Twenty-Five Years of Gene Therapy for ADA-SCID: From *Bubble Babies* to an Approved Drug

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Twenty-five years have passed since first attempts of gene therapy (GT) in children affected by severe combined immunodeficiency (SCID) due to adenosine deaminase (ADA) defect, also known by the general public as *bubble babies*. ADA-SCID is fatal early in life if untreated. Unconditioned hematopoietic stem cell (HSC) transplant from matched sibling donor represents a curative treatment but is available for few patients. Enzyme replacement therapy can be life-saving, but its chronic use has many drawbacks. This review summarizes the history of ADA-SCID GT over the last 25 years, starting from first pioneering studies in the early 1990s using gamma-retroviral vectors, based on multiple infusions of genetically corrected autologous peripheral blood lymphocytes. HSC represented the ideal target for gene correction to guarantee production of engineered multi-lineage progeny, but it required a decade to achieve therapeutic benefit with this approach. Introduction of low-intensity conditioning represented a crucial step in achieving stable gene-corrected HSC engraftment and therapeutic levels of ADA-expressing cells. Recent clinical trials demonstrated that gamma-retroviral GT for ADA-SCID has a favorable safety profile and is effective in restoring normal purine metabolism and immune functions in patients >13 years after treatment. No abnormal clonal proliferation or leukemia development have been observed in >40 patients treated experimentally in five different centers worldwide. In 2016, the medicinal product Strimvelis™ received marketing approval in Europe for patients affected by ADA-SCID without a suitable human leukocyte antigen–matched related donor. Positive safety and efficacy results have been obtained in GT clinical trials using lentiviral vectors encoding ADA. The results obtained in last 25 years in ADA-SCID GT development fundamentally contributed to improve patients' prognosis, together with earlier diagnosis thanks to newborn screening. These advances open the way to further clinical development of GT as treatment for broader applications, from inherited diseases to cancer.

Keywords: gene therapy, ADA-SCID, clinical trial, primary immunodeficiency, adenosine deaminase

INTRODUCTION

ADENOSINE DEAMINASE (ADA) deficiency is a rare, autosomal recessive severe combined immunodeficiency (SCID; OMIM#102700).[1] ADA-SCID is caused by mutations in the *ADA* gene and represents the cause of 10–15% of all SCIDs, with an overall prevalence in Europe ranging between 1:375,000 and 1:660,000 live births. In 1972, it became the first immunodeficiency for which a specific molecular defect was identified, at both the genetic and biochemical level. The importance of ADA for immune function was revealed when it was discovered that the enzyme was absent in several immunodeficient patients being considered for bone-marrow transplantation (BMT). This finding showed the crucial role of the enzyme involved in purine metabolism in the development of immune system, which was later confirmed by studies in murine models of immunodeficiency.[2] The main features of the disease are impaired differentiation and functions of T, B, and natural killer (NK) cells, predisposing patients to develop severe, opportunistic, and recurrent infections and failure to thrive.[1] Similarly to other SCIDs, ADA-

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SCID is a fatal disease, usually leading to death in the first year of life, if not treated. Among SCIDs, it is one of the most difficult to handle clinically due to the non-immunological abnormalities (mainly neurodevelopmental, behavioral,[3] skeletal, and hepatic), which occur as a consequence of the systemic metabolic defect.[4] However, in the last years, the prognosis for ADA-SCID infants has improved greatly.

AVAILABLE TREATMENTS FOR ADA-SCID

At present, treatment options for ADA-SCID include allogeneic hematopoietic stem-cell transplantation (HSCT), enzyme replacement therapy (ERT), or autologous *ex vivo* gene therapy (GT).[5,6]

HSCT from a human leukocyte antigen (HLA)-matched related sibling or family donor without conditioning is recommended as first-line curative treatment, being associated with good survival and effective immune reconstitution. Current evidence[6,7] supports HSCT as early as possible after diagnosis in order to prevent the development of infections and to reduce systemic adenine metabolite-related toxicity, but this therapeutic option is only available for <25% of patients.

Allogeneic HSCT from alternative donors (matched unrelated, haploidentical) is still associated with an increased risk of morbidity and mortality, with survival falling significantly as HLA matching decreases, ranging from 67% to 43%, albeit improving in most recent years.[8,9] New approaches have been recently developed to ameliorate survival and immune reconstitution in these transplant settings, such as improved methods for graft manipulation (*e.g.*, selective α/β T-cell depletion[10]) and improved supportive measures, but no specific outcome data for ADA-SCID patients have been reported to date.

Newly diagnosed patients are usually treated with weekly intramuscular injections of ERT with exogenous polyethylene-glycol-conjugated bovine ADA (PEG-ADA). PEG-ADA has proved a very useful way to stabilize patients as a bridging therapy to promote immune recovery before a definitive procedure can be implemented. This treatment is licensed in the United States but not in Europe where it is provided under special provisions. While PEG-ADA is highly effective in the short term, rescuing the immunological defects through extracellular detoxification, these effects are often partial and poorly sustained over time. ERT efficacy tends to decrease over time, with an often inadequate long-term immune reconstitution[11] and survival rate of 78% at 20 years.[5] Moreover, it does not prevent chronic complications and can amount to extremely high costs if maintained long term. A cohort of patients (from infant age to young adults) was reported to continue PEG-ADA as chronic treatment in the absence of a matched donor.[1,11]

Because of the limitations of these therapies, gene correction of autologous hematopoietic stem/progenitor cells (HSPC) has been explored as a potentially curative treatment for ADA-deficient SCID in the past 25 years and has now become an approved treatment in Europe.[12]

RATIONALE FOR GT IN ADA-SCID

ADA-SCID was the first inherited disease in which *ex vivo* gene transfer in peripheral blood lymphocytes (PBL) or HSPC was performed. Some evidence supported the use of autologous gene-corrected cells for ADA-SCID.[13] First, *ADA* gene is a ubiquitously expressed housekeeping gene, with no need of fine regulation and therefore suitable to be expressed under viral promoters as those present in gamma-retroviral vectors (RV). Moreover, *ADA* cDNA could be easily cloned and expressed by such vectors, being relatively small (1.5 kb). Second, relatively low enzymatic levels allow normal immune functions in healthy individuals carrying a normal *ADA* gene. Third, normal or gene-corrected cells have a strong selective survival advantage over ADA-deficient cells because they can more easily eliminate toxic metabolites from inside the cell. This was demonstrated both in HSCT and in preclinical GT models.[14] Thus, even a relatively low amount of correction and/or of engrafted HSPC might have resulted in successful therapy. In addition, a decline in revertant T lymphocytes during ERT was observed in a delayed-onset patient with mosaicism for a "second-site suppressor" of a splicing mutation,[15] stressing the importance of the discontinuation of ERT in GT preparatory phase in order not to compromise the selective advantage of genetically corrected cells. Finally, the target tissue, hematopoietic cells, is relatively easy to obtain and to culture *in vitro* before reinfusion in the patients.

ADVANTAGES AND DISADVANTAGES OF GT OVER OTHER TREATMENTS

GT has several potential advantages over other existing treatment options, and is suitable for patients who do not have an available HLA-identical sibling donor.[13] With respect to HSCT, autologous transplantation of gene-corrected HSPC is potentially applicable to all patients and immediately available, with no delay due to donor search.

Moreover, using autologous gene-corrected HSPC abolishes the risk of rejection and graft-versus-host disease due to HLA mismatches or minor antigen incompatibility. Finally, use of immunosuppressive prophylaxes or high-dose conditioning regimens are not required in the GT setting, thereby decreasing treatment-related organ toxicity, prolonged myelosuppression, and increased infection risk.

GT has also a great advantage over ERT. A single injection of gene-corrected HSPC may be sufficient to treat a patient lifelong, avoiding the need for chronic replacement therapy with its extremely high costs and burden on patients' quality of life. Moreover, there is evidence from studies in mice and HSCT that endogenous ADA is more effective than exogenously administered ADA.[14] However, GT can also present some peculiar limitations and risks. Being an autologous procedure, low autologous bone marrow (BM) HSPC content or pre-existing chromosomal alterations in BM progenitors may lead to the exclusion of patients from the procedure.[16] Moreover, the efficiency of gene transfer and correction represents an important limiting factor for the procedure outcome. Finally, a potential serious adverse event related to gene transfer can be represented by insertional mutagenesis caused by RV "turning on" cellular proto-oncogenes adjacent to where they integrate into the target cell chromosomes, leading to the appearance of clonal expansion or even leukemic proliferation. This event is known to have occurred in some patients treated with RV GT for X-linked SCID,[17] Wiskott–Aldrich syndrome (WAS),[18] and chronic granulomatous disease (CGD)[19] who developed acute leukemia or myelodysplasia after the procedure. So far, the cumulative experience of five different centers performing various GT for ADA-SCID did not reveal the occurrence of such complications, indicating that it has a favorable risk–benefit profile.

GT PILOT STUDIES

More than 25 years ago, fundamental preclinical proof-of-concept about GT feasibility and adequate safety profile was provided by the pioneering work of researchers in Italy and in the United States. In the early 1990s, several GT pilot studies were initiated using gamma-RV that were previously evaluated in murine[14,20] and primate[21] models as well as in ADA patients' BM cells,[22] in which *ADA* cDNA transgene expression was regulated by retroviral long terminal repeats (LTR). These studies differed considerably in terms of target cells, transduction procedures, and clinical protocols. Patients who were eligible for GT were chosen among those lacking a matched familiar donor or displaying inadequate immune reconstitution after ERT. All patients enrolled in these studies continued to receive PEG-ADA in order to avoid the risk of a further deterioration of immune function, and did not receive any preparatory conditioning regimen.

PBL GT

Based on the observation that patients receiving BMT achieved immune reconstitution by sole engraftment of donor T cells, PBL GT was used initially for the treatment of ADA deficiency. Nine ADA-SCID patients have been reported to be treated with multiple infusions of gene-corrected PBL in three different clinical trials.[23–27] Autologous T lymphocytes were transduced with gamma-RV derived from Moloney Murine Leukemia Virus (MMLV), veiculating *ADA* gene cDNA. The marker gene coding for Neomycin resistance, NeoR, was inserted inside the vector for identification and selection of transduced cells. The available follow-up reported in the literature extend to >15 years in the first-treated children, with no adverse events or toxicity observed. The majority of patients showed improvements in immune function, and transduced T cells persisted >12 years after infusions of gene-corrected lymphocytes were discontinued, including functional decade-long surviving genetically engineered T memory stem cells,[28,29] which were demonstrated to be able to persist in circulation long term. This demonstrated the safety and the therapeutic potential of PBL GT with the sustained engraftment of ADA-expressing T cells.[27] However, the real therapeutic impact was difficult to evaluate because all subjects continued to receive ERT, which might have impaired the selective growth advantage for gene-corrected cells. Indeed, in one patient in whom PEG-ADA was discontinued at San Raffaele Telethon Institute for Gene Therapy (SR-Tiget) in Milan (Italy), gene-corrected T cells progressively replaced the defective cells, resulting in restoration of normal T cell function and antibody responses to neoantigen. However, infusion of T cells was not sufficient to allow full correction of the metabolic defect,[26] likely because of the limited mass of detoxifying cells.

HSPC GT

A parallel strategy was developed aimed at infusing BM or umbilical cord blood (UCB) progenitors to patients without any preparative conditioning.

Autologous HSPC have been always considered the optimal target cells for long-term, full correction of ADA-SCID defect. The rationale for GT in HSPC was based on the assumption that the engineered HSPC would stably engraft, self-renew, and differentiate in multiple lineages, including mature lymphocytes. These newly generated gene-corrected lymphocytes should produce adequate levels of endogenous ADA enzyme to sustain their growth and function, independently from exogenous sources. On the other hand, production of ADA in all hematopoietic lineages should contribute to an overall improvement in systemic detoxification.

Eight patients have been treated with engineered HSPC in the first clinical trials[24,30,31] after collection of BM- or UCB-derived cells followed by transduction and infusion without any myeloablative conditioning. In particular, the patients enrolled at SR-Tiget received multiple infusions of BM progenitors and peripheral T lymphocytes, transduced with similar vectors but distinguished by a different marker gene.[24,32] This first study reported the progressive expansion of circulating corrected T cells, derived from the infusion of engineered PBL or of BM precursors differentiating in lymphoid lineage. Moreover, it showed that *ex vivo* transduced BM progenitors were able to differentiate *in vivo* into multiple lineages, even if not achieving therapeutic levels of ADA expression.[24] Two years after the procedure, BM-derived corrected cells were present at a low level and thereafter were no longer detected.

In a study conducted at the Children's Hospital of Los Angeles in three ADA-SCID patients, CD34+ progenitor cells were collected at birth from the umbilical cord of newborn babies, transduced, and re-infused after 4 days. In these patients, a 4-year follow-up indicated the presence and the expression of ADA in BM and peripheral blood (PB).[31,33] Furthermore, lymphocytes derived from gene-corrected cord blood HSPC accumulated over time, still being detectable at least 8 years after the procedure,[34] suggesting T cell lineage selective advantage. However, due to the fact that no conditioning was administered, the level of engraftment of transduced cells was not sufficient to produce therapeutic ADA expression.[33] Moreover, the procedure was performed with concomitant PEG-ADA administration.

Together, the results of these early studies showed the feasibility and safety rationale of BM HSPC infusion, but they resulted in low vector marking of progenitor-derived cells with insufficient ADA production, and failed to provide clear clinical benefit over and above that of ERT, which patients continued to receive, supporting the need for the implementation of new and improved clinical trials.

GT WITH AUTOLOGOUS BM CD34+ CELLS FOLLOWING NON-MYELOABLATIVE CONDITIONING: THE SR-TIGET CLINICAL PROTOCOL

Subsequent studies using autologous CD34+ cells transduced with gamma-RV introduced some key changes, including PEG-ADA withdrawal prior to BM harvest to enhance the selective advantage of corrected cells and, most importantly, the use of reduced-intensity conditioning with the alkylating agent busulfan to promote engraftment of HSPC.

In particular, a breakthrough was achieved in 2000, when these changes, combined to improved transduction protocol for BM HSPC, were introduced in two successful pilot studies that were started at Hadassah Hospital (Jerusalem, Israel) and SR-Tiget (Milan, Italy). Notably, the transduction protocol was optimized by the use of a retroviral supernatant produced in conditions adapted for human CD34+ cells, and cells were transduced in the presence of retronectin and of a culture medium containing a cytokine cocktail (including FLT3-ligand, stem-cell factor, thrombopoietin, and IL-3) that preserved the ability to engraft efficiently into immunodeficient mice and give rise to B and T cell progeny.[35–37]

The use of non-myeloablative conditioning regimen with busulfan in advance of GT was introduced to favor corrected cells' engraftment, making space for the transduced progenitors in patients' BM.[37] The rationale for its adoption originated from gene-marking studies in animal models and from transplantation experiences in the field of hematological pediatric disorders. In particular, busulfan, used alone, was chosen as chemotherapeutical agent based on its wide use in pediatric settings, including SCID patients, and its effects on primitive HSPC. The administered dose represented approximately 25% of the total dose usually used in totally myeloablative protocols. Compared to the combination of cytoreduction or myeloablative chemotherapy and pre-/post-HSCT immunosuppression, this regimen showed low toxicity in the post-GT clinical course. Initially, two ADA-SCID patients, who lacked an HLA-identical sibling donor and could not access to PEG-ADA therapy, were enrolled in the pilot clinical trials. Since none of them received concurrent ERT, the efficacy of GT as single treatment could be fully assessed, exploiting at the same time

the growth advantage for ADA-transduced cells. Thanks to the new protocol, GT resulted in engraftment sustained by gene corrected CD34+ BM progenitor cells, with multi-lineage differentiation, increase of lymphocyte counts, improvement of cellular and humoral responses, including antigen-specific responses, and a substantial reduction of toxic metabolites.[37] These results represented the first demonstration of the clinical efficacy of HSPC GT for ADA-SCID.

DEVELOPMENT OF ADA-SCID GT AT SR-TIGET: FROM CLINICAL TRIALS TO EUROPEAN UNION MARKETING APPROVAL OF STRIMVELIS™

Following these initial studies, results were confirmed and extended in a Phase I/II pivotal clinical trial with a long-term follow-up component and a compassionate use program, conducted at SR-Tiget (Milan, Italy) for a total of 18 patients treated according the same strategy, including the pilot studies.[38,39] Children with ADA-SCID lacking a healthy HLA-identical sibling donor were enrolled. In addition, patients for whom ERT was not a lifelong therapeutic option or for whom PEG-ADA treatment administered for at least 6 months was ineffective or harmful were eligible.

Preliminary results from the first 10 treated patients were reported in 2009,[38] showing improvement in immune functions, ranging from partial to full reconstitution, with adequate systemic detoxification in most patients. Recently, results of all 18 patients treated with this approach were reported[39] for a median follow-up period of 6.9 years (range 2.3–13.4 years at data cutoff of May 2014) with 100% survival. The first patient treated is alive and well and has now reached 16.5 years of follow-up (P. Stepensky, pers. commun.). GT resulted in sustained lymphoid reconstitution[40] with evidence of long-term gene correction in T lymphocytes, improvement of immune functions, restoration of functional thymopoiesis, and effective metabolic detoxification due to restoration of ADA activity in the majority of patients, who remained off ERT (15/18). Most could stop immunoglobulin replacement therapy, showing ability to mount specific antibody response after vaccination and/or infections (*e.g.*, chickenpox). Multi-lineage engraftment of gene-corrected cells stably persisted throughout long-term follow-up in the majority of patients, indicating that, although at low levels, correction of multipotent stem cells was achieved. The treatment provided clinical benefit to patients, with significant and sustained reduction in severe infection rate after GT, continued physical growth, and improved quality of life. On the other hand, there was no indication that GT had an impact on incidence of neurological issues[39] typically present in ADA-SCID patients.[3] On the whole, the safety profile was in line with what can be expected in an ADA-SCID population receiving busulfan conditioning and undergoing immune recovery. Importantly, no serious adverse events were attributable to GT, and no events indicative of myelodysplasia or leukemic transformation of transduced cell clones were reported. All but one patient had viral integrations' insertion site within and/or near potentially oncogenic loci (*e.g.*, *CCND2*, *LMO2*), but clones with these inserts have been present for several years and are stable.[41] An abdominal adipose tumor was found in one patient[42] but was not related to GT.

Based on the positive and promising safety and efficacy data collected from these 18 ADA-SCID children treated from 2000 to 2011,[39] *ex vivo* HSPC GT for the treatment of ADA-SCID, based on the single infusion of an "autologous CD34+ enriched cell fraction containing CD34+ cells transduced with a gamma-RV encoding for the human *ADA* cDNA sequence," was approved for licensure by the European Medicines Agency (EMA) in May 2016, under the commercial name of Strimvelis™ (GlaxoSmithKline [GSK]). This advanced therapy medicinal product (ATMP) is the first *ex vivo* HSPC GT to receive regulatory approval anywhere in the world. Its market approval in the European Union (EU) arrived 25 years after the first pioneering GT trials in ADA-SCID patients.[12] This fundamental milestone was achieved thanks to a joint effort among GSK, the Italian Telethon Foundation, and San Raffaele Scientific Institute, at SR-Tiget, where the medicinal product was originally developed. The alliance signed by these three stakeholders in 2010 was crucial to prepare for the launch of this new medical product, providing the expertise, economic resources, and infrastructure required to complete nonclinical studies[43] and clinical development and to establish pharmaceutical production. The biotech company MolMed S.p.A. produced vector and cells under Good Manufacturing Practices and applied its know-how in product development to reach robustness and suitability for commercial supply. Despite the complexity and the many challenges faced by such a highly innovative product, EU marketing was approved by the EU Commission within 12 months. The Italian Medicines Agency (AIFA) approved Strimvelis™ pricing and reimbursement in Italy in <2 months through an accelerated procedure. Strimvelis™ is now available for ADA-SCID patients lacking a

suitable HLA-matched related donor and is currently administered only at San Raffaele Hospital in Milan due to the short product shelf life. The first patient was treated with commercial Strimvelis™ in March 2017. Long-term monitoring after GT (>10–15 years) will be performed to follow up patients treated with the experimental and approved product. GSK is implementing a prospective long-term observational study according to EMA recommendations in order to monitor the long-term risks of insertional mutagenesis, oncogenesis, immunogenicity, and hepatic toxicity, as well as the persistence of efficacy of ADA-SCID GT over time.

Despite being based on the first generation of vectors for HSPC GT, the safety and efficacy track record of Strimvelis™ represents a model for ATMP development, from early clinical experimentation to drug registration and marketing approval. The relevant results of this process go well beyond the benefit of ADA-SCID patients and include development of strategies to support ATMP quality assessment, specifications, and manufacturing, as well as its administration to patients in the hospital, and, finally but not less notably, the policies adopted for establishing its cost and reimbursement.

GT WITH AUTOLOGOUS BM CD34+ CELLS FOLLOWING NON-MYELOABLATIVE CONDITIONING: POSITIVE RESULTS IN OTHER CLINICAL TRIALS

The positive outcome and the key role of non-myeloablative conditioning in favoring multilineage engraftment of gene-corrected HSPC and achieving therapeutic benefit were also reported by subsequent GT studies for ADA-SCID performed at other centers using other gamma-RV.[44–47]

In 2006, researchers from Great Ormond Street Hospital (GOSH) in London, United Kingdom, reported the success of GT for ADA-SCID based on the adoption of a different conditioning (single dose of melphalan, instead of busulfan) and a different RV in an ADA-SCID patient[44] with an inadequate response to ERT, which was ceased before GT. Two years after the procedure, metabolic and immunological corrections were maintained, with cellular immunity reconstitution, recovery of thymopoiesis, and systemic detoxification, with no adverse events related to gene transfer. Subsequently, seven additional patients were treated with the same approach and are all reported to be alive. Four patients remained off ERT and discontinued immunoglobulin replacement.[6] Immune recovery and metabolic correction in the first six patients treated were reported in 2011[45] (all but one received conditioning with melphalan), showing engraftment of gene-corrected cells resulting in immune reconstitution in four subjects. All showed effective metabolic detoxification and remained infection-free. None developed any leukemic side effect.

Another clinical trial was conducted in the United States at the National Institutes of Health and Children's Hospital of Los Angeles.[46] Ten ADA-SCID patients were enrolled, and two slightly different RV were used to transduce CD34+ cells. Four subjects were treated with no myelosuppression and continued to receive ERT throughout the procedure. Only transient, low levels of gene marking were detected in two older patients who did not show sustained improvement in immunological parameters during the follow-up, while some gene marking persisted in two younger patients. On the contrary, six additional patients withdrew ERT and received low-dose busulfan before GT. This approach led to improved immunological and metabolic outcome in three patients who remained off ERT. Two subjects restarted ERT due to poor gene marking and immune reconstitution. Another patient experienced a prolonged pancytopenia[16] following busulfan conditioning, which was related to a pre-existing cytogenetic abnormality, indicating an important limitation to be considered for autologous HSPC gene transfer. This subject underwent allogeneic HSCT 8 months after GT. Subsequently, the clinical safety and therapeutic efficacy of this last GT approach were evaluated in a Phase II study performed in the same centers,[47] enrolling 10 ADA-SCID patients between 2009 and 2012. All subjects, except for an adolescent, remained off ERT with normalized PB mononuclear cell ADA activity, improved lymphocyte counts, and normal proliferative responses to mitogens. Three patients discontinued immunoglobulin replacement therapy. None developed lymphoproliferative disorders or other GT-related complications. These results further confirmed the therapeutic efficacy and favorable safety profile of HSPC GT in this disease.

Finally, the results of a Japanese clinical trial based on infusion of autologous gene-corrected BM CD34+ cells in two ADA-SCID children with no cytoreductive conditioning[48] strengthened the importance of preparatory regimen to ensure adequate HSPC *in vivo* engraftment. In spite of PEG-ADA discontinuation to enhance the selective advantage of gene-corrected cells, delayed, partial immune reconstitution and moderate detoxification of purine metabolites were observed, allowing only temporary ERT withdrawal.

Autologous *ex vivo* lentiviral GT for ADA-SCID

From first proof-of-concept in humans, vector technology has advanced considerably in the past 25 years. In particular, human immunodeficiency virus–based lentiviruses have been adopted as the vectors of choice for new programs of HSPC gene transfer because of their advantageous safety and efficacy profile. Self-inactivating lentiviral vectors (LV) appear to have a more favorable genome insertion profile and a higher gene transfer efficiency in comparison to LTR gamma-RVs.[49] Following several years of preclinical studies,[50,51] clinical studies using LV are currently ongoing for ADA-SCID.[6,52] They are based on the use of a self-inactivating LV with a codon-optimized human cADA gene under the control of the short form elongation factor-1α promoter (LV EFS ADA). The vector showed decreased transformation potential in *in vitro* immortalization assays compared to the gamma-RV used in previous British and U.S. studies,[51] and in the murine model allowed good immune reconstitution of treated $ADA^{-/-}$ mice. Currently, Orchard Therapeutics in partnership with GOSH and Children's Hospital Los Angeles are developing an *ex vivo* LV GT to restore normal gene function in patients with ADA-SCID by conducting a Phase I/II clinical trial to assess the safety and efficacy of a LV EFS ADA, combined with low-dose busulfan conditioning. Results in the first five patients treated were reported at the 16th Biennial Meeting of the European Society for Immunodeficiencies (ESID) in 2014,[52] showing significant immunological and metabolic recovery, with improved T cell counts and normalization of PHA responses. Integration site analysis showed some expansions but no persistence of expanded clones. All patients were clinically well, and the procedure was well tolerated with no significant adverse events. As of March 2016, >30 patients, some of whom were identified by newborn screening, have been treated with this approach in London and Los Angeles, with promising efficacy results and no associated toxicity.[53,54] Survival was reported to be 100% with a follow-up of 2–49 months, with evidence of immune reconstitution in most patients[6] in the absence of persistent clonal dominance or insertional mutagenesis.[54] Of note, in these current LV GT trials, ERT is continued through the first month after GT based on findings in the murine model, demonstrating equal or even improved engraftment of gene-corrected cells with 1 month of ERT after GT compared to no ERT,[6,55] and on the assumption that ERT in the very early follow-up after GT may help in maintaining a detoxified environment that may improve engraftment. This is in contrast to previous experience in autologous GT with gamma-RV,[37,38] which foresees stopping ERT 1–2 weeks before HSC harvest in order to produce a lymphopenic state/environment to drive production of *de novo* lymphocytes from gene-corrected progenitors, also exploiting their selective advantage.

Safety of GT for ADA-SCID

Since 1990, >80 ADA-SCID patients have been treated with GT worldwide in different centers[6] with experimental GT based on RV or LV, and to date no genotoxic event has been observed. The cumulative experience gained in the past 15 years from HSPC GT with RV indicates that GT for ADA-SCID has a favorable risk–benefit profile, since no events of insertional oncogenesis or leukemic proliferation have been observed over a long period of follow-up. This is consistent with the results of detailed analyses of retroviral integration sites in cells from ADA-SCID patients treated with LTR-intact gamma-RV performed in past years, documenting a polyclonal pattern of vector integrations and T-cell repertoire,[56] lack of *in vivo* skewing for potentially dangerous insertions,[38] and the absence of transcriptional perturbation of cellular proto-oncogenes.[57] Interestingly, Cooper *et al*.[58] recently reported a significant correlation between the intensity of busulfan conditioning and CD34+ cell dose with clonal diversity and T-cell repertoire in a cohort of ADA-SCID patients treated with RV-GT, underscoring the relevance of cytoreductive conditioning in this type of GT setting.

The observed integration pattern included various common IS near proto-oncogenes such as *LMO2*, *MECOM*, *BCL2*, and *CCND2*, together with indications of mild mutagenic clonal disturbances that could be interpreted as molecular evidence of genotoxicity from gamma-RV GT. However, these findings did not lead to any gene dysregulation or frank malignant phenomena so far.[41,53,56–58] Indeed, these clones remained stable over several years and never dominantly expanded or degenerated in dysplasia. Only a benign clonal dominance was reported in a patient, likely due to a gene-corrected NK cell-mediated response to chronic Epstein–Barr virus viremia.[58]

These findings are in sharp contrast with those from GT trials for other primary immune deficiencies, such as X-linked SCID, WAS, and CGD, presumably thanks to peculiar disease-specific factors that may have contributed to this different outcome and toxicity profile (*e.g.*, lower T-cell replicative stress during immune reconstitution in the thymus), suggesting that disease background may

be a critical factor in influencing leukemogenic potential of RV GT.

Current limitations and future challenges

Results obtained so far with GT for ADA-SCID are very promising, but additional improvements could be achieved in the near future.

Conditioning has been demonstrated to be fundamental for appropriate engraftment of gene-corrected cells.[59] The low-intensity conditioning used in GT trials for ADA-SCID so far has been well tolerated by patients. However, to reduce the risk of long-term sequelae of chemotherapy, novel conditioning approaches are currently under investigation. They are based on less harmful monoclonal antibodies, targeted to HSPC in order to deplete them selectively in the BM (*e.g.*, immunotoxin CD45-SAP or anti-*c*-Kit antibodies).[60] Potentially, these strategies could allow robust, multi-lineage engraftment of autologous gene-corrected HSPC with minimal toxicity to non-hematopoietic tissues. This will be of particular relevance for more compromised patients, with pre-existing organ damage (lung, liver) or for those with a milder phenotype, for which risks of conventional conditioning regimen would not be acceptable.

A current limitation of GT is represented by its present availability in only few centers worldwide. This is mainly due to the highly specialized expertise required to conduct clinical trials and the use of cells manufactured with a short shelf life. Studies are ongoing to evaluate the efficacy and the safety of a cryopreserved formulation of LV-based product (ClinicalTrials.gov #NCT02999984), with the aim of allowing patients to be treated in a higher number of clinical centers with expertise in HSCT on a wider geographical area. Autologous HSPC to be corrected could be shipped to centralized pharmaceutical facilities for standardized manufacturing, processing, and quality controls, and then frozen and shipped back to the patient for re-infusion.

CONCLUSIONS

A crucial milestone toward the goal of delivering new medicines for orphan diseases has recently been reached by GT research. Autologous transplantation of gene-modified HSPC has shown to provide clinical benefit for ADA-SCID patients when combined with reduced intensity conditioning and ERT withdrawal. More than 50 ADA-SCID patients have been treated to date in various centers with different gamma-RV GT and are all reported to be alive.[6] In most patients, engraftment of gene-corrected cells led to effective immunological and metabolic correction, without the need of PEG-ADA or allogeneic HSCT. Importantly, to date, no clinical events of abnormal clonal proliferation and/or leukemic transformation have been observed, but safety monitoring will be continued long term in all patients.

The clinical management of ADA-SCID patients has changed, thanks to the progress in GT for ADA-SCID and the increased rate of early diagnosis due to more widespread newborn screening for SCIDs. Following these results, ESID and EBMT have recently updated the guidelines recommending GT as the first option to be considered for all ADA-SCID patients lacking an HLA-identical sibling donor,[6,7] while allogeneic HSCT from unrelated or haploidentical donors or long-term ERT are subsequent alternative options.

The advances in GT for ADA-SCID and other primary immune deficiencies have now opened the way to exploit HSPC GT as a treatment for a broader list of applications, from inborn error diseases to cancer.

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AUTHOR DISCLOSURE

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