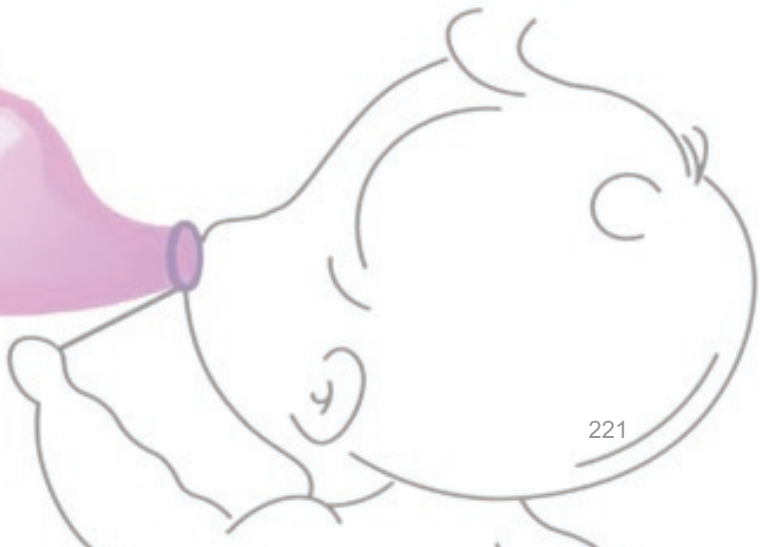
From stem cells to functional lymphocytes: cell differentiation and gene therapy implementation for RAG-SCID

García Pérez, L.

Chapter 8

General Discussion



Severe Combined Immunodeficiency (SCID) is a devastating immune disorder affecting infants lacking a functional immune system, in particular T cells. Infants with SCID will die within the first year of life unless effective treatment is given. Curative treatments are limited and confined to allogeneic hematopoietic stem cell (HSC) transplantation and emerging autologous stem cell gene therapy. Hence, for this thesis I aimed at gaining more knowledge about normal immune development, with detailed focus on the transcriptional network orchestrating T-cell development. In addition, I focused on developing efficient and safe gene therapy approach to correct Recombinase-activating gene (RAG) immune defect, in conjunction with improving related protocols by developing novel tools and conditioning regimens. These findings and implications for the current gene therapy field will be extensively discussed in this chapter.

In **Chapter 2**, a deeper understanding of the key transcription factor network for successful early T-cell development was shown. A novel functional hierarchy of transcription factors was described: Notch signaling induces Tcf1 expression which subsequently has two target genes, *Gata3* and *Bcl11b*, that accomplish a division of labour with Gata3 suppressing non-T cell lineages and Bcl11b inducing the expression of T-cell specific genes. Understanding of this network may reveal potential new mutations or gene dysregulations that can cause SCID related phenotypes.

An overview of the global procedure and steps to successfully develop gene therapy for immunodeficiencies from the bench to the clinic have been described in **Chapter 3**, where I discussed development, efficiency, safety and regulatory hurdles across the process. Importantly, we present autologous lentiviral-based HSC gene therapy as an efficient and safe therapy to correct RAG-SCID deficiency. Successful preclinical development of gene therapy for RAG1-SCID described in **Chapter 4** substantiates the first phase I/II clinical trial worldwide for RAG1-SCID. In **Chapter 5,** we focused on the pre-clinical development of gene therapy to correct the immune RAG2 deficiency which seems to be more challenging due to the crucial role of RAG2 expression levels.

Corrected HSCs with the proper transgene expression are infused back into the patient. Therefore, it is essential to have/get a thorough understanding of the obtained gene therapy product. A novel method for single-cell characterization of the transduction efficiency and transgene expression in the product was introduced in **Chapter 6**. The use of a Branched DNA technique allowed to study the heterogeneity within the gene therapy product and to reconsider the actual proportion of potential therapeutic cells and the underestimated transgene vector copy number.

Successful outcome of HSCT and autologous gene therapy highly depends on proper cell engraftment. To achieve suitable cell engraftment and immune recovery, conditioning regimen prior transplantation is needed. However, these chemotherapy conditioning regimens are entailed with high toxicities leading to short and long-term side effects. Therefore, in **Chapter 7**, I described our efforts to develop reduced toxicity regimens by combining reduced chemotherapy with clinically approved mobilizing agents. However, the focus in this field is moving towards antibody-based regimens that have been proven to drive successful engraftment with reduced toxicity.

IN DEPTH KNOWLEDGE ON T-CELL DEVELOPMENT

T-cell development has been studied predominantly in murine models due to the limited accessibility to human thymic material and lack of genetic loss-of-function human models. Availability, improvement and extensive use of human *in vitro* assays and humanized NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} / SzJ) mouse model has invariably led to substantial advances on human T-cell development knowledge. Although human and murine T-cell development are comparable with respect to the major developmental stages, murine T-cell development remains better characterized. In both cases, the more immature double negative (DN) CD4$^-$CD8$^-$ cells develop into immature single positive cells (ISP; CD8$^+$CD3$^-$ in mice and CD4$^+$CD3$^-$ in human) followed by the double positive (DP) CD4$^+$CD8$^+$ stage and finally become either CD4$^+$ or CD8$^+$ single positive (SP) T-cells after selection. However, multiple subpopulations have been described within the early DN stage in the mouse that can be easily differentiated by a combination of markers. Contrarily, the thymic seeding populations and the division of the DN stage in the human thymus are still poorly defined as murine markers are not applicable to human T-cell progenitors [1, 2]. Recent efforts to uncover human T-cell development have been made using state-of-the-art single cell RNA sequencing (scRNA-seq) to generate a comprehensive transcriptome profile of the diverse populations present in the human thymocytes and human thymic stroma [3-7]. As nicely reviewed by Liu C. et al [8], scRNA-seq in human thymic biopsies has remarkably been able to identify and characterize early thymic progenitors previously more challenging to find because of the cellular scarcity of these populations [3, 6]. In addition, scRNA-seq technology is being applied to refine conventional and unconventional (iNKT cells, ILCs, γδT cells) human T-cell developmental trajectories [4, 9, 10].

T-cell development consists in a complex, multistep progression from HSC through the different developmental stages. Proper T-cell development is orchestrated by an intricate network of transcription factors. Therefore, in **Chapter 2** we focused on understanding the role of the major transcription factors (Tcf1, Gata3 and Bcl11b) in the more immature stages of murine T-cell development. Considering that Notch signalling is required for T-cell development and given that the first T-cell specific target gene is *Tcf7* [11], which encodes Tcf1, we investigated the process of T-cell lineage commitment in Tcf1-deficient mice. We showed that seemingly “committed” DN3 T cells lacking Tcf1 have a promiscuous expression and chromatin accessibility and can dedifferentiate into immature cells that can give rise to non-T cell lineages, including B cells and myeloid cells. Tcf1 is not only required for initiating the T-cell commitment process, but its expression is additionally needed to maintain lineage fidelity. Given that both *Bcl11b* and *Gata3* are key target genes for Tcf1 and our finding that the constitutive expression of Bcl11b in Tcf1-deficient cells fully rescued T cell development, we suggest the following transcription factor network with: Tcf1 expression activating Gata3 and Bcl11b (in collaboration with Notch signalling) and then a division of labor between Bcl11b and Gata3, with Gata3 suppressing non-T cell lineages and Bcl11b inducing the expression of T cell–specific genes. scRNA-seq on murine thymus has also provided evidences of an ordered sequence of gene expression transitions in early T-cell precursors leading to T lineage commitment. At least three transient regulatory states are within the T-cell developmental progression of the same precursor cell and not representing cells of different lineages,

giving new insights into the controlled transition from multipotency to T-cell commitment [12].

The development and application of advanced techniques such as ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) or ChIP-seq (Chromatin immunoprecipitation followed by sequencing) is revealing new insights into the epigenetic landscape of thymocytes. The transition between the DN2 stage and DN3 stage, associated with T-cell commitment, is not only associated with widespread changes in genome-wide transcriptional patterns, but also in abrupt global changes in chromatin accessibility [13]. Successive transformations of chromatin organization are observed across T-cell development, constraining cells into new chromatin states towards irreversible commitment [14-16]. Substantial shifts in epigenome organization have been also observed in crucial developmental stages during human T-cell development [17]. As in murine T-cell lineage commitment, the chromatin regions of genes supporting stemness and alternative non-T cell lineages switch into a closed landscape. An additional drastic change occurs during branching between αβ and γδ T cells, where substantial changes in chromatin accessibility occurs in immature γδ T cells while αβ T cell landscape remains stable [17]. A better understanding of the chromatin and transcriptional network involved in the success of T-cell development in the thymus is essential to understand potential defects or dysregulations related to immunodeficiencies such as the more recent reported SCID cases caused by defective Bcl11b expression [18]. In addition, better knowledge of normal T-cell development may help to elucidate and enlighten the underlying genetic defect of the 20% of “unknown” SCID forms.

SUCCESSFUL PRE-CLINICAL DEVELOPMENT OF LENTIVIRAL-BASED GENE THERAPY FOR RAG SCID

Vector and transgene suitability

Gene therapy for immunodeficiencies has over 2 decades of experience in using integrating RNA vectors like γ-retroviral vectors (γRV) and lentiviral vectors (LV). First attempts to correct ADA and X-linked SCID were accomplished using γRV with efficient stable integration of the transgene as well as providing a successful correction of the disease and a long-lasting therapeutic effect in most of the treated patients. However, some X-linked SCID patients treated with the gene therapy product developed leukemia caused by insertional mutagenesis of the therapeutic vector [19-23]. With the appearance of these adverse effects, the field move towards developing a new generation of safe vectors with decreased risk of insertional mutagenesis. Self-inactivating (SIN) vectors lacking potent enhancers in the LTRs, for both gamma-retroviral and lentiviral vectors were further developed. For LV, this SIN property combined with the well-known 3rd generation LV system resulted in generation of replication-deficient LV [24-26]. Additional vector modifications have been introduced to ensure both safety and efficiency like insulators, polyadenylation signals, the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and codon optimization [27, 28]. Thanks to all the advances made in vector design, SIN lentiviral vectors are the safest to date with improved transgene expression and a highly reduced genotoxicity and side adverse effects compared to γ-retroviral

vectors [29, 30]. Along, alpha retroviral vectors have been designed as an additional safer alternative supporting long-term transgene expression in transplanted HSC with lower genotoxicity [31-33]. Although less used than SIN-LV, SIN alpha retroviral vectors has substantial potential as new candidate vector platform for gene therapy [34].

To correct RAG1 and RAG2 deficiency, we made use of these safer SIN-LVs carrying a codon optimized version of the gene (**Chapter 4** and **Chapter 5** respectively). Codon optimization was used to enhance transgene expression and stability aiming to reduce the total number of transgene integrations and therefore reduce the risk of insertional mutagenesis. Clinically applicable vectors including various promoters were tested to study and achieve optimal transgene expression for each specific case. RAG1 correction was only consistently achieved employing the stronger promoter (MND), while RAG2 required a more modest expression driven by PGK promoter. This difference in RAG expression level requirement for successful T- and B-cell development was in line with the native expression of RAG1 and RAG2 in the bone marrow and the thymus (www.immgen.org, [35]). Interestingly, higher RAG2 expression driven by the MND promoter seems to be detrimental for B-cell development in the bone marrow (**Chapter 5**), potentially due to the impairment of the RAG recombination activity by RAG2 surplus [36]. The different promoter choice for RAG1 and RAG2 highlights the importance to assess the optimal transgene expression per therapeutic gene, as the different deficiencies have different expression requirements.

While a phase I/II clinical trial was recently opened (EudraCT Number: 2019-002343-14) to provide an alternative therapeutic treatment for patients with RAG1-SCID using our SIN LV MND-c.o.RAG1 vector (www.recom.eu), additional safety and efficiency studies need to be performed to further develop a safe LV-based alternative to correct RAG2-SCID. Better understanding of RAG2 expression levels and its relation to immune recovery is needed to develop a safe therapy.

***In vitro* and in vivo modeling**

Both *in vitro* and *in vivo* models are being used to explore immune cell development. In **Chapter 2** we make use of the *in vitro* OP9-DL1 co-culture system [37] to study the T-cell developmental block caused by Tcf1 deficiency. The OP9-DL1 system is an efficient tool for pre-clinical validation of gene therapy in cells from γc deficient patient as a readout of the correction of T-cell development [38]. This assay is very sensitive to subtle differences in cytokines and labile contents of culture media, making it a delicate assay [38, 39]. These last hurdles have been overcome by the generation of an artificial thymic organoid (ATO) system based on a stroma cell line expressing DL1 that efficiently initiates and sustains normal stages of T-cell development from human stem cells, enhancing the positive selection thanks to the 3D structure and the new stroma cell line used [40]. This ATO system has allows to precisely reveal the T-cell developmental blocks from patient HSCs with gene defects causing T-cell lymphopenia such as X-linked SCID, RAG-SCID or Reticular Dysgenesis [41]. A more complex system that better recapitulates the intricacies of human T-cell development is provided by artificial human thymic organoids that can be derived from iPSCs [42]. Such a system can also support the later stages of human T-cell differentiation. However, these *in vitro* assays are focused on T-cell development being

mainly applicable for $T^-B^+NK^+$ SCID phenotypes. The *in vitro* study of more complex SCID phenotypes like RAG1- and RAG2-SCID remains challenging. The development of a useful *in vitro* models to investigate T, B and NK cell development in one assay is crucial for pre-clinical development of SCID therapies.

To study cell engraftment and multilineage reconstitution, we have used immunodeficient mouse models. Fortunately, proper murine models exist recapitulating the human SCID phenotype caused by RAG1 and RAG2 deficiency. We extensively use the RAG1 -/- mouse model [43] and the RAG2-/- mouse model [44] in **Chapter 4** and **Chapter 5** respectively to study efficiency and safety of the gene therapy. Most SCID mouse models are good and mimic human phenotypes as ADA-SCID [45], X-linked (IL2rg)-SCID [46], Artemis-SCID [47]. These mouse models have been principally used to retrieve crucial information for gene therapy like HSC homing, long-term stability, biodistribution or toxicology of the therapy. Unfortunately, other SCID mouse models such as X-linked (IL7r)-SCID does not reproduce the human setting as the mouse model has an extra B cell block that is not observed in humans [48].

The most recent advance for the field was the development of “humanized mice” such as the NSG mouse model, an immunocompromised mouse model where human patient cells can be xenografted and directly tested *in vivo*. This model allows sustained engraftment of human $CD34^+$ stem cells isolated from cord blood, bone marrow or mobilized peripheral blood in adult mice, developing high levels of functional lymphoid (T and B cells) and myeloid cells [49]. Thanks to this model, in **Chapter 4**, we proved for the first time the correction of RAG1-SCID by gene therapy in human patient cells. Furthermore, alternative conditioning with chemotherapy like busulfan represents a suitable alternative offering simple, convenient, individual, weight-adjusted and less-toxic conditioning regimen as discussed in **Chapter 7**. Busulfan is indeed an attractive and effective alternative conditioning model that allows an improved human immune reconstitution, especially T-cells, and better well-being and survival of the mice which is extremely important when working with precious patient material like SCID patient material (**Chapter 4**). So far, busulfan-conditioned NSG mice prior transplantation of gene therapy corrected patient HSCs is the closest model to the clinical protocol before jumping into a clinical trial.

Safety assessment on preclinical studies

Before the clinical implementation of any gene therapy medicinal product, the medicine regulatory agencies like the EMA (European Medical Agency) or FDA (Food and Drug Administration) require a risk assessment including toxicology, biodistribution, integration and genotoxicity studies [50, 51]. Toxicology (full organ pathology) and biodistribution (absence of the vector in non-immune organs) are assessed by *in vivo* studies with the appropriate animal model. Another minimal requirement is to perform integration studies. Unfortunately, some patients from the first-generation clinical trial for X-linked SCID developed T-lymphocyte acute lymphoblastic leukemia due to retroviral insertions near proto-oncogenes [21-23]. Since then, continuous progress to develop a robust technique to detect integration patterns have been made [52-54]. Linear Amplification mediated DNA (LM-PCR/LAM-PCR/nrLAM-PCR) together with sequencing allow a quantitative and qualitative measurement of clonal kinetics for pre-clinical studies and patient follow up; making it a

robust method to understand vector integration pattern of new vectors and potential therapies as well as to detect possible malignancies derived from retroviral insertion. A key point to reduce potential insertion mutagenesis is to keep the transgene integration per cell close to one. Targeting the integration of the transgene cassettes into safe genomic harbors using genome editing techniques could be a potential new approach to reduce insertion mutagenesis due to RV/LV random integration [55]. *In vivo* and/or *in vitro* genotoxicity studies are also highly advisable. One such i*n vivo* assay is based on the degree of tumor onset acceleration and follow up upon transplanting gene-corrected cells on a tumor prone Cdkn2$^{-/-}$ mouse model [56]. Powerful *in vitro* assays are convenient and shows good sensitivity, without requiring inducing leukemias or tumor growth in an animal model. The *In vitro* Immortalization (IVIM) assay [30, 57] is based on the findings suggesting that insertional mutagenesis induce competitive growth advantages *in vivo* [58]. Currently, an advanced version of this *in vitro* system is being developed: "Surrogate Assay for Genotoxicity Assessment" (SAGA), integrating a molecular read-out, which enhances reproducibility, sensitivity, and reduces assay duration [59]. Although *in vitro* assays are powerful to detect genotoxicity, the readout is myeloid skewed as myeloid-inducing differentiation medium favoring the readout of selective myeloid mutants. In addition, IVIM and SAGA are short-term assays which are not suitable as a readout for delayed onset genotoxicity that also occurs. Efforts are being made to develop and adapt these assays in the case of SCID therapy to gain a more relevant B- or T-cell mutants readout i.e., in a lymphoid rather that myeloid background [30, 60]. A last step will be to adapt these assays to human cells instead of murine cells.

For our pre-clinical development of RAG1 and RAG2 LV-based gene therapy we performed an extensive safety assessment using most of the techniques mentioned. Insertion site analysis revealed polyclonal insertions of MND-c.o.RAG1 in murine and human cells (**Chapter 4**) and an oligo/polyclonal insertion pattern of our PGK-c.o.RAG2 in murine cells without insertions near known oncogenes (**Chapter 5**). Safe toxicology and biodistribution as well as no clonal expansion was observed with the chosen vectors. Therefore, MND-c.o.RAG1 LV and PGK-c.o.RAG2 LV (full assessment ongoing) present a safe profile, suitable for further development and implementation in clinical trial. These assays were designed as safety readout of the vector type per se (RV vs LV). However, we observed that the transgene can also drive differences in the genotoxicity and insertion profile. When comparing the MND-c.o.RAG1 vector (**Chapter 4**) and the MND-c.o.RAG2 vector (**Chapter 5**) with the same pCCL backbone and promoter but different transgene, we invariably observed adverse safety concerns for the MND-driven c.o.RAG2 vector compared to our safe MND.c.o.RAG1 vector. The MND-c.o.RAG2 vector showed a more oligoclonal insertion profile as well as the development of immortalized clones in the IVIM assay. In conclusion, these safety assays are not only a readout for vector backbone safety but also seem useful to assess the safety of a transgene.

Recently reported Suspected Unexpected Serious Adverse Reaction (SUSAR) in patients treated with gene therapy (Strimvellis™ and LentiGlobin) forced the temporary suspension of the clinical trials to investigate whether or not the adverse effects were caused by the therapy. In November 2020, Orchard Therapeutics' gene therapy product Strimvelis™ was

linked to a patients' leukemia [61]. Strimvelis ™, which has been approved since 2016 by the EMA to treat ADA-SCID, consist of autologous $CD34^+$ enriched cell fraction transduced with γRV that encodes for the human ADA cDNA sequence. This leukemia case currently under investigation, resembles the leukemia cases that developed in the first-generation γRV trials for X-linked. The company stated that "Preliminary findings suggest this diagnosis may be attributable to an insertional event related to treatment with Strimvelis™"; however, the potential causal relationship is still under investigation. As discussed elsewhere, although ADA-SCID can be cured by cross correction and therefore transduced cells can have clinical benefit, the risk of insertional mutagenesis by integrations near the LMO2 locus remain [39, 62]. Therefore, Orchard Therapeutics is also developing OTL-101, which uses a lentivirus to insert a functional copy of the ADA gene into a patient, envisioning a safer approach to avoid potential insertional oncogenesis [63]. In addition, Bluebird Bio announced in February 2021 that the company has placed its Phase 1/2 (HGB-206) and Phase 3 (HGB-210) studies of LentiGlobin gene therapy for sickle cell disease (SCD) on a temporary suspension due to a reported SUSAR of acute myeloid leukemia (AML) [64]. After thorough analysis, Bluebird Bio reported that "In addition to our earlier findings of several well-known genetic mutations and gross chromosomal abnormalities commonly observed in AML in this patient, our latest analyses identified the integration site for the vector within a gene called VAMP4. VAMP4 has no known association with the development of AML nor with processes such as cellular proliferation or genome stability. Moreover, we see no significant gene misregulation attributable to the insertion event," providing evidence that the vector is unlikely to have played a role in the AML case [65]. A second SUSAR of a myelodysplastic syndrome (MDS) in a patient from the HGB-206 trial was also reported and is currently being investigated, although this SUSAR might be related to the conditioning regimen rather than the gene therapy. In addition, BluebirdBio decided to suspend marketing of ZYNTEGLO™ although no cases of hematologic malignancy have been reported in any patient who has received this treatment for β-Thalasemia because it uses the same BB305 lentiviral vector used in LentiGlobin gene therapy for SCD. Taking into account these adverse events in the gene therapy field the EMA's Pharmacovigilance Risk Assessment Committee released new safety information for healthcare providers for the use of Strimvelis™ [66], although the advice can be extended to any gene therapy product. Long-term follow-up of patients treated with gene therapy, monitoring for possible cancerous changes and late adverse effects are extremely important.

Clinical trial for SCID

Phase I/II, open labelled, clinical trials of autologous HSC gene therapy to treat SCID (ADA, X-linked, Artemis and RAG1) are ongoing worldwide. These clinical trials take place in specialized institutes where patients are referred to. A patient/newborn would be eligible for gene therapy and therefore can be part of the clinical trial if the patient has a confirmed SCID diagnosis, lack an HLA identical related donor for allogeneic HSCT and shows good clinical conditions (or being treated to control infections). To receive the pioneering treatment the patient/newborn, together with the family, needs to relocate to the location of the specialized institution. Most of the time this means the family have to temporarily move to another city and even country to proceed with the treatment. There, the whole

procedure takes place (collection of HSCs, cell transduction and infusion of the cell product) and the patient stays for follow up for several months until the immune system recovery is safely achieved. To reduce the inconvenience of being relocated for several months, a new approach to this kind of clinical trial is being established. RECOMB is a multinational, multicentre clinical trial to treat RAG1-SCID, working on implementing a new idea where the cells will travel while the patient and families can stay close to home. The patient's HSC will be collected and sent to the transduction specialized site (for RAG1-SCID; LUMC, The Netherlands). Subsequently, the genetically modified HSC will be returned to the participating clinical center and transplanted to the patient. A key advantage of this protocol is that the patients and their families will not need to travel across Europe for treatment; instead, only the cells will be shipped. Altogether, this will bring more comfort to the families during this already unpleasant and stressful period.

CHARACTERIZATION AND CLINICAL IMPLEMENTATION OF GENE THERAPY PRODUCT

One key parameter for the success of gene therapy is to achieve the appropriate transgene expression in the therapeutic cells. Low transduction efficiency and expression in the therapeutic cells could lead to non-correction of the disease, while a high number of transgene insertions can lead to serious adverse effects as described previously. Therefore, it is important to confirm suitable transgene insertion and expression in the gene therapy product, ideally prior infusion, to allow for a safe immune reconstitution. As discussed in **Chapter 3**, ideally one transgene copy per cell is desired. The standard method to control transgene insertion is by Quantitative Polymerase Chain Reaction (qPCR) in the isolated DNA, by detecting a vector sequence relative to a household gene. Detection of stable transgene insertion into the DNA is measured after culturing the cells at least for 9 days. Although determining the transgene copy number in the gene therapy product is one of the main product release criterions, no standardized protocols across lentiviral-based gene therapy treatment sites have been established. Standardization of the duration of cell culture before determining the vector copy number (VCN), as well as of the vector sequences and household gene amplified, could be implemented to set a global protocol for HSC based lentiviral gene therapy field. In addition, the measured VCN by qPCR is detected in the overall gene therapy product, while different cell subpopulations might be differentially transduced.

Therefore, in **Chapter 6** we introduce, describe and validate a novel method to detect transgene expression at the single-cell level by flow cytometry. This DNA branched method, the PrimeFlow™ assay, has suitable specificity, sensitivity and reproducibility. In addition, this assay can be customized and easily adapted to other therapeutic transgenes, especially for codon optimized transgene that are being broadly implemented. The branched DNA technique can be used as a gene therapy tool for virus titration, promoter strength assays and to study the gene therapy product heterogeneity. Importantly, this DNA branched assay allows an accurate and extensive characterization of the product, defining the actual proportion of targeted therapeutic cells and reconsidering the actual VCN in these therapeutic cells. Robust predictors of the

transplantation outcome and immune recovery of gene therapy cell products are still lacking as the VCN detected in the bulk of the gene therapy product does not always correlate with the cell engraftment and graft duration. A more robust understanding and extensive characterization of the infused therapeutic product will allow to better study, understand and correlate key variables of the transplantation outcome and immune recovery of the patients treated with gene therapy.

In most ongoing clinical trials using lentiviral-based gene therapy, the analysis of the gene therapy product is essentially performed after the product is already infused into the patient; cells are transplanted freshly after transduction. A limited portion is kept *in vitro* for analysis up to 2 weeks later. To avoid the transplantation of non-suitable gene therapy products, a new approach will allow transplanting the gene therapy product after confirming the compliance with all criteria, including an appropriate VCN. After *ex vivo* transduction, the gene therapy product is frozen down until the product release tests have been finalized. Only when the product complies to all release criteria, it will be transplanted into the patient. This approach additionally allows for the therapeutic cell product to travel from the production site to the gene therapy sites instead of the patient and families, making the treatment more accessible and comfortable for the newborn families.

FUTURE PERSPECTIVES OF GENE THERAPY

Enhancing HSC availability and targeting

In the clinical setting, the enriched $CD34^+$ fraction, a mix of progenitors and long-term HSCs, is used for HSCT and gene therapy manipulation. The easy accessibility and isolation of these cells from bone marrow aspirates or mobilized peripheral blood makes it an easy target population to manipulate. However, obtaining a sufficient number of cells for *ex vivo* manipulation and successive transplantation and achieving appropriate gene correction for cell therapy remain two fundamental challenges in the field.

HSPCs for therapeutic use are accessible thanks to the improved protocols for collection and isolation. However, the limited number of HSPCs to make a therapeutic product remains a challenge. Hence, enormous efforts have been placed into improving HSC culture protocols to successfully maintain and even expand HSC *ex vivo*, and therefore, help to overcome the shortage of primary material. HSC have the potential to undergo symmetric and asymmetrical cell division. To expand HSC population *ex vivo* approaches resulting in symmetric cell division and self-renewal without further differentiation are desired [67]. Combination of growth factors and cytokines like SCF (Stem Cell Factor), TPO (Thrombopoietin), FLT3-Ligand, IL-3 (Interleukin 3) and IL-6 are frequently used in HSC culture. Although widely used in culture, this combination seems to regulate survival and proliferation of short-term HSCs rather than long-term HSCs [68]. Newer compounds with potential to expand long-term HSCs *in vitro* have been found through library screening on human enriched $CD34^+$ cells. Stemregenin1 (SR1) molecule supports HSPC expansion *in vitro*, with an increase of multipotent progenitors rather than long-term HSC, leading to faster recovery of neutrophil and platelets *in vivo* [69-71]. Other recently identified compounds to expand human HSPC cells *in vivo* and supports expansion *ex vivo* are CPI203, a BET inhibitor that acts at the epigenetic level [72, 73], and C2968 (Chrysin), an antioxidant small

molecule [74]. Prostaglandin E2 (PGE2) [75] and UM171 [71, 76] not only result into *in vitro* HSPC expansion and enhanced long-term repopulation potentials, but interestingly enhance gene transfer in HSCs [75, 77, 78]. In addition, UM171 is being tested on an ongoing clinical trial for allogeneic stem cell transplantation (NCT02668315).

The common readout of HSC expansion is the total number of HSPCs, however, a more extensive characterization of the different subpopulations with advanced flow cytometry panels and the use of conventional and spectral flow cytometers will give a better insight into the expansion of long-term HSC or more progenitor cells. Additionally, further testing on transduction efficiency of HSC with clinically relevant vectors need to be further investigated as well as the molecular mechanisms underlying expansion of LT-HSCs and how is this linked to multilineage differentiation potency. The recent advances in HSC expansion *in vitro* are promising for application not only for allogeneic HSCT but also to be implemented in gene therapy and gene-editing protocols.

Transduction enhancers (TEs) have been shown to be valuable compounds in achieving appropriate gene transduction and expression in the gene therapy product by boosting VCN in primary HSCs. Protamine sulfate, a transduction-promoting polycation, have been extensively used in the gene therapy field to efficiently enhance gene transfer into HSCs with low toxicity [79] as we used to ensure appropriate transduction with our RAG1 and RAG2 LVs in **Chapter 4 and 5** respectively. Various TE compounds have been discovered and developed more recently to further increase lentiviral transduction efficiency, VCN and transgene expression in a broad range of primary cells, even murine T-cells for which lentiviral transduction with protamine sulfate was still less successful [80]. TEs can enhance entry into the cell of interest by increasing co-localization (i.e. RetroNectin [81, 82]) or by reducing the repulsion (i.e. Protamine Sulfate [79], LentiBOOST [83] or Staurosporine [84]) between the target cells and the viral particles. Alternatively, TEs can enhance post-binding/entry mechanisms by affecting intracellular processes (for example Prostaglandin E2 (PGE2) [75], Cyclosporin and Rapamycin [85]). Interestingly, PGE2 (as described above) has additional beneficial effects in promoting self-renewal and transplantation efficacy of HSPCs. As TEs possess distinct mechanisms to enhance LV transduction in HSPCs, combinatorial TE application has also been tested, yielding even more potent effects [84-86]. When compared to previously established TEs such as protamine sulfate, this variety of novel TEs achieved higher transduction efficiency in all HSPCs subpopulations including the long-term repopulating HSCs, without changing viability, integration sites pattern, global gene expression profiles, *in vivo* toxicity or differentiation capacity *in vitro* (colony-forming assay) and *in vivo* (NSG mouse model). TEs have been tested in both murine and human cells as well as healthy and patient donor cells and are already manufactured in a GMP-compliant manner, facilitating their application in clinical protocols. With this approach, the transduction efficiency of LVs can be maximized, requiring less virus per product and enabling to the use of one batch for more patients as well as allowing the reduction of transduction protocols from 2 transduction hits to one [86, 87]. In addition, enhancing transduction efficiency by using TEs will prevent the production of gene therapy with low transduction efficiency and transgene expression that would then be unsuitable for treatment. The use of TEs may additionally allow for getting reliable effects to achieve

the correct VCN in the gene therapy product across patients. Altogether, this strategy makes gene therapy more accessible because of reduced production costs of viral vectors per patient which will help to implement gene therapy as a standard protocol.

The gene therapy field is continuously evolving; an alternative approach has been developed by further narrowing the isolation of HSPCs to ideally a pure long-term HSC population with a clinically relevant method. A GMP-compliant platform based on immunomagnetic-based cell sorting has been developed to purify large cell numbers of $CD34^+CD38^-$ cells, quickly and with good recovery [88]. This $CD34^+CD38^-$ population is more enriched for long-term HSCs, decreasing the number of cells needed for transduction *ex vivo* and transplanted back into the patient; reducing the amount of therapeutic viral supernatant needed [89]. However, myeloid reconstitution after purified $CD34^+CD38^-$ transplantation was delayed [89]. Individual clone tracking follow up of patients treated with autologous HSC LV-based gene therapy revealed that the first wave of immune reconstitution is accomplished by progenitor cells present in the bulk HSPCs [90] which can explained the delayed in myeloid immune reconstitution when more purified HSC populations are transplanted. Potentially, a gene therapy product including a mix of purified corrected $CD34^+CD38^-$ (or even long-term HSCs) with an untransduced fraction of the progenitor $CD34^+$ subpopulations could lead to a satisfactory and quick post-gene therapy recovery.

Gene addition vs gene editing approach for SCID and immunodeficiencies

To correct RAG1 and RAG2 immune deficiency, we used a gene addition approach as discussed in **Chapter 4** and **Chapter 5** respectively. With this approach, a correct version of the defective genes RAG1 or RAG2 (in this case codon optimized) under the control of a constitutive promoter is integrated semi-randomly into the DNA of HSPCs using a lentivirus as a vehicle. While to correct RAG1 deficiency only the strongest promoter, MND, consistently lead to successful immune reconstitution (**Chapter 4**), RAG2 expression seems to be more modest and regulation tighter. The assumed advantage of RAG proteins is the RAG1/2 complex is only active as heterodimer. Therefore, even if one RAG protein is expressed constitutively, the activity of the complex will be properly regulated in the normal fashion by the counterpart protein. However, an excess of RAG2 protein adversely affect B cell development (**Chapter 5**), potentially due to disruption of the proper activity of the RAG complex. Hence, RAG2 deficiency will benefit from direct correction in the genome conserving its physiological expression. Indeed, genome editing approaches are predominantly attractive to correct diseases where the defective gene is under strict transcriptional regulation like RAG2, IL7Rα, JAK-3 or CD40L, leading to preserve physiological expression patterns and prevent severe adverse side effects [91].

Genome editing platforms with programmable artificial nucleases are being explored to enhance precision in the cell and gene therapy field (See **Figure 1**). Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and more recently RNA-based CRISPR/Cas9 nucleases (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) are being developed to precisely target into a predetermined sequence of the genome a DNA double-stand break (DSB) or a nick. ZFN and TALEN nucleases are based on protein-DNA interactions to mediate the DSB and

therefore requires complex protein engineering for each new target. However, CRISPR/CAS9 nuclease comprising high-fidelity Cas9 variants and Cas9 nickase-based techniques relies on a RNA-guided endonuclease (RGN) used to recognize the complementary protospacer DNA target in the genome. This RGN approach provides additional simplicity, easier programming, lower cost and the potential to multiplex editing. These engineered nucleases induce a site-specific chromosomal DSB into the chosen and unique DNA sequence in living cells triggering the distinct endogenous repair pathways. DSB in the cells can be repair by Non-Homologous End Joining (NHEJ) or by Homology-Directed Repair (HDR). While the end-to-end ligation by NHEJ frequently introduces small insertions/deletions ("indels") leading to functional inactivation of the targeted gene, HDR allows the introduction and the precise replacement of a desired sequence by delivering a homologous DNA template. Correction of a mutation in the genome entails the introduction of an exogenous template DNA bearing homology to the sequences flanking the DSBs and the activation of the HDR machinery [92-94].

Genome editing to treat SCID and other Primary Immunodeficiencies poses an attractive option to re-establish physiological expression and regulation of the defective gene having a significant impact in both safety and efficacy. Notable advances in gene editing have been made for X-linked SCID [95, 96]. Targeted gene correction of the *IL2RG* locus in both murine HSCs and human (healthy and patient) HSCs have been successfully obtained using donor DNA template and a ZFNs approach [97, 98] or more recently a CRISPR/Cas9 based strategy [99]. Gene-edited HSCs from both innovative strategies sustained normal hematopoiesis and support multilineage lymphoid development with a selective growth advantage over non-edited cells. Although relevant levels of gene editing have been reasonably achieved in HSCs by homologous recombination, clinical trials using gene editing in HSC are nowadays confined to gene deletion strategies like the disruption of the Bcl11a erythroid enhancer for β-Thalassemia and Sickle cell disease patients (reviewed by Zittersteijn et al. (2021) [94]).

Gene editing in HSCs is still in its infancy and the field is facing some challenges to improve the strategy. A crucial challenge in targeting HSCs comes from their quiescent status, the poor accessibility to target sequences [100, 101], the limited efficiency of the HDR machinery and the insufficient uptake of the DNA template. Additionally, HSCs are prone to differentiation and apoptosis in response to the DSBs, damaging the survival and the self-renewal capacity of the cells. Efforts to increase gene editing efficiency are being focused on improving HSC expansion *in vitro*, inducing cell cycle without triggering cell differentiation and enhancing HDR levels by for example blocking transient P53 pathway activation [102]. Base editing is emerging as new genome-editing approach without involving HDR, a key current limiting factor of gene editing in HSCs. Without making DSBs, Cas9-base editing directly modifies point mutations (adenosine and cytosine) in non-dividing cells [103]. A major safety concern of these state-of-the-art techniques is the off-target modifications produced and the on-target adverse effects reported [104-106] that can potentially cause genotoxicity to the progeny. Therefore, assessment of off-target and on-target events should be better defined and reduced to a minimum. Unfortunately, no genome-editing selection method is available to detect and enrich for edited-HSCs, which

due to the low editing efficiency achieved in the HSPC product may lead to low cell engraftment capacity, limited functional immune reconstitution and overall unsuccessful transplantation.

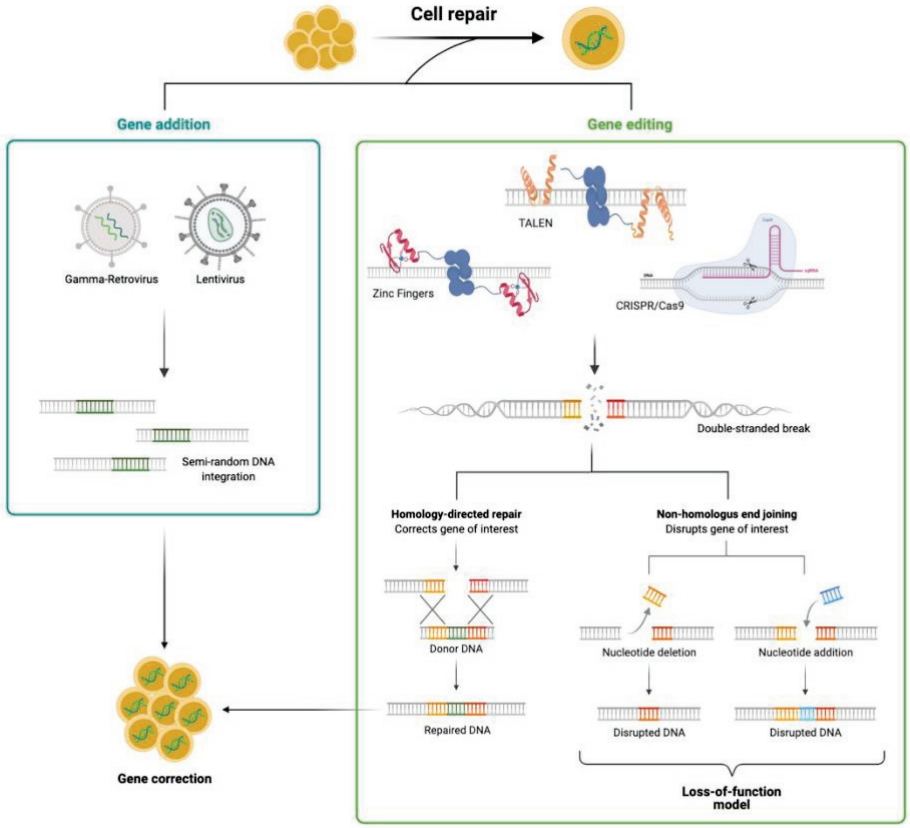


Figure 1: Gene addition vs gene editing approach overview for gene correction on HSCs. *Gene correction of HSCs by gene addition is by semi-random integration into the DNA using retroviral or lentiviral vectors. Gene editing can be achieved by programmable artificial nucleases (Zinc Fingers, Transcription activator-like effector nuclease TALEN or CRISPR/Cas9). A double-strand break is induced into a specific sequence in the genome by the nucleases, triggering the homology-directed repair or the non-homologous end joining repair signals. Repaired DNA can be achieved through homologous recombination and a donor DNA template. Non-homologous end joining repair leads to a loss-of-function model of the targeted gene.*

Gene editing is an interesting approach to correct RAG deficiency (both RAG1 and RAG2) as their expression is strictly regulated during specific stages of B- and T-cell development. RAG genes are not expressed in HSPCs, presenting a packed chromatin and therefore resulting in difficulty targeting of the genes. Cas9 and DNA donor template delivery for HRD is therefore probably lacking efficiency resulting in a low frequency of edited cells. Additionally, for RAG1 and RAG2 multiple unique mutations have been identified in

patients (more than 150 and 50 respectively) [107] making it virtually impossible to implement a base-editing approach to correct RAG deficiencies. Therefore, gene addition and gene-editing by HDR of the full-length RAG genes realistically are the most suitable approaches to result in RAG immune correction, taking into account continuous improvement of the class of medicines [95], it remains to be explored whether the gene editing approach will be more beneficial than "conventional" gene addition therapy for SCID, particularly for RAG-SCID.

New approaches for SCID modelling

As mentioned previously, one main challenge in HSC studies as well as clinical application is the limited number of patient cells available. Therefore, there is a need to find suitable solutions to the shortage of patient cells to study pre-clinical efficiency and safety of novel therapies in human cells.

Human induced pluripotent stem cells (iPSCs) generated from somatic cells from patients can provide a good approach to model SCID *in vitro*. iPSCs generated by the overexpression of the Yamanaka factors [108, 109] can be differentiated *in vitro* into T-cells [110-112]. Successful iPSCs modelling for X-linked chronic granulomatous [113, 114], X-linked SCID [115], JAK3-SCID [116], WAS [117], RAG1-SCID [118, 119] and RAG2-SCID [120] have been generated allowing the study of the immune phenotype caused by the genetic mutation as well as enabling preclinical efficacy and safety studies. However, as these diseases are rare disorders the procurement of patient somatic cells is still limited.

Alternatively, $CD34^+$ cells enriched from cord blood is the major source of HSCs available for research. Ideally, immunodeficient-related gene mutations (i.e., gene knock-out) can be generated in cord blood HSPCs using gene editing tools such as CRISPR/Cas9, allowing the generation a platform with patient-like HSCs from healthy cord blood enriched $CD34^+$ cells. Patient-like edited HSCs can be used to study *in vitro* and *in vivo* immune development as mentioned previously, by OP9 or thymic organoid system and NSG xenotransplantation. This approach could be used to further study the immune phenotype of known genes (*IL2rγ, RAG1, RAG2, Artemis*, ...) in more detail. Indeed, a novel source of patient-like HSCs will also allow more extensive pre-clinical efficiency and safety testing of novel therapies in human cells, now usually confined to none or limited testing in human cells before clinical implementation. In addition, this platform could be used to study potential T-cell related developmental genes. As the study in murine *Tcf1*-knock-out (KO) model in **Chapter 2**, ideally human *TCF1* KO $CD34^+$ could be generated to study the role and importance of TCF1 in human T-cell development. Generated human TCF1 KO HSCs can then be investigated *in vitro* (OP9 system or thymic organoids) and *in vivo* (NSG mice). Similarly, the role of GATA3 and Bcl11b in human cells could also be studied, as well as other interesting genes involved in human immune cell development, by implementing this cord blood $CD34^+$ cell derived gene editing platform.

Manufacturing challenges in gene therapy

For clinical implementation of research-based gene therapy treatment, the manufacturing of both the lentiviral starting material as well as the gene therapy medicinal product need to be scaled up and translated to a GMP (Good Manufacturing Practice)-compliant manufacturing process. Good technology transfer from the research group to the

manufacturer is crucial when this process is outsourced to a Contract Manufacturing Organization. Otherwise, (parts of) the manufacturing process can be performed in the academic environment when proper GMP-compliant cleanroom facilities with suitable equipment are available. To bridge the gap between research and GMP production, collaboration between knowledgeable departments and qualified personnel is essential. To comply with GMP guidelines is not easy and research-based protocols may need to be adapted to the new demands such as producing larger volumes or the use of more sophisticated methods to ensure a high-quality product as discussed in **Chapter 3**.

As the gene therapy field is rapidly reaching several clinical trials, the production of highly concentrated and purified large-scale virus batches is in demand. Scalability issues remain a crucial challenge for lentivirus production. A research-based transient transfection protocol on adherent cells is used, which become costly as high-quality raw materials that are required are expensive. The main problem with adherent cells in the scaling-up is the huge surface area and the laborious manipulations needed. From a single research culture flask, large-scale protocols are adjusted to multi-layer flasks, allowing a higher surface to culture cells in the same space. However, the increase in LV production persists modest as cell density is still “low”. To overcome the lack of scalability, different systems that increase cell density by extending the surface to adhere have been developed, such as hollow fiber bioreactors [121] or fixed-bed bioreactors [122]. Another approach in development is the adaptation of adherent cells to suspension cultures, achieving greater cell density and easier scaling-up. To achieve such manufacturing, the use of a stable lentiviral producing cell line would be ideal, as cell lines are easy to scale up and adapt to serum-free medium and culture in suspension. Nevertheless, suitable GMP-grade lentiviral producing stable cell lines are not yet available [123-126]. Concentration and purification processing of large amount of lentiviral supernatant produced is crucial to achieve high purity and high titer viral batches. Various methods have been established for this downstream processing for which a streamlined combination of many techniques is being used. These methods such as anion exchange chromatography [127, 128], size exclusion chromatography [129], affinity absorption chromatography [128] or tangential flow filtration [130] are different and more sophisticated than research-based methods, but are legitimately suitable for lentivirus downstream processing.

Protocols to successfully isolate human $CD34^+$ cells and transduce them with the GMP-grade LV also needs to be adjusted from a research laboratory setting to be able to manufacture a suitable medicinal product for clinical use under GMP compliance. The isolation and transduction process is adapted to reduce contamination risk. Therefore, close system purification instruments have been available and used to successfully enrich large numbers of cells like CliniMACS (Milteny Biotec) or Prodigy (Milteny Biotec), an updated semi-automated version including a transduction protocol [131, 132]. In addition, high-quality grade available transductions enhancers have improved the efficient transduction of high number of cells, creating a unique potential to improve the availability and standardization of HSC gene therapy.

The last bottleneck in manufacturing is the extensive quality control required for both the lentiviral starting material and the gene therapy medicinal product. Extensive

characterization of each GMP grade product batches is needed to reduce potentially adverse effects of the therapy. Thorough assessment of the purity and microbial contamination (e.g. endotoxin, bacteria, yeast, mycoplasma, toxic agents and residual host cell protein and DNA free), safety (e.g. Replication competent lentivirus negative and residual plasmid negative) and potency (e.g. transgene identity, viral titer, cell numbers, viability, transduction efficiency, product stability) is requested from both the therapeutic lentiviral batches and the individual gene therapy products before final clinical use.

IMPROVING ALLOGENEIC AND AUTOLOGOUS GENE THERAPY TRANSPLANTATION OUTCOME

Continuous improvement of the gene therapy product has captured profound attention as a key part of the gene therapy procedure. However, advances on other essential steps of the whole transplantation protocol can also provide a massive impact on the success of the transplantation outcome.

Advantages of newborn screening

An improved HSCT outcome, better survival and lower morbidity rate is observed in SCID patients with early diagnosis and early treatment [133, 134]. Newborn screening allows for the detection and identification of immunodeficiency before severe infections and deterioration of the infant health would occur. In addition, curative treatment can be administered earlier, which is associated with better recovery. A higher survival after successful HSCT and cost-effectiveness has been demonstrated after HSCT before the age of 3.5 months [134, 135].

Newborn screening for SCID and severe T-cell lymphopenia is performed using dried blood spot samples routinely collected from newborns. This test is based on quantification by qPCR of TRECs, a small piece of DNA known as T-cell receptor excision circles. These TRECs are formed in the thymus during T-cell receptor gene rearrangement and are, therefore, a marker for the number of naïve newly formed T-cells. TRECs quantification by qPCR is implemented as an indicator of intrinsic T-cell development and maturation, avoiding the detection of maternal circulating T-cells, that can mask the deficiency during the first months of life. Patients with SCID or severe T-cell lymphopenia are characterized by undetectable/very low levels of circulating TRECs. The TREC assay therefore represents a suitable strategy to successfully identify asymptomatic SCID patients before the onset of infectious complications, to provide an early treatment and consequently improve the quality of life of the young patients [136, 137].

In addition, newborn screening for SCID has revealed novel genes causing immunodeficiencies such as Bcl11b [18] and allows for the detection of a higher number of cases that were previously missed. When the TREC assay test positive (low counts), the patient is referred to a pediatric specialist for investigating the underlying defect by immuno-phenotyping and genotyping for SCID. This screening program is yielding incomparable information about disease incidence, spectrum and outcomes; however, a fraction of SCID patients remains for whom the underlying genetic defect is unknown. To note, newborn screening for SCID is also accompanied by a number of secondary and incidental findings influenced by the TREC cut-off value like the diagnosis of other

syndromes with T-cell impairment (DiGreorge syndrome, Trisomy 21, Noonan syndrome or Ataxia Telangiectasia). A follow-up protocol with regard to these incidental findings (Ataxia telangiectasia) should be established in parallel [138]. Additionally, a second tier newborn screening test for SCID is being developed to reduce the number of control samples, recalls and false positives [139].

Altogether, the TREC assay became the first assay that is capable of identifying the presence of an immune disorder in the setting of large scale newborn screening. In addition, it is the first high-throughput DNA-based newborn screening assay [140, 141]. The TREC assay for immunodeficiencies remains a major regulatory and logistical challenge but it is slowly, successfully being implemented in many countries around the globe like United States [142 143-145], Israel [146], New Zealand, Taiwan [147], Canada (several provinces), Australia (some regions) and some European countries like Spain (Catalonia) [148], Iceland, Norway [139], Switzerland (2019), Germany, Sweden [149], Denmark [150] or The Netherlands [151]. As Newborn screening for SCID is broadly being implemented and unfortunately countries are using its own definitions (cut-off values, screening algorithm and referral policies) there is now an emerging need to uniform (inter)national registration of screen-positive cases and screening terms.

Towards a reduced, less toxic, conditioning regimen prior to transplantation

Patient conditioning prior to HSCT is needed to get suitable cell engraftment and immune reconstitution. However, current conditioning regimens are linked to short- and long-term toxicity, which is even more prominent for young patients/babies. SCID patients are not only under one year old at the time of transplantation, but also usually present with comorbidities and a delicate health status. Therefore, there is a need to improve pediatric conditioning regimens and develop reduced toxicity regimens that will improve the HSCT outcome without toxicity, especially for more delicate SCID forms such as RAG deficiency or the radiosensitive Artemis form.

In **Chapter 7** we attempted an alternatively approach to reduce the current busulfan-based chemotherapy conditioning regimen. Our approach consisted in reducing the busulfan dose by combining it with clinically approved mobilizing agents used to mobilize HSCs from the bone marrow to the peripheral blood. While an interesting reduction of the HSC compartment was observed in bone marrow with the novel combinations including G-CSF or Plerixafor, no significant difference were achieved in the transplantation outcome. A similar approach employing a combination of G-CSF and Plerixafor as conditioning agents to efficiently mobilize HSCs before transplantation was also tested in a small pilot trial (6 SCID patients). Although well-tolerated, donor T-cell development was observed but no donor myeloid nor B cell engraftment [152], showing that HSC mobilization is not enough to allow proper donor HSC engraftment in bone marrow.

Reduced busulfan-based conditioning may also be insufficient in some forms of SCID like the RAG1/2 SCID where there is a more prominent occupancy of bone marrow niches by (B-cell) precursors cells blocked in development. In RAG1/2 SCID, lymphoid precursor cells completely occupy bone marrow and thymic niches which highly compete with transplanted cells leading to poor immune reconstitution [153, 154]. For this patient group, a greater risk of graft failure and poorer T- and B-cell reconstitution have been reported in

the absence of conditioning compared to other SCID forms [155-157]. Depletion of the early B-cell compartment in the bone marrow prior transplantation can also be an interesting therapeutic option that may lead to improve B-cell engraftment and development after HSCT. Furthermore, T-cell reconstitution can be improved and accelerate by additionally transplanting *in vitro* cultured T-cell precursors [158].

Recent and promising developments of antibody-based conditioning are extremely attractive to the field, as they ideally will provide the ability to achieve donor chimerism without the use of toxic chemotherapeutic agents. A diverse range of antibodies drug conjugated (ADC) targeting stem cell markers such as CD45 and CD117 have been developed and offered potential to deplete HSCs in bone marrow without toxicity *in vitro* and *in vivo*. Anti-CD45 antibody have been shown to effectively deplete HSPC when conjugated to Saporin, a ribosome inactivating protein lacking the cell-entry domain and toxic only upon receptor-mediated internalization. A single dose anti-CD45-Saporin pre-conditioning of immunocompetent mice enables efficient HSC engraftment and rapid B- and T-cell recovery [159]. As CD45 not only selectively target hematopoietic progenitors but also all leucocytes, a profound lymphodepletion can occur which will enhance the susceptibility to opportunistic infections until immune recovery [159]. However, it can be a promising candidate to target autoreactive T-cells in combined immunodeficiency with granuloma and/or autoimmunity (CID-G/AI) patients or to target blocked B-cell precursors in RAG deficient bone marrow patients that usually hamper HSCT outcome and immune recovery [160]. Alternatively, anti-CD117 (c-kit receptor) antibody drug conjugates specifically depletes host HSC with no effect on differentiated progenitor or mature cell lineages and therefore preserve immunity. Naked anti-CD117 in murine studies result in an effective and safe single-agent approach to use as conditioning regimen leading to donor engraftment [161]. However, to improve potency, anti-CD117 was combined with low-dose irradiation [162], CD47 antagonism [163] or more recently, conjugated to Saporin [164]. With the high specificity to deplete HSC together with the minimal toxicity observed, anti-CD117 is being translated into the clinic as a potential nonmyeloablative conditioning strategy. An ongoing clinical trial for SCID patients using the naked humanized anti-CD117 antibody version (NCT0296306) shows that the antibody is able to safely clear human HSC niches and facilitate donor HSC engraftment [165].

CONCLUDING REMARKS

Altogether, the work described in this thesis moves towards a regular enforcement of gene therapy treatment for immunodeficiencies for which potential improved/to improve milestones during the overall procedure are described in Figure 2. In contrast to allogeneic HSCT, gene therapy is seen as a pharmaceutical drug named Advanced Therapy Medicinal Products (ATMPs) and entered via legislation into the pharma world. Although understandable, this has made development of gene therapy-based medicinal products much more complex, with long regulatory procedures and increased costs. Although pharmaceutical companies are needed for drug development, registration, pharmacovigilance, commercialization and wider distribution, this process comes with a price tag. The pricing for a curative medicine that is administered only once to a given

patient is still subject to much debate. Fair pricing that societies can afford (for instance via health insurance reimbursement) must be balanced against the profits needed by biotech and pharma companies to survive. This is imperative if we don't want the uncurative diseases from the past that can now be elegantly treated with stem cell-based gene therapy, to become unaffordable for patients.

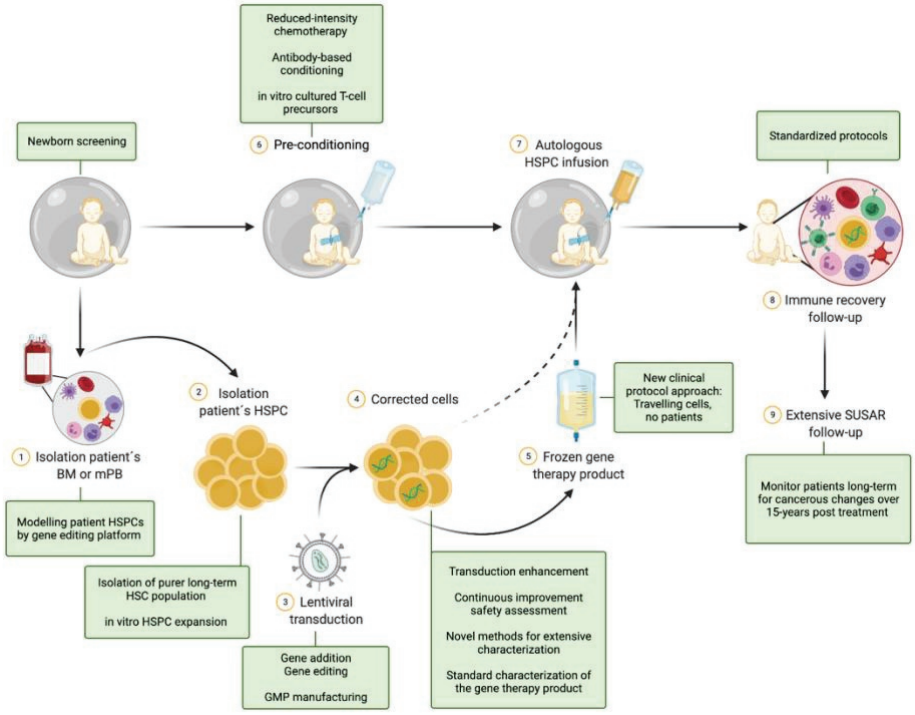


Figure 2: Overview of the autologous HSC gene therapy treatment and potential improved/to improve milestones during the overall procedure.

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