase enzyme as demonstrated by phenotypic restoration and previous work [14].

Tissue-Specific Vectors for WAS and XLA

Ectopic expression of the WASP or Btk genes can, potentially lead to citotoxicity [116] and/or unknown secondary effects. Therefore, several groups have developed tissue-specific SIN-retroviral vectors for GT of WAS and XLA immunodeficiencies. By directing gene expression only to those cell types that naturally express the gene, we can minimize citotoxicity (deleterious effects due to protein expression in cells that do not express it naturally) and genotoxicity.

Most vector development for WAS GT has focussed in SIN LVs. Several groups have developed SIN LVs expressing WASP cDNA through PGK [64, 117] or the WAS endogenous promoter [64, 66]. Both vectors effectively provide correction of the immune and cytoskeleton defects of WASP WKO mice [117, 118]. However if a vector must be chosen nowadays for WAS GT, this must be the WASp-promoter-driven LV. The WASp-promoter-driven LVs are highly haematopoietic-specific [64, 66] and, in addition we have shown that transgene expression of transduced HSCs are regulated in a similar way as endogenous WAS [119]. Moreover WASp-promoter driven LVs are able to express WASP protein levels close to those found in normal cells [66, 120] and avoid deleterious effects of over-expression in non-target cells [116]. Taken together this work has led to the preparation of two clinical trials for WAS GT based on WASp-promoter-driven LVs [120, 121].

As for WAS gene therapy, LVs have also been the vector of choice to replace gammaretroviral-based vectors for XLA GT. In addition, since Btk is expressed throughout the B-lineage, several groups have developed B-lineage restricted LVs [122-125]. These LVs were able to retaining the B-cell specificity in progeny derived from transduced human HSCs using eGFP as a marker gene. Recently, Moreau et al. [126], demonstrated preferential long-term Btk transgene expression in transduced human CD34+ B-cell progeny. Although vector efficiency may need to be improved, the B-lineage-specific expression of Btk is an important improvement for XLA GT since Btk is involved in multiple signalling pathways affecting proliferation, differentiation and apoptosis. However the presence of the strong enhancer activity of Eμ MAR (present in Moreau vector) represents a potential genotoxicity risk. It is therefore required to assess the transformation potential of these LVs before translation into clinic.

Physiologically-Regulated Vectors for X-HIGM1

The data showing abnormal proliferative responses in the first attempt to reconstitute CD40L expression in animal to models [92] stopped further investigations aiming GT of this disease. In addition, the appearance of leukemias in the SCID-X1 clinical trials has prompted the few groups working on X-HIGM1 GT to focus on safety. The aim is to obtain
physiologically regulated CD40L expression in order to avoid deleterious effect derived from the unregulated CD40L expression observed in previous work. In these direction Dr. Crystal’s group corrected a CD40L mutation while preserving the natural regulation of CD40L using pre-mRNA trans-splicing [127]. Trans-splicing is a process by which two different pre-mRNAs are joined (by the cellular splicing apparatus) correcting the defect at the mRNA level, leaving intact the genomic defect [128]. The authors developed a lentiviral vector encoding a RNA, called trans-splicer, harbouring a hybridizing domain, a spacer and the corrected version of the CD40L upstream of the hybridizing domain. HSCs transduced with this lentiviral trans-splicer and transplanted into lethally irradiated CD40L-knockout recipient mice were able to correct CD40L mRNAs. This correction was sufficient to achieve partial restoration of immunoglobulin switching, regulated CD40L expression after CD3 stimulation and attenuation of Pneumocystis carinii pneumonia. Importantly, the treatment did not induce lymphoproliferative disease. Recently, another group tackled X-HIGM1 GT in a rather different way. Yamada et al. used the human artificial chromosome (HAC) vector harbouring a CD40L genomic fragment to express physiologically-regulated expression of CD40L [129]. The author demonstrated endogenous expression of the transgene through its own promoter in Jurkat (T cell line) and U937 (a myeloid cell line) upon microcell-mediated chromosome transfer (MMCT). However both strategies, transsplicing and HAC are only partially effective and their safety profiles must be still determined once they reach similar efficiencies as lentiviral reconstitution by gene addition.

**FUTURE DIRECTIONS**

New vectors must consider improving two principal safety aspects: 1- genotoxicity (genomic alteration due to vector integrations) and 2- ectopic/unregulated expression of the transgene. Genotoxicity can be defined as harmful actions on the integrity of the genetic material. In the worst scenario, genotoxicity can cause cell transformation and tumor development [7]. Strategies to minimize or eliminate genotoxicity problems can be grouped in those based in retroviral vectors (Fig. 2) and those that do not (Fig. 3). In this section we will discuss novel approaches still under development and therefore still not studied in detail for PID GT.

**Improving Safety of Retroviral-Based Vectors**

The almost random insertion pattern of retroviral vectors has two sides, 1- possible deleterious effects of vector enhancer elements in host cell gene expression (genotoxicity) and 2- The effect of chromosomal position in vector expression such as transcriptional silencing. Therefore several gene therapy laboratories have been trying to isolate the vector from the host chromatin. Insulators are genetic elements near chromatin domain boundaries that function as barriers against repressive effects of neighbouring heterochromatin or prevent inappropriate activation of a promoter by nearby heterologous enhancers [130]. Felsenfeld and colleagues have shown that the 5’HS4 element can protect against position effects and provide enhancer blocking function [131]. There are several other chromosomal elements that function as insulators in different organisms (reviewed in [132]). Different insulators have been used in various gene therapy vectors [132, 133] including retroviral and lentiviral vectors [134-138].

The introduction of insulators in the vectors backbone decreased genotoxicity [136, 138, 139] and gene silencing [134, 135, 137, 140, 141]. These important results have pushed gene therapist to incorporate these elements in therapeutic vectors, specially when the strategy involved gene transfer into stem cells [142-144].

Although enhancer interactions are the most frequent form of retroviral insertional mutagenesis, read-through transcripts (not stopping at the poli-A site contained in the vector) could be also an important safety problem [145, 146]. Some groups have shown that the inclusion of upstream polyadenylation enhancer elements improve the efficiency of 3’ end mRNA processing in gamma-retroviral and lentiviral SIN vectors [146]. The resulting vectors have also improved titer and transgene expression in target cells. Still, whether stronger poli-A signals in retroviral vectors will result in a biosafety improvements must be demonstrated in the future.

**New Strategies for Stable Gene Modification**

Several groups have focus in target vector integrations to certain non-deleterious DNA locations (Fig. 3). One of such strategies is based on the ability of the Rep78 or Rep68 proteins of adeno-associated virus (AAV) to direct integration of AAV-ITS-containing sequences into the AAVS1 region of chromosome 19 [147]. However, the most logic way to achieve this, the use of AAV-derived vectors, has been hindered by the limited DNA cargo capacity of AAV particles. Alternatively, this technology has opened the possibility of integrate large DNA sequences in a site-specific fashion, minimizing genotoxicity by using hybrid vectors (based on plasmids, baculovirus, adenovirus or herpes simplex virus) containing AAV-ITS-sequences and rep78/68 genes. These hybrid vectors can potentially accommodate genetic material of more than 30,000 base pairs (see [148] for review). However, although promising, hybrid vectors have several limitations that preclude their use for PID GT: first, the site-specific integration occur, at best in 50% of stable expressing cells [149, 150] and second, it has never been demonstrated their efficiency to modify HSCs. Another way to achieve site-specific integration uses phiC31 integrase [151, 152], able to insert large DNA elements (over 10Kb in size) containing an attB sequence into genomes containing attP sites (Fig. 3). This integrase recognizes sequences in the human genome termed pseudo-attP sites [153] that can mediate efficient phi C31-integrate-mediated integration [151, 152]. However, although stable transduction of different cell types have been achieved [154], no results have been shown in HSCs and, in addition several authors have found imprecise integration into pseudo-attP sites resulting in DNA deletions [151, 155].

Although site-specific integration can reduce mutagenic potential, out-target integration (30-50%) and unexpected DNA re-arrangements are still problems of these strategies. An alternative is to deliver DNA that can be kept stably without integration into the host DNA. HAC are able to deliver full genomic loci harbouring all regulatory sequences [156]. HAC act as stable episomal elements in dividing cells,
including haematopoietic [129] and embryonic cells [157] and could therefore be useful to achieve stable and physiologically regulated expression of therapeutic genes. However stable HAC delivery into HSCs has not been achieved so far, limiting its use for PID GT.

In any case, the ideal GT strategy for primary immunodeficiencies should aim the in situ correction of chromosomal mutations encountered in patients. This is exactly the objective of new strategies that use cellular homologous recombination machinery to modify surgically any selected sequence using either Zinc-Finger Nucleases (ZFNs) [158] or meganucleases [159] (Fig. 3). Both strategies achieve up to 10,000 times enhancement of cell homology-recombination mechanism allowing efficient in situ gene correction. However, only ZFNs have been used to study PID GT and have managed to modify stably HSCs although with low efficiency. ZFNs combine the non-specific cleavage of endonucleases with the specific recognition properties of zinc finger proteins (ZFPs) [160] to cut at the desired chromosomal locus inside the cells [161-163] (Fig. 4). Once the target DNA has been cut, the cells’ own homologous-directed-repair (HDR) mechanism uses the delivered DNA to correct the gene defect. Recent results have shown that ZFNs can be used to create targeting frequencies from 5- 50% in the absence of selection in several cell lines [162-167] including primary T cells [168] and HSCs [167]. However, although very promising, ZFNs are still under development and several aspect must be solved before this technology can be applied in clinical trials such as the reduction of off-target double strand breaks and ZFN-associated genotoxicity.

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ABBREVIATIONS

PID = Primary immunodeficiencies
GT = Gene Therapy
CT = Clinical Trial
HSCs = Haematopoietic stem cells
SCID-X1 = X-linked severe combined immunodeficiency

Fig. (4): DNA surgery by Zinc Finger Nucleases (ZFNs). To achieve site-specific gene correction, we need to deliver inside the target cells (a) three different players; two ZFNs recognizing adjacent DNA targets and a DNA molecule harbouring the correct version of the gene (b, bold sequence). Each Zinc Finger Nuclease (ZNF) consists of two functional domains: the non-specific cleavage domain of FokI endonuclease, and the zinc finger domain with the specific recognition properties of zinc finger proteins (b). Since FokI functions as a dimer, it will be active only when both ZFNs are bound to their target sequences as illustrated (c). Once active, the FokI endonuclease domain digests the DNA and creates a double-strand break (d), promoting cells’ own homologous-directed-repair (HDR) mechanism. The presence of high amounts of donor DNA allows using this sequence to correct the gene defect (e).
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ADA-SCID = Adenosine deaminase immunodeficiency
X-CGD = X-linked chronic granulomatous disease
WAS = Wiskott-Aldrich Syndrome
XLA = X-linked agammaglobulinemia
XHIGM-1 = X-linked hyper IgM syndrome
MOI = Multiplicity of infection
yc = Common cytokine-receptor gamma chain
LTR = Long Terminal Repeat
PG-MDF = Polyethylene glycol-megakaryocyte differentiation factor
RBCs = Red blood cells
G-CSF = Granulocyte colony-stimulating factor
SIN = Self-inactivated
MLV = Murine leukemia virus
LV = Lentiviral Vectors
SIV = Simian immunodeficiency virus
HIV-1 = Human immunodeficiency virus type I
HAC = Human artificial chromosome
AAV = Adeno-associated virus
ZFNs = Zinc-Finger Nucleases
HDR = Homologous-directed-repair
ADA = Adenosine-deaminase
btk = Bruton’s tyrosine kinase gene
ITS = Integration target site

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