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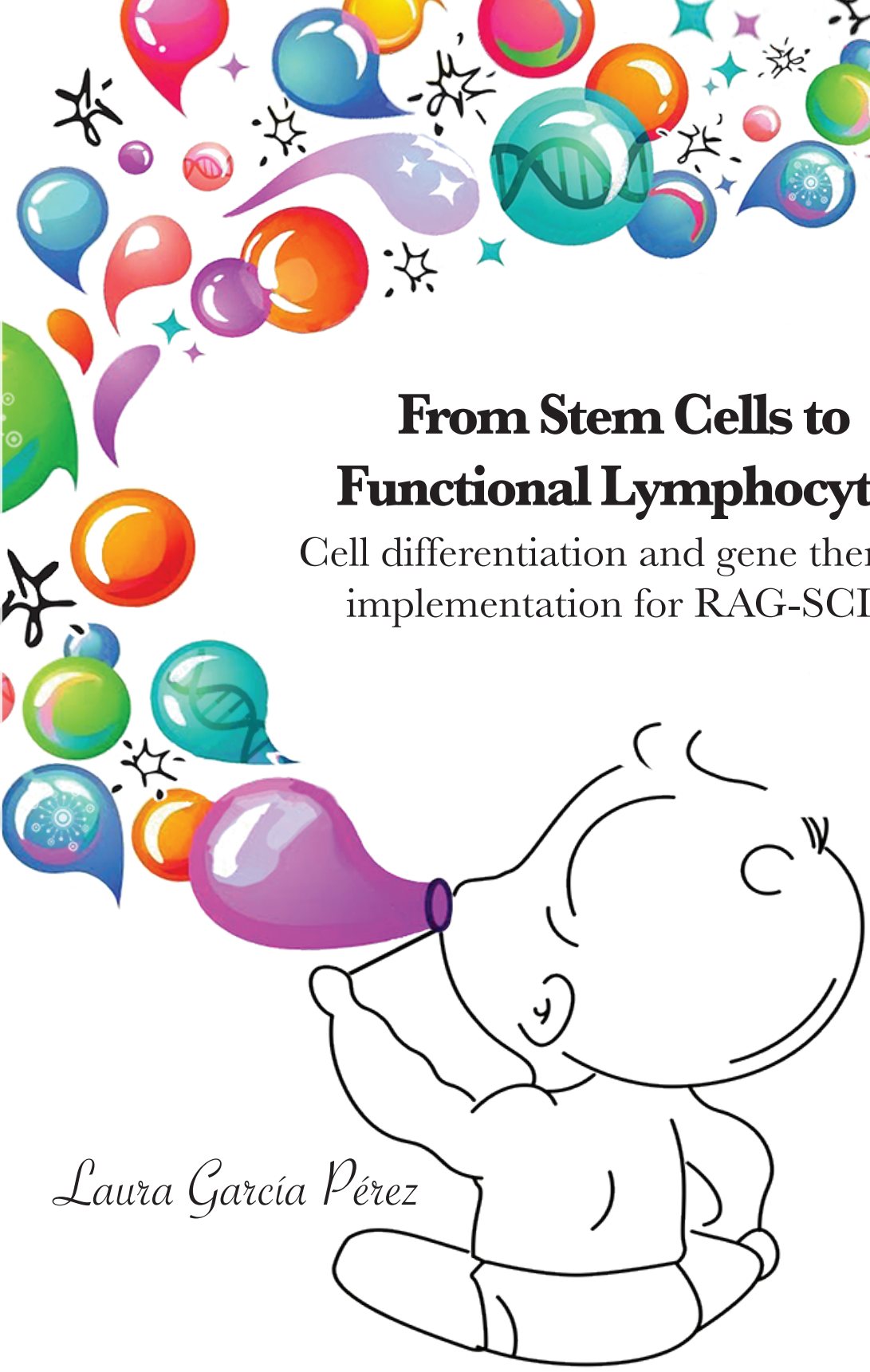

Laura García Pérez

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Cell differentiation and gene therapy implementation for RAG-SCID

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Cover: Severe Combined Immunodeficiency is also commonly called the "bubble baby disease". Babies affected with the disease need to live in a sterile environment symbolize by the bubble. The development of gene therapy to treat the disease is progressing successfully as described in this thesis allowing to reconstitute a normal immune system and therefore get out of (burst) the bubble. The development of these new complex therapies allows to progressively burst the bubbles into life.

From Stem Cells to Functional Lymphocytes: Cell differentiation and gene therapy implementation for RAG-SCID

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A mis padres y hermano por su apoyo y confianza

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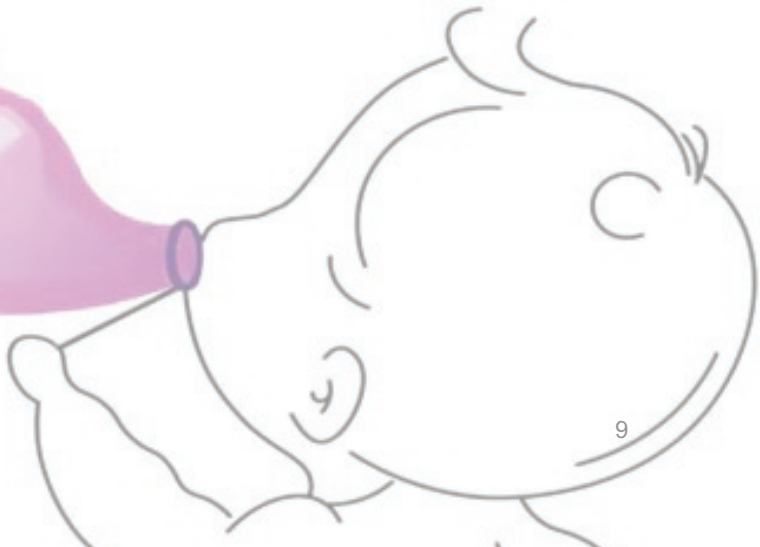
General Introduction



Our body and organs are continuously exposed to a vast array of both pathogenic and nonpathogenic microbes and toxic or allergenic substances that threaten normal homeostasis. The immune system is a complex mechanism of defence to prevent or limit infections and to guarantee homeostasis and repair, consisting of an interactive network of lymphoid organs, cells and humoral factors. An essential feature of the immune system is the ability to precisely discriminate between the pathogen and the host cells, eliminating the threats while limiting the damage to own tissues.

The immune system can be divided into the innate and the adaptive responses, determined by the speed and specificity of the response. Importantly, the synergy between them is essential for an effective response. The first line of defence is established by physical and physiological barriers like skin and mucosa. The innate response represents the first line of immune defence, based on non-specific responses and guided by the digesting activity of neutrophils and macrophages attacking the invading pathogens. Likewise, components of this innate system contribute to the activation of the adaptive response, the hallmark of the immune system which is characterized by the exquisite specificity for its target antigens. This response is based on the antigen-specific receptors expressed on the surfaces of T and B lymphocytes and have the ability to adapt and remember the confronted pathogens. Activated B cells are responsible of an antibody-mediated response where specific secreted antibodies bind and inactivate antigens, while T cells induce apoptosis of infected cells in direct cell-mediated response. Importantly, each cell type of the immune system performs a unique role, and their development is tightly regulated by the expression of many transcription factors and genes.

The disruption of the lymphoid development can lead to severe illness know as immunodeficiency. Severe Combined Immunodeficiency (SCID) represents a group of devastating rare and inherited immunodeficiencies characterized by a vastly reduced or utter absence of the adaptive immune system. The over 20 different genetic defects underlying the various SCID phenotypes revealed the functional importance of specific proteins in lymphoid development like the Recombinase Activating Gene (RAG) proteins. Effective treatment options are limited to allogeneic hematopoietic stem-cell transplantation and the innovative promising autologous stem cell-based gene therapy which has shown significant safety and efficacy in correcting the immunodeficiency.

In this thesis, the successful development of gene therapy for RAG-SCID and related transplantation protocols together with a better understanding of key factors involved T-cell development are described. Therefore, the normal and defective development of the adaptive immune system from hematopoietic stem cells (HSCs) is discussed in this introduction together with the gene therapy approach established for the effective treatment and clinical implementation.

HEMATOPOIESIS

The blood system contains many different specialized cell types, including the red blood cells, platelets and leucocytes (also called white blood cells). The white blood cells are made up of both the innate including dendritic cells, monocytes, granulocytes, innate lymphoid cells (ILCs), and the adaptive immune responses comprised of B and T lymphocytes. All blood cell lineages arise from hematopoietic stem cells (HSC) mainly residing in the bone marrow (BM) [1]. It involves a highly controlled process of self-renewal to maintain the pool of HSCs, proliferation, differentiation and specialization steps (Overview hematopoiesis in **Figure 1**). The formation of the blood cellular compartment is a lifelong process lead by the continuous development and turnover of blood cells. The blood system is a highly regenerative and plastic tissue, with a flexible hematopoietic process in space and in time. It is estimated a production of $1{,}4.10^{14}$ mature blood cells per year from an estimated 3.000-10.000 adult HSCs [2].

Hematopoietic stem cells

HSCs are a rare heterogeneous population mainly residing in the BM which is the primary site of hematopoiesis after birth in adult mammals [3]. Specialized niches within the BM provide a favorable microenvironment for the process of their unique dual capacity of self-renewal and multipotency [4]. Self-renewal is defined as the ability to give rise to HSC itself while maintaining undifferentiated features which is important for the maintenance of the HSC pool. Multipotency is known as the ability to differentiate into all blood cell types. The long-term repopulation capacity of the entire hematopoietic system is the fundamental criterion to define a true HSC. Long-term HSCs give rise to short-term HSCs subsequently generating multi-potent progenitors (MPPs), while progressively losing self-renewal and multilineage differentiation potential [5]. In addition, the continuous advances in the development of innovative flow cytometry platforms and monoclonal antibodies have favored the characterization of the HSCs populations by the combination of specific surface markers, facilitating the purification of these specific subsets of cells.

Murine HSCs were first described by Becker et al. (1963) [6], describing the clonal origin of a population of hematopoietic cells. At the beginning of the 1990s, Jordan and Lemischka [7] proposed a model where single stem cell clones are sufficient to maintain lifetime hematopoiesis in an animal model and suggested that the hallmark of the long-term reconstitution system may arise from mono- or oligoclonality. Suitable markers to characterize stem cell subpopulations were identified, allowing the purification of the murine cells of interest [8, 9]. The most widely known murine HSC population is the LSK population, standing for lack of lineage markers (B220, Mac-1, Gr-1, CD3, CD4, CD8, and Ter119), and the presence of Sca1 and c-Kit. LSK comprise a heterogeneous population with different subpopulations distinguished as long-term ($Thy1^{lo}LinSca1^{+}cKit^{+}CD38^{+}CD34^{-/lo}Slam^{+}$) and short-term ($Thy1^{lo}Lin^{-}Sca1^{+}cKit^{+}CD38^{+}CD34^{+}Slam^{-}$) populations [10, 11]. In parallel, Weissman and co-workers (1992) [13] isolated a candidate population in human fetal BM ($Thy1^{+}Lin^{-}CD34^{+}$) that was enriched for the clonogenic activity that established long-term and multilineage capacity. CD34 is the main marker to define human HSCs, consisting of a pool (mix) of populations that represents around 1%

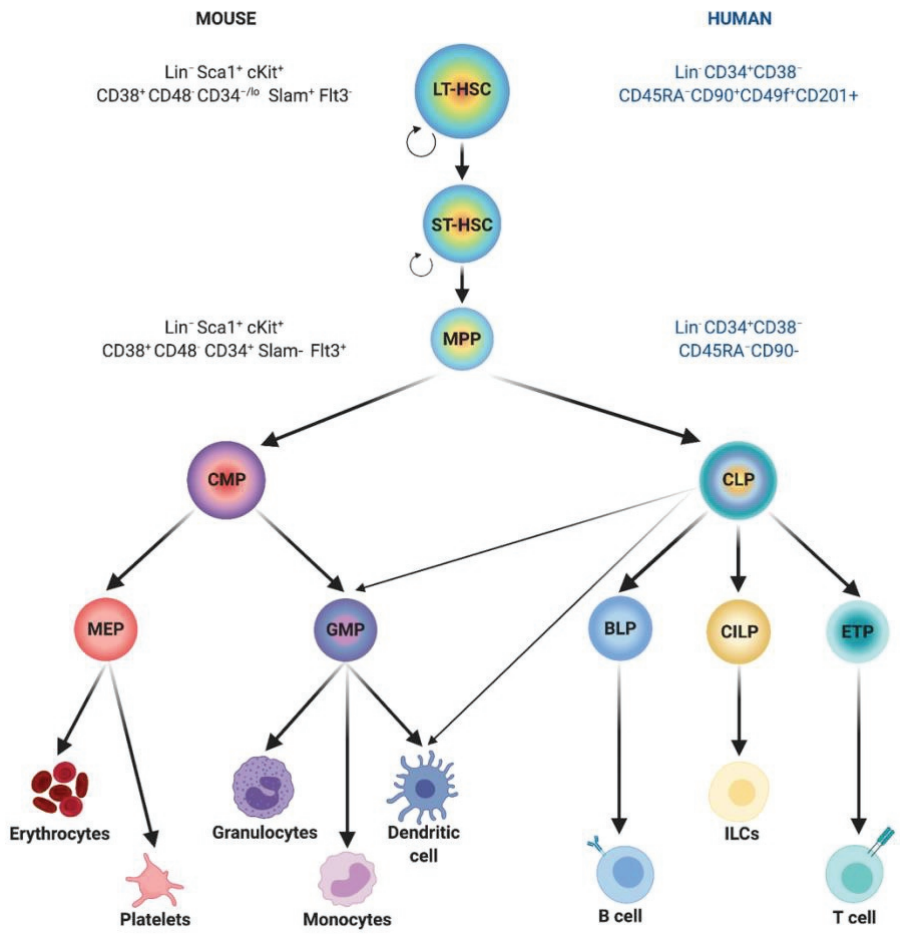


Figure 1: Overview of the classic model of hematopoiesis. *All blood cell lineages arise from hematopoietic stem cells (HSC), a heterogenous rare population that have the unique dual capacity of self-renewal and multipotency to maintain the pool of HSCs, and to differentiate into all blood cell types respectively. Long-term HSCs give rise to short-term HSCs (ST-HSC) subsequently generating multi-potent progenitors (MPPs), while progressively losing self-renewal and multilineage differentiation potential and can be distinguish by different cell surface marker combination both in mouse and in human. MPPs differentiate into two main branches: common myeloid and common lymphoid progenitor (CMP and CLP), that give rise to more committed progenitors like the Megakaryocyte-Erythroid Progenitors (MEP) or the Granulocyte-Monocyte Progenitors (GMP) and the B-cell-biased Lymphoid Progenitors (BLP), the Common Innate Lymphoid Progenitors (CILP) or the Early Thymic Progenitors (ETP) respectivelly. Subsequently, all blood cell lineages mature (Adapted form Tajer et al. (2019) [12]; Created with BioRender.com).*

of total BM cells. HSCs have been further phenotypically redefined as $CD34^+CD38^-$ cells [14] and further divided into subpopulations based on the expression of CD90/Thy1 and CD45RA [15] and CD49f. Therefore, the first full phenotypic definition of human HSCs was proposed by the laboratory of John Dick (2011) [16] as $CD34^+CD38^-CD45RA^-CD90^+CD49f^+$,

where single defined HSCs were highly efficient in generating long-term multilineage grafts in NOD scid gamma (NSG) mouse models. Recently, new HSC markers have been identified like EPCR/CD201, which is also fairly reliable to detect HSCs in culture[17].

In the clinical setting, the $CD34^+$ fraction, also known as hematopoietic stem and progenitor cells (HSPC), with a mix of progenitors and long-term populations (similar to murine LSK) are used for transplantation or gene therapy manipulation. HSPCs and their regenerative capacity is used as a therapeutic treatment for a variety of hematological disorders, such as leukemias [18], lymphoma [19], hemoglobinopathies [20, 21] or various immunodeficiencies including SCID [22, 23]. The principal advantages of using the total HSPC population are the easy accessibility, isolation and purification of these cells from multiple sources [24, 25]. HSPCs can be harvested from the bone marrow by direct puncture or nowadays, preferably by mobilization followed by leukapheresis. HSPCs are mobilized with two mobilizing agents (G-CSF and Plerixafor) from the bone marrow to the peripheral blood that is then collected, containing an enriched portion of HSCs [26]. CD34 enriched cells from umbilical cord blood have been also used in clinical settings for transplantation [27, 28]. HSPCs can efficiently be administered to the treated patient by infusion, where HSPCs will naturally home to the BM and achieve a therapeutic effect.

Lymphoid lineage

Both antigen-specific lymphocytes (B and T cells) and innate lymphocytes lacking antigen specific receptors arise from the common lymphoid progenitor (CLP) in primary lymphoid organs. While B and ILCs differentiate and mature in the adult bone marrow; T cells develop in the thymus where CLPs derived from the bone marrow migrate, seed the specific niche and definitively commit and mature into T lymphocytes. Mature cells leave the primary organs respectively, enter the circulation and occupy peripheral lymphoid organs such as the spleen and the lymph nodes, where specific immune responses are generated [29] .

Natural killer (NK) cells

Re-categorized as innate lymphocytes (ILCs), NK cells comprise around 15% of all circulating lymphocytes, representing the 3rd largest lymphoid population. NK cells, the prototypic ILCS, were first described in 1975 [30] and have been classified as group 1 ILCs playing an important role in the early innate response, preventing infections by viruses, intracellular pathogens or certain tumour cells. As a crucial component of the innate immune system, NK cells can be found circulating in blood and within tissues. NK cells are characterized as having a large lymphocyte-like morphology. However, they lack myeloid and dendritic cell phenotypical markers as well as the adaptive immune rearranged antigen-specific receptors and co-receptor complexes except for the IL-7 receptor. Their unique granular cytoplasm containing cytotoxic proteins provides NK cells with potent cytolytic functions, recognizing infected cells through the expression of various families of innate receptors and killing infected cells by the release of the granules. NK cells also enhance other immune cell responses by the secretion of cytokines like interferon-γ and tumor necrosis factor-α [29, 31, 32].

Both murine and human NK cell development in the bone marrow essentially requires the expression of the transcription factor Id2 in the CLP to repress the B- and T-cell fates, as

well as IL-15 cytokine for NK maturation [33-36]. In mouse, CLP differentiates into pre-NK progenitor cells ($Lin^{-}Flt3^{-}CD27^{+}CD244+CD127^{+}CD122^{-}$) which further develop into re-defined NK progenitors expressing CD122, immature NK stage with NK1.1 marker expression and finally becoming mature NK cells, highly expressing NK1.1, CD49, CD43, CD62L and KLRG1 [37-39]. In humans, NK progenitor cells ($Lin^{-}CD34^{+}CD38^{+}CD123^{-}$ $CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$) develop into an intermediate NK-cell precursor ($CD34^{+}IL15R^{+}$), that through IL-15 signalling differentiate into mature NK cells with CD3 negative and CD56 and CD16 positive surface expression. Up to four different NK subsets have been recently re-defined based on their surface markers expression level, transcription factors, their granule content and their cytotoxic activity: circulating $CD56^{bright}$ cells, canonical $CD56^{dim}$ cells, adaptive $CD56^{dim}$ cells and tissue-resident $CD56^{bright}$ NK cells [40-43].

B-cell development

B cells were for the first time identified in 1965 by Cooper et al [44]. Within the common CLP compartment, the B cell-biased lymphoid progenitor (BLP) cells have the B-cell differentiation program initiated [45, 46]. Early B cells develop from BLPs through different stages divided based on their differential expression of cell surface markers and their immunoglobulin (Ig) rearrangement process and surface expression during development (**Figure 2**). BLPs progress to the pre-pro-B cell stage, marked by the expression of the B220 marker ($B220^{+}CD43^{+}CD19^{-}cKit^{low}Flt3^{low}IL7R^{+}$) in mice and $CD34^{+}CD38^{+}CD10^{+}$ markers in human. Early pre-pro-B cells proliferate in response to Interleukin 7 or stem cell factor (SCF) cytokines and develop into murine pro-B cells expressing CD19 marker ($B220^{+}CD43^{+}CD19^{+}ckit^{low}IL7R^{+}$) or human pro-B cells ($CD34^{+}CD10^{+}CD22^{+}CD19^{-}$) rearranging IgH. After V(D)J rearrangement of the IgH during this stage, the pre-B cell receptor (pre-BCR) is expressed on the pre-B-cell surface together with murine B220 and CD19 or human CD19 and $CD20^{dim}$, entering a high proliferative cell stage. Further differentiation continues with the rearrangement of the composed heavy (H) and light (L) chain, resulting in mature BCR expression at the immature B-cell stage (mouse $B220^{+}CD19^{+}IgM^{+}$ and human $CD19^{+}CD20^{high}IgM^{+}$). Immature B cells transition from the bone marrow niche to the periphery becoming transitional B cells expressing both IgM and IgD on the cell surface. Transitional B cells develop to naïve B cells in the periphery where cells can directly encounter antigens through BCR binding and secrete antibodies in response to an antigen. In response to this antigen-specific stimulation, naive B cells differentiate into plasmablasts, plasma cells and memory B cells through somatic hypermutation, affinity maturation and class-switching recombination within the germinal center. Throughout B-cell development two important rearrangement checkpoints take place: the pre-BCR checkpoint at the pre-B cell stage where only cells presenting a successful IgH rearrangement continue through maturation and further rearrangement; and a positive selection of effective mature BCR at the cell surface of immature B cells leading to B cell survival [47-49].

B cell commitment is one of the best-understood models of cell differentiation in the hematopoietic system with a well-established core of contributing specific transcription factors with E proteins (E2A)[50, 51], the early B-cell factor 1 (EBF1)[52], Foxo1 [53] and Pax5 [54]

as the main players. This transcription factors lead B cell commitment in a hierarchical as well as combinatorial manner. Briefly, E2A activates Foxo1 expression, and together they induce EBF1 expression. Foxo1 and EBF1 upregulate Pax5 leading to the activation of the B-cell gene expression program and to commit to the B cell fate from the pro-B cell stage [55, 56].

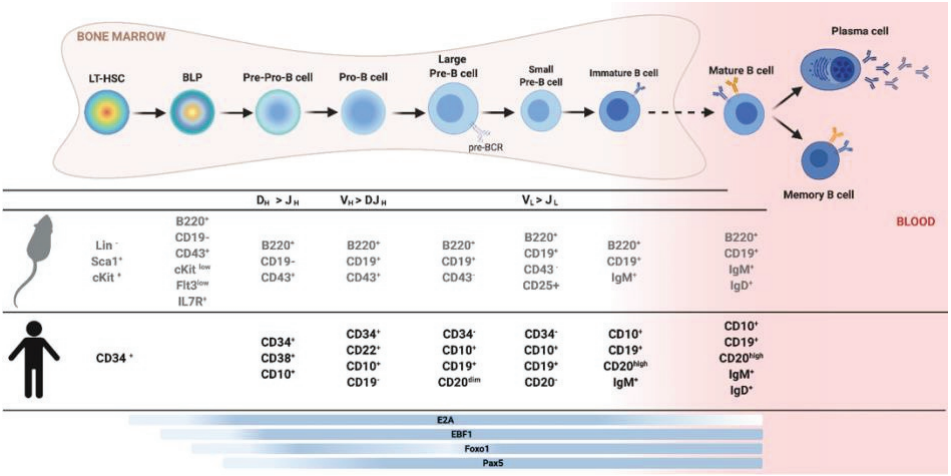


Figure 2: Schematic overview of B cell development in the bone marrow. *Murine and human B cells develop in the bone marrow (BM) from the B-cell-biased lymphoid progenitors (BLP) cells, progenitor cells with the B-cell differentiation program initiated. Cells develop through successive comparable stages described according to the surface markers expressed for mouse and human. The successive ordered V(D)J recombination stages to form B cell receptor (BCR) are also illustrated. In the lower part, contributing specific transcription factors in B-cell development in the BM are represented, (light shade represents low expression and darker shade high expression). (Adapted from the thesis of K.Pike-Overzet and A-S. Wiekmeijer; Created with BioRender.com)*

T-cell development

T cells develop within the specialized microenvironment of the thymus. A few bone marrow hematopoietic progenitors enter circulation and migrate into the thymus every day to maintain continuous T lymphopoiesis [57]. In mouse, one major progenitor population migrating to the thymus contains lymphoid-primed multipotent progenitors (LMPPs), defined as $Lin^{-}Sca1^{+}ckit^{+}Flt3^{+}$. In humans, three potential thymic progenitor seeding population have been described ($CD34^{hi}CD45RA^{hi}CD7^{+}$, $Lin^{-}CD34^{+}CD10^{+}CD24^{-}$ and $Lin^{-}CD34^{+}CD10^{-}CD45RA^{+}CD62L^{hi}$ cells) indicating that multiple population can be involved in early thymic seeding [58-60].These early thymic progenitor cells extensively proliferate while triggering the T-cell differentiation program in response to extracellular signals provided by the thymic stroma cells including Notch and WNT signalling and cytokines such as SCF, FLT3 ligand or IL-7 [61, 62].

In both human and mouse, T-cell development within the thymus is a highly complex process involving successive stages in which the expression of CD4 and CD8 co-receptors occurs in distinct microenvironments. Via a series of progressive developmental stages (**Figure 3**), T cell precursors differentiate from double negative cells (DN; $CD4^{-}$

CD8$^-$), into Intermediate immature single-positive cells (ISP; CD8$^+$CD3$^-$CD4$^-$ in mouse and CD4$^+$CD3$^-$CD8$^-$ in humans), then into double-positive cells (DP; CD4$^+$CD8$^+$) and finally into single-positive mature cells (SP; CD8$^+$CD4$^-$CD3$^+$ or CD4$^+$CD8$^-$CD3$^+$). Within the DN stage, developing thymocytes can be further subdivided into four phenotypically distinct stages of differentiation (DN1, DN2, DN3 and DN4) characterized by the expression of several membrane molecules, like CD44 and CD25 in mouse and CD38 and CD1a in humans [57, 63-66]. Early developing T lymphocytes (ETP, DN1 and to a lesser extend DN2) are not T-cell fate restricted, allowing alternative lineages such as B, ILCs, dendritic and myeloid cells to develop. However, developing cells gradually and irreversibly lose this alternative lineage potential development while acquiring a T-cell specific transcriptional program until fully commitment to the T-cell lineage from the DN3 stage followed by TCR rearrangements [67, 68]. V(D)J rearrangement of the TCRβ occurs at the early DN3 stage (DN3a) favored by the arrest in cell cycle. DN3b cells expressing pre-TCRβ and properly signaling through undergo proliferation, survival and differentiation i.e. β-selection. Cells differentiate further into DN4, ISP and DP stages where during another arrest in cell cycle, TCRα chain rearrange leading to mature TCRαβ assembly and expression on the cell surface. Functional TCRαβ is exposed to positive selection (recognition of self-MHC molecules) and negative selection (absence of self-antigens reactivity). Successfully cells will definitely differentiate into CD4 T helper cells or CD8 cytotoxic T cells [69-71].

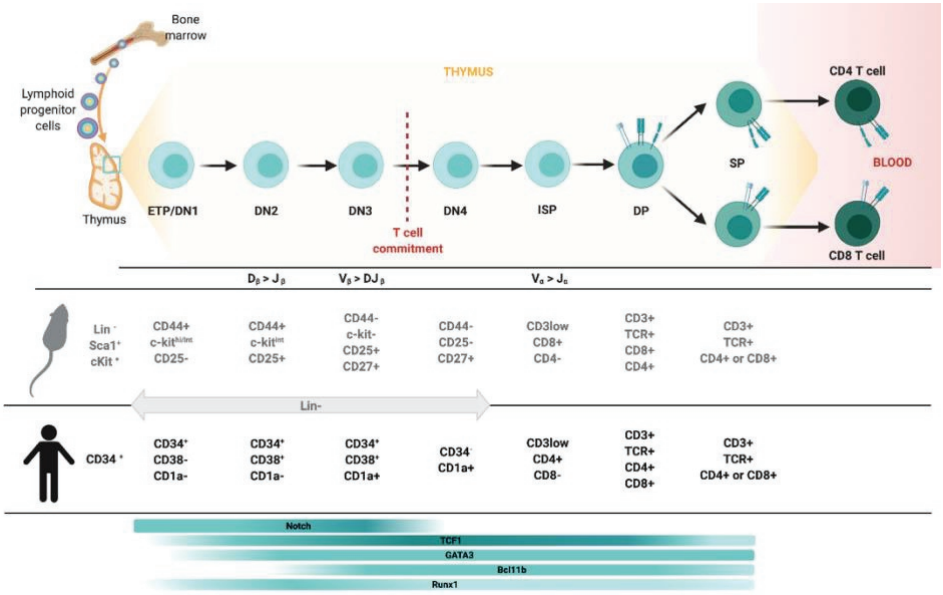


Figure 3: T-cell development in the thymus overview. *T cells develop within the specialized microenvironment of the thymus. Few bone marrow hematopoietic progenitors migrate from the bone marrow and seed the thymus. Subsequent developmental stages are described for mouse and human according to the surface marker expression and the V(D)J recombination process of the T cell receptor. Key transcription factor expression is depicted in the thymus (light shade represents low expression and darker shade high expression) (Adapted from the thesis of K.Pike-Overzet and A-S. Wiekmeijer; Created with BioRender.com).*

T-cell maturation is orchestrated by a set of transcription factors with specific gene expression profiles along the different maturation stages, starting by the well-defined Notch signalling that establishes T cell identity and including E2A, TCF1 (encoded by Tcf7), GATA3, Bcl11b and Runx1 [72]. Notch signalling helps to inhibit alternative lineage potential of earlier T cell stages [73, 74] and activates the expression of important transcription factors such as TCF1 (encoded by Tcf7) and GATA3. These crucial regulatory genes drive T-cell specification by combining a restrictive role for alternative linages and a positive regulation of T-cell identity genes [75-80]. Later activation of Bcl11b is associated with T-cell commitment and successful β-selection [81-84]. Although the main transcriptional players of early T-cell specification and maturation have been identified, the precise roles, regulation and interactions are not fully understood [85, 86].

V(D)J recombination

B and T lymphocytes recognize foreign antigens through their antigen specific receptors, the B-cell receptor (BCR) also known as Immunoglobulin (Ig) and the T-cell receptor (TCR) respectively. A diverse repertoire of BCRs and TCRs on mature B and T cells are generated during lymphopoiesis. Ig contains two Ig heavy chains, encoded by the IgH heavy locus, together with two IgL light chains encoded by the Igι or Igκ loci. Alternatively, TCR predominantly consist of a TCRα chain paired with TCRβ chain expressed in circulating T cells, while below 15% of these cells contains a TCR with a TCRδ chain paired with TCRγ. These antigen-specific receptors have the capacity to recognize a vast variety of antigens due to the high diversity degree of the receptor binding specificity generated from a substantial limited number of gene segments by V(D)J recombination process of the receptor loci. The V(D)J recombination is a lineage-specific, tightly regulated process resulting in the joining of V (variable), D (diversity) and J (joining) gene segments through a series of hierarchical ordered, somatic and site-specific DNA rearrangement steps [87]. Essentially, a D segment rearranges with a J segment, followed by the rearrangement between the created DJ segment and a V segment. In the absence of a D segment, V directly rearranged to a J segment. While IgH, TCRβ and TCRδ chains are encoded by a combination of V, D and J segments; IgL, TCRα and TCRγ loci contain only V and J. Accordingly, each receptor consisting of the combination of two chains needs to successfully complete 3 recombination events joining V, D and J (two events) for one chain and V and J (one event) for the other chain [88, 89].

The V(D)J recombination process (**Figure 4**) during the G1 phase of the cell is initiated by a double-strand break (DSB) produced at specific homologous recombination signal sequences (RSS). All competent V, D and J gene segments are flanked by two types of RSS depending on the spacer length (12 or 23). Following the 12/23 rule, only RSS with differing spacer length efficiently recombine. Recombination-activating gene 1 and 2 (RAG1 and RAG2) recognize properly spaced RSS sequences, bind to them, cleave the DNA between the RSS and the coding sequence and, without dissociation, the RAG complex forms a hairpin. Re-joining of the coding segment is mediated by other factors recruited to the coding ends. Ku heterodimer binds to the hairpin ends, both preserving them from digestion as well as recruiting the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), the nuclease Artemis, polymerases and the DNA ligase IV to finally

attach the C-Non-homologous End Joining (C-NHEJ) reconstituting the rearranged gene. While Artemis opens the DNA hairpin by making a single-strand nick, the two compatible ends are joined by the DNA ligase IV and XRCC4 complex forming the coding segment. An extra layer of diversity, the junctional diversity, is introduced during the last step of non-homologous end joining contributing to the overall TCR and BCR diversity repertoires. The diversity is increased by the omission of nucleotides due to the exonucleolytic cleavage and by the insertion of random numbers of P- and N-nucleotides at the junction site between gene segments. Palindromic sequences (P-nucleotides) are incorporated in asymmetrical opened hairpins. In addition, up to 20 non-templated N-nucleotides can be added to the single-stranded ends by the terminal deoxynucleotidyl transferase (TdT). Not only the coding segment is joined, but also the blunt ends are precisely joined by the same complex forming the signal joint containing the coupled RSS and the unused V, D and J segments, also known as circular excision products (TRECs or KRECs) [29, 90, 91].

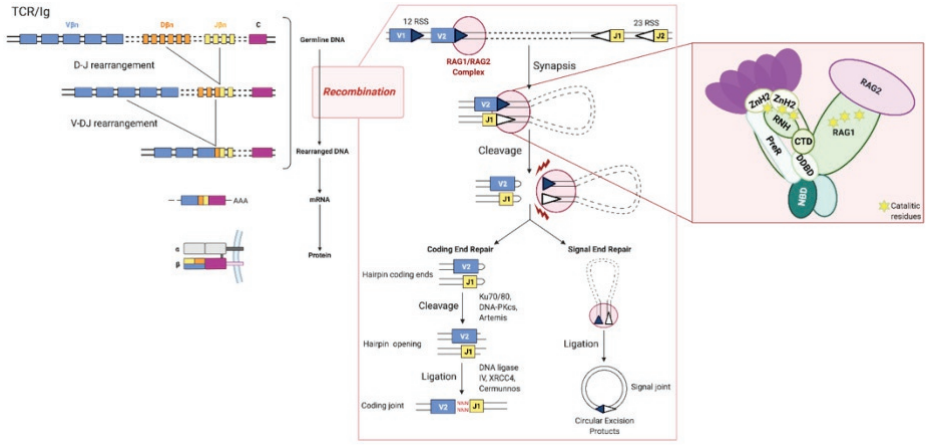


Figure 4: Schematic overview of TCR/Ig gene rearrangement by V(D)J recombination and highlighting the structure of the RAG1/2 recombinase complex. *Highly diverse repertoire of B-cell receptors (BCR) and T-cell receptors (TCR) on mature B and T cells are generated during lymphopoiesis from a substantial limited number of gene segments in the germline DNA. Diversity is generated by the V(D)J recombination process of the receptor loci, a lineage-specific, tightly regulated process that combines a series of hierarchical ordered, somatic and site-specific DNA rearrangement steps. The Diversity D segment rearranges with a Joining J segment, followed by the rearrangement between the created DJ segment and a Variable V segment. Rearranged DNA is transcribed into mRNA and translated into protein. V(D)J recombination is initiated by a double-strand break (DSB) produced at specific homologous recombination signal sequences (RSS) by proper recognition by RAG1/2 recombinase complex. RAG1/RAG2 complex binds to the RSS, cleave the DNA between the RSS and the coding sequence and, without dissociation, the RAG complex forms a hairpin. Rejoining of the coding segment is mediated by Ku70/80, DNA-PKcs and Artemis proteins. Two compatible coding ends are joined by the DNA ligase IV and XRCC4 complex forming the coding segment. The blunt signal ends are joined by the same complex forming the signal joint containing the coupled RSS, also known as circular excision products (TRECs or KRECs for T- and B-cell rearrangement respectively). RAG1/RAG2 complex form Y-shaped structure heterotetramer composed by 2 units of each RAG1 and RAG2. RAG1 is composed by a nonamer-binding domain (NBD), a dimerization and DNA-binding domain (DDBD), a PreRNase-H (PreR), a catalytic RNase-H*

region (RNH) which includes the three essential active sites residues and two zinc-finger domains (ZnH2) interacting with the RAG2 protein- RAG2 forms a ring-like structure with a folded six-bladed beta-propeller fold with two fundamental domains: the competent core domain essential for the interaction with the RAG1 protein and the high-affinity DNA cleavage activity. (Adapted from Kim et al (2015) [92]and Notarangelo et al. (2016) [91] Notarangelo 2016); Created with BioRender.com)

Recombinase Activating Gene proteins: RAG1 and RAG2

The core of the recombinase machinery involved in the recognition of the RSS segments and the DNA cleavage is composed of two lymphoid-specific proteins RAG1 and RAG2. Encoded by the recombination-activating genes *RAG1* and *RAG2* respectively they are tightly regulated at the early stages of T- and B-cell development. RAG genes are located side by side on the human 11p13 chromosome, containing one protein-coding exon each [91, 93]. RAG proteins are highly conserved throughout evolution, with related sequences found in other animal genomes, such as a 90% homology between human and mouse amino acid sequences. A transposable element which underwent strong selective pressure to promote a controlled DNA end joining and DNA repair seems to be the origin of the RAG recombinase, with RAG2 involved in the emergence of the 12/13 rule [94].

The human RAG1 is composed by a full-length protein of 1.043 amino acids (1.040 amino acids for the murine protein) with a catalytic core between the 387 and 1.011 residues. RAG1 protein is composed of several domains. Briefly, a nonamer-binding domain (NBD) and the dimerization and DNA-binding domain (DDBD) are connected by a flexible linker and are crucial for DNA interaction and anchoring to the RSS sequence [95]. In addition, RAG1 has a PreRNase-H and a catalytic RNase-H region which includes the three essential active sites residues (D600, D708 and E962) essential for the catalysis and the double-strand DNA break. It also harbours two zinc-finger domains which are crucial for homodimerization and interaction with RAG2 protein. Finally, regulatory regions have been identified with ubiquitylation-dependent regulatory processes linked to the N-terminus of the RAG1 protein involved in the nuclear import, histone modulation and DNA repair [96].

The human RAG2 protein includes 527 amino acids, with a minimal core region with catalytic function of 1-350 residues. RAG2 acquires a ring-like structure with a folded six-bladed beta-propeller fold. The RAG2 protein comprises two fundamental domains: the competent core domain essential for the interaction with the RAG1 protein and the high-affinity DNA cleavage activity as stabilizer of the RSS-binding and the C-terminal non-core domain which contain multiple regulatory motifs. This non-core domain includes residues responsible of the demethylation of the Igκ locus in the early B cells, a PHD finger that is implicated in regulating chromatin accessibility and in regulating cell cycle-dependent recombination activity by means of RAG2 degradation at the G1-to-S transition and finally a “hinge” region involved in post-cleavage DNA stabilization and DNA repair [97].

The RAG complex is formed by two units of each RAG1 and RAG2. The formed heterotetramer creates a Y-shaped structure as was revealed by crystal and cryo-electron microscopy [92] (**Figure 4**). The RAG1 units form the branch by their NBD domains binding. The RAG1 zinc-finger regions on the top contact with the RAG2 core protein, forming the tip of the Y structure. The active site is located in the middle region of the Y-shaped

structure, within the arms, where it contacts the RAG2 protein. Following DNA binding at the DDBD region, at the bottom of the arms, both arms come together [91, 92].

RAG proteins are tightly regulated during B- and T-cell development at the transcriptional and post-transcriptional level. RNA levels of RAG proteins in sorted cells showed restricted expression to the B- and T-cell lineage. RAG expression is first needed for the IgH or TCRβ chain rearrangement at the pro-B-cell stage and the DN T-cell stage respectively. A second RAG expression wave is involved at later developmental stages (pre-B cell and DP T-cell stages) for IgL and TCRα rearrangement respectively. RAG activity is also post-transcriptionally regulated mainly by cis-regulatory elements, the control of the RAG2 protein subcellular localization and most importantly to the regulated degradation of RAG2 protein in the transition to the S phase of cell cycles, containing the RAG recombinase activity to non-replicative cells [98].

SEVERE COMBINED IMMUNODEFICIENCY (SCID)

Severe combined immunodeficiency (SCID) is a rare life-threatening inhered disorder of the immune system with an estimated incidence of 1-2 per 100.000 live births [99-101]. All forms of SCID are characterized by the absence of functional T cells due to their developmental arrest in the thymus, often accompanied by deficiency in B cells and/or NK cells. The lack of a functional adaptive immune response leads to failure to thrive of the affected infant, associated with severe and recurrent opportunistic infections and other metabolic abnormalities that are invariably fatal within the first year of life unless effective treatment is provided. SCIDs are the most severe forms of primary immunodeficiencies and represents a real pediatric emergency for which curative treatment options are limited and confined to allogeneic HSC transplantation (HSCT) [102, 103] and autologous HSC gene therapy [104, 105]. More than 20 different genes have been shown to be causative of SCID [106, 107], but still a lot of affected infants (around 20%) remain without a known genetic cause [101, 108]. Based on the main pathways affected by the molecular defect, SCID can be classified according three major types (**Figure 5**). The cell metabolism disorder type of SCID that affect highly proliferating cells such as immature thymocytes. Adenosine deaminase (ADA) deficiency is the prototype disease for this subtype, but other deficiencies have also been found, for instance purine nucleoside phosphorylase (PNP) deficiency. A second type of SCID is formed by V(D)J recombination deficiencies and TCR abnormalities. In these types of SCID the recombination machinery that is responsible for V(D)J) recombination of T cell receptor (TCR) and immunoglobulin (Ig) genes is affected. Examples are recombination-activating gene-1 (RAG1), RAG2 deficiency and Artemis mutations [109, 110]. The third major type of SCID concerns the cytokines signaling associated disorders mainly due to defects in the IL2Rγ chain (which is also termed the common gamma-chain). Deficiencies in JAK3 and IL7Ra are much rarer but also fall into this category.

Cell metabolism disorders

Deficiency in genes involved in cell metabolism such as adenosine deaminase (ADA) [111], purine nucleoside phosphatase (PNP) [112, 113] or adenylate kinase 2 (Ak2) [114] cause SCID characterized by abnormal accumulation of toxic nucleoside products [115]. These

deficiencies have an autosomal recessive pattern of inheritance leading to a $T^-B^-NK^-$ SCID phenotype.

The ADA gene encodes an enzyme that catalyzes the deamination of adenosine and deoxyadenosine, converted respectively to inosine and deoxyinosine in an irreversible manner. ADA is predominantly expressed in the lymphoid system, where it plays a key role in immune differentiation and maturation. More than 70 mutations have been described in ADA patients [116], which lead to the massive accumulation of the substrates converted to deoxyadenosinetriphosphate (dATP), resulting in lymphotoxicity [115, 117, 118]. The accumulation of these toxic metabolites alters the lymphocyte signaling pathways, serving as danger signals and triggering lymphocyte apoptosis affecting in particular tissues and cells characterized by rapid proliferation [119]. The 13% of total SCID patients affected with this deficiency can benefit from enzyme replacement therapy using PEG-ADA, HSCT or gene therapy [120-122].

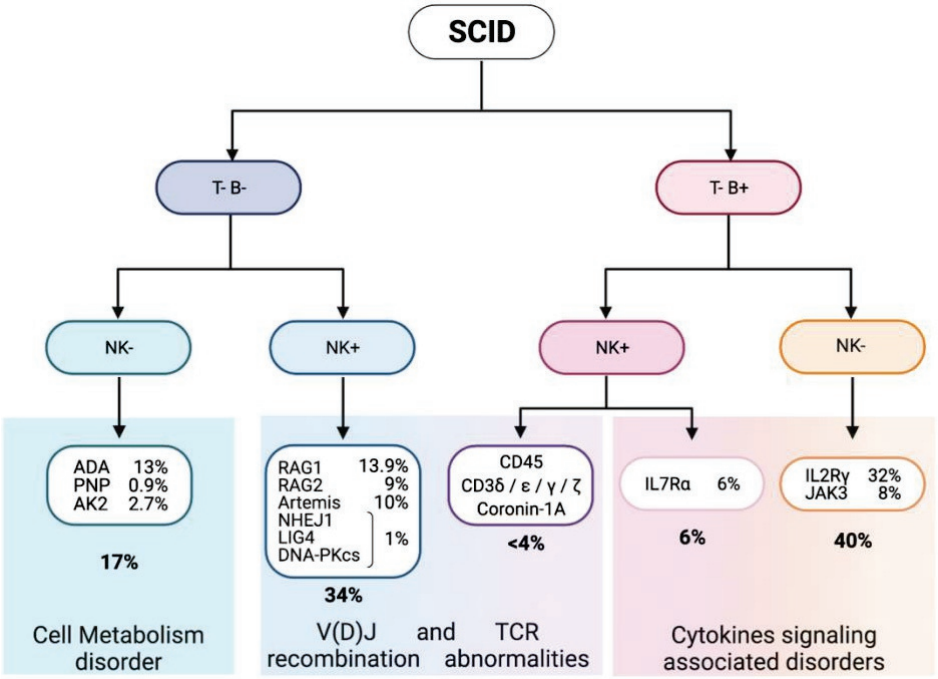


Figure 5: Severe Combined Immunodeficiency (SCID) classification. *Classification of SCID according to 3 major types of deficiency based on the main affected pathways: 1) SCID concerning metabolic enzymes, with a T-B-NK- immune phenotype, affects highly proliferating cells such as Adenosine deaminase (ADA), Purine nucleoside phosphorylase (PNP) or adenylate kinase 2 (AK2) deficiency. 2) SCID formed by recombination deficiencies and T-cell receptor (TCR) abnormalities such as recombination-activating gene-1 (RAG1), RAG2 and Artemis deficiencies or CD45, CD3 and Coronin-1A deficiencies, resulting in a T-B-NK+ or T-B+NK+ immune phenotype, respectively. 3) The common cytokine signaling associated SCIDs deriving to a T-B+NK- or NK+ immune phenotype, are mainly caused by defects in the IL2Rgamma chain, the Janus Kinase 3 (JAK3) or the IL7-Receptor alpha gene. (Data retrieved from genetically confirmed SCID patients transplanted in Europe in the 2006-2014 period based on the SCETIDE registry, submitted). (Created with BioRender.com)*

Defects on the PNP protein cause an extremely rare form of SCID accounting for 1% of the total, with the same phenotype as described for ADA deficiency [111]. In this case, the gene encodes an enzyme that catalyzes the conversion of guanosine, deoxyguanosine, inosine, and deoxyinosine to their respective purine bases [115, 123]. The elevated intracellular accumulation of deoxyguanosine triphosphate results in an increased sensitivity to DNA damage and apoptosis due to the inhibition of the mechanisms of DNA synthesis and repair, especially in T cells during selection [124]. Allogeneic HSCT is the only treatment available to correct this deficiency.

Finally, AK2 deficiency causes reticular dysgenesis (RD) and represents the most severe form of SCID occurring in almost 3% of infants with SCID. The immunodeficiency derives from a defective survival of the hematopoietic lineage precursors. Encoded AK2 regulates adenine nucleotide interconversion within the mitochondrial intermembrane space [125], involved in the maintenance and monitoring of cellular and mitochondrial energy homeostasis. RD is associated with the absence of both innate and adaptive immunity, as mutations in the AK2 gene impair myeloid and lymphoid lineage development [114]. To date, the only effective treatment for RD as for PNP deficiency is allogeneic HSCT.

V(D)J recombination and TCR abnormalities

V(D)J recombination is a complex process that occurs in early B- and T-cell development leading to a functional Igs and TCRs respectively. The deficiency in genes involved in the recombination process like *RAG1/2* [126], *DCLRE1a* [127], *PRKDC* [128], *NHEJ1* [129] or *LIG4* [130] lead to the T-B-NK+ form of SCID with an autosomal recessive trait characterized by the absence of functional TCR and Igs. The genes involved are mostly related to DNA repair resulting in increased sensitivity to radiation and chemotherapeutic agents for these patients [127, 131]. In addition, gene mutations that suppress early TCR signaling like CD45, CD3 or Coronin-1A are associated with selective T-cell development and function abnormalities ($T^-B^+NK^+$ SCID phenotype). To date, the main effective treatment is allogeneic HSCT for most of the deficiencies, with emerging gene therapy options for RAG1 [132] and Artemis [133].

RAG1 and RAG2 proteins form a heterodimer complex at the beginning of the V(D)J recombination process mediating the binding and cleavage of the DNA. Deficiency of one of these proteins is associated with a limited production of T and B cells, associated with the absence of V(D)J recombination causing cell apoptosis. RAG deficiency bears a broad clinical phenotypic spectrum correlating with the different mutations and related recombination activity [134] and represents 14% (RAG1) and 9% (RAG2) of total SCID patients.

Artemis is a nuclear protein encoded by *the DNA cross-link repair enzyme 1c (DCLRE1C)* gene. Essential for V(D)J recombination and DNA repair, Artemis enables end joining and opening of the hairpin loops after DNA nicking of RAG complex. Gene mutations cause T- and B-cell maturation arrest at their respective rearrangement checkpoints [135]. DNA-PKcs, DNA dependent protein kinase encoded by the *PRKDC* gene, regulates Artemis promoting the endonucleolytic activity essential for during V(D)J recombination [136, 137]. Deficiency of the DNA-PKcs causes a comparable SCID phenotype to Artemis deficiency. During V(D)J recombination, non-homologous end joining (NHEJ1) proteins and DNA ligase IV (LIG4)

are involved in the final DSB repair [138]. Deficiencies of these proteins cause profound T and B cell lymphopenia, in line with the previous SCID, representing up to 11% of the total SCID cases.

CD45 (leukocyte common antigen), the molecule that marks lymphocytes, is encoded by the *PTPRC* gene [139, 140]. CD45 is a transmembrane tyrosine phosphatase expressed on hematopoietic cells and precursors (except RBCs and platelets) and required in both TCR signaling and T-cell development in the thymus. CD45 deficiency impairs T-cell maturation, with normal TCRγδ chains but a severe decline of TCRαβ cells. Although B cells are present and with increased numbers, they are non-functional partially due to the lack of germinal centers within the lymph nodes [141]. The TCR is associated with the multimeric CD3 complex consisting of different subunits. All CD3 δ chain, γ chain, ε chain, and ζ chain are required for TCR signaling and T-cell differentiation, importantly for T-cell proliferation of DN cells. Mutations in all the different CD3 chains have been reported in SCID patients (up to 2%), causing the developmental arrest of T-cell development when reaching the DP transition. Moreover, the central tolerance and regulatory T-cell development can be compromised due to the impairment of the crosstalk between thymocytes and the thymic epithelial cells, leading to autoimmune manifestations [142-145]. Finally, Coronin-1A (*CORO1A*) is an actin-binding protein expressed in hematopoietic and immune cells. Proper regulation of actin proliferation of the cytoskeleton is essential for chemotaxis and activation, playing an important role in thymic egress to the secondary lymphoid organs. Coronin-1A deficiency is related with the lack of peripheral T cells, while normal size thymus is observed compared to any other SCID form [146-148].

Cytokine signaling associated disorders

Cytokine signaling associated disorders are the most frequent forms of SCID, with defects on the common gamma chain (IL2R**γ**), Janus Kinase 3 (JAK3) and the IL-7 receptor **α** chain (IL7R**α**) as prototypic deficiencies. The therapeutic options for these forms of SCID are HSCT and for IL2Rγ deficiency, gene therapy.

Located on the X chromosome, the gene encodes the common γ chain of the interleukin 2 receptor (IL2R). This transmembrane protein [149] is involved in IL-4, IL-7, IL-9, IL-15 and IL-21 signaling, all critical for the development and function of lymphocytes [150-152]. Known as X-linked SCID ($T^-B^+NK^-$), IL2Rγ deficiency comprises 32% of all cases with around 200 different pathogenic mutations reported. The absence of intracellular signaling through the IL-7 and IL-15 pathway causes the lack of both mature T cells and NK cells respectively. Although B cells are present in normal or even increased numbers, they are functionally abnormal as their Ig production is often impaired due to the absence of T cells needed for Ig class-switching [153].

Deficiency in the JAK3, downstream of the IL2Rγ complex, acting as a transducing element [154], leads to an autosomal recessive SCID (8% of the total SCID) with a similar clinical phenotype to the observed in the L2Rγ deficiency ($T^-B^+NK^-$) [155]. JAK3 is primarily expressed in lymphoid and myeloid cells, being essentially involved in the differentiation of hematopoietic precursors. JAK3 plays a key role in both early T- and NK-lymphocytes differentiation programs, but not in the B-cell program. Comparable to X-linked SCID,

abnormal B cells are present with impaired class switch recombination and subsequent defective antibody production [156-158].

On the other hand, mutations of the interleukin 7 receptor α chain (IL7Rα) trigger a selective T-cell deficiency (T-B+NK+) [159]. With an autosomal recessive transmission, IL7Rα deficiency accounts for around 6% of all SCID patients [160]. The encoded protein is almost exclusively expressed on the lymphoid lineage, required during early stages of T-cell development and involved in thymocyte survival, proliferation and maturation of T cells in the periphery [161].

Leaky and Ommen Syndrome SCID

Frequently, diverse mutations in the same gene lead to different clinical phenotypes, developing into leaky SCID or Omenn Syndrome. Apart from the SCID clinical phenotype, atypical leaky SCID is associated with immune dysregulation and autoimmunity, generalized severe itchy rashes, enlarged lymph nodes and liver, splenomegaly and chronic diarrhea. Few patients can present an Omenn Syndrome phenotype characterized by elevated IgE serum levels and eosinophil count as additional common features. In these cases, a small T-cell population develops but does not provide adequate protection from infections; instead, oligoclonal T cells expand in the periphery. Over-activated T cells cause inflammation and damage similar to autoimmune disease. Leaky SCID and Omen Syndrome is mostly generated due to hypomorphic mutations reported already for a variety of SCID genes like *JAK3* [162], *IL7Rα* [163, 164], *RAG1/2* [91, 165, 166], *LIG4* [137], *Coronin 1A* [148], *AK2* [167] or *DCLRE1C (Artemis)* [168].

Modeling SCID

While B-cell developmental arrests are commonly visualized by flow cytometry and Ig repertoire analysis, thanks to the availability of BM aspirates [128, 130, 169], this information remains more difficult to elucidate for T-cell development as thymic biopsies are not routinely taken, especially not from SCID patients. To overcome this hurdle, different genetic mouse models have been developed to mimic the different forms of SCID described in humans, like ADA-SCID [153, 170, 171], X-linked (IL2rg)-SCID [172, 173], Artemis-SCID [174-176] or RAG1/2-SCID [177, 178] including hypomorphic forms [179-182]. Importantly, most of these mice present a similar immunodeficient phenotype as found in humans. Unfortunately, other SCID mouse models, such as IL7Rα-SCID, do not reproduce the human phenotype, because the mouse model has an extra B-cell block not observed in humans [183]. Recent functional experiments using bone marrow stem/progenitor cells from SCID patients in NSG xenograft mouse model [184] allow to provide previously unattainable insight into human T-cell development and contributes to functionally identify the arrest in thymic development caused by three major types of SCID (IL2Rγ, IL7Rα and Artemis) as this data was largely missing due to the non-availability of thymic biopsies. This xenograft model showed earlier blocks in thymic differentiation than proposed before. Although the humanized mouse model is suitable to recapitulate most of the human SCID phenotypes, murine enzymes can complement and overcome human deficiency in SCIDs that result from lacking certain metabolic enzymes (like ADA) [185]. An overview of the different developmental blocks causing SCID is depicted in **Figure 6**. However, the availability of primary SCID HSCs to performed xenograft experiments is restricted due to the low

disorder incidence. Alternatively, 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 [186, 187] can be differentiated in vitro into T cells [188-190]. Successful iPSCs modelling X-linked chronic granulomatous [191, 192], X-linked SCID [193], JAK3 SCID [194], Wiskott-Aldrich Syndrome (WAS) [195], RAG1 SCID [196, 197] and RAG2 SCID [198] have been generated allowing to study the immune phenotype caused by the genetic mutation as well as enabling preclinical efficacy and safety studies.

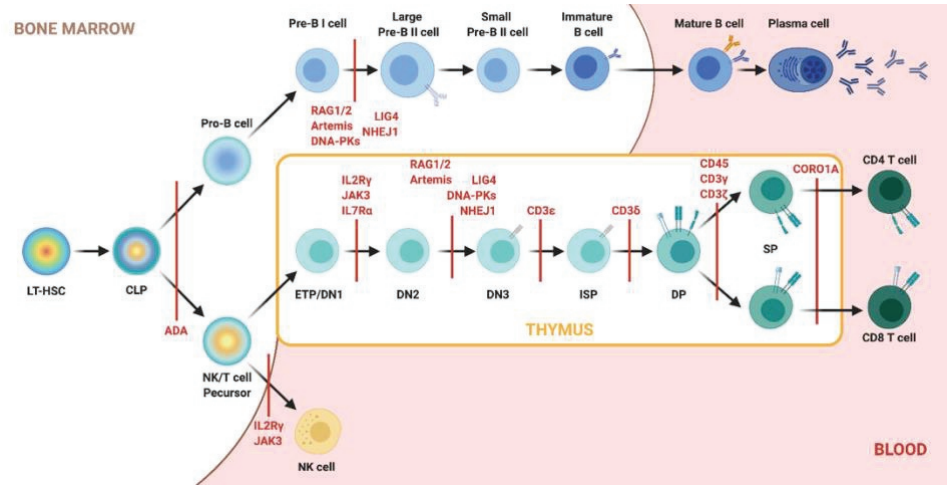


Figure 6: Schematic representation of the developmental blocks in B, T and NK cell development in SCID. *Lymphoid development blocks caused by the different genes are indicated. B–cell developmental arrests are commonly visualized by flow cytometry and Immunoglobulin repertoire analysis on available patient bone marrow aspirates. T-cell development block are derived from murine data, on different genetic mouse models and xenograft models that have been developed to mimic the different forms of SCID described in human. Diverse mutations in the same gene can lead to different clinical phenotypes, developing into a leaky block. In that case, like RAG1/2, the earliest block is depicted in the figure. (Adapted from the thesis of K.Pike-Overzet); Created with BiorenRender.com)*

THERAPY FOR SCID

Although allogeneic HSCT remains the prevailing therapeutic treatment for SCID, and for a long time the only treatment, gene therapy has been explored for the last 3 decades as an alternative treatment (see **Figure 7**). In addition, ADA-SCID patients can benefit from enzyme replace therapy using bovine PEG-ADA, although it is very costly as it involves lifelong administration and requires appropriate monitoring [199].

Newborn screening for SCID

An improved HSCT outcome, better survival and lower morbidity rate, is observed in SCID patients with early diagnosis and early treatment [200, 201]. Newborn screening (NBS) is a suitable strategy to successfully identify SCID patients early, before the onset of infectious complications [202, 203].

The implementation of the TREC assay within the NBS program remains a major regulatory and logistical challenge but is slowly being included in many countries. The first TREC assay as part of the NBS program was initiated in Wisconsin in 2008 [209] and proceed state-by-state until by the end of 2018, all states of United States had NBS for SCID up and running [101, 210, 211]. Outside the United States TREC assay is currently implemented in Israel (2015) [212], New Zealand, Taiwan (2102) [213], Canada (several provinces), Australia (some regions) and some European countries like Spain (Catalonia, 2017) [214], Iceland (2017), Norway (2018) [215], Switzerland (2019), Germany (2019), Sweden (2019) [216] or Denmark (2020) [217]. Pilot studies are being performed in additional countries such as Spain (Andalusia) [218], France [219], Finland, Italy, Poland or The Netherlands [220]. The TRECs assay allows an early detection identifying asymptomatic SCID patients, protection from infection and early treatment. In addition, NBS for SCID has revealed novel genes causing SCID, like Bcl11b [221]. Altogether, the TREC assay became the first immune disorder for which NSB was possible, as well as the first high-throughput DNA-based NBS assay [222, 223].

Additional aspects of SCID can be addressed in a similar way. B lymphopenia can be identified by detecting the kappa recombination excision circle (KREC) formed during IGK locus rearrangement during B cell development [206]. The KREC quantitative assay can be performed by PCR, similar to TRECS and has been included is some pilot studies [216, 218].

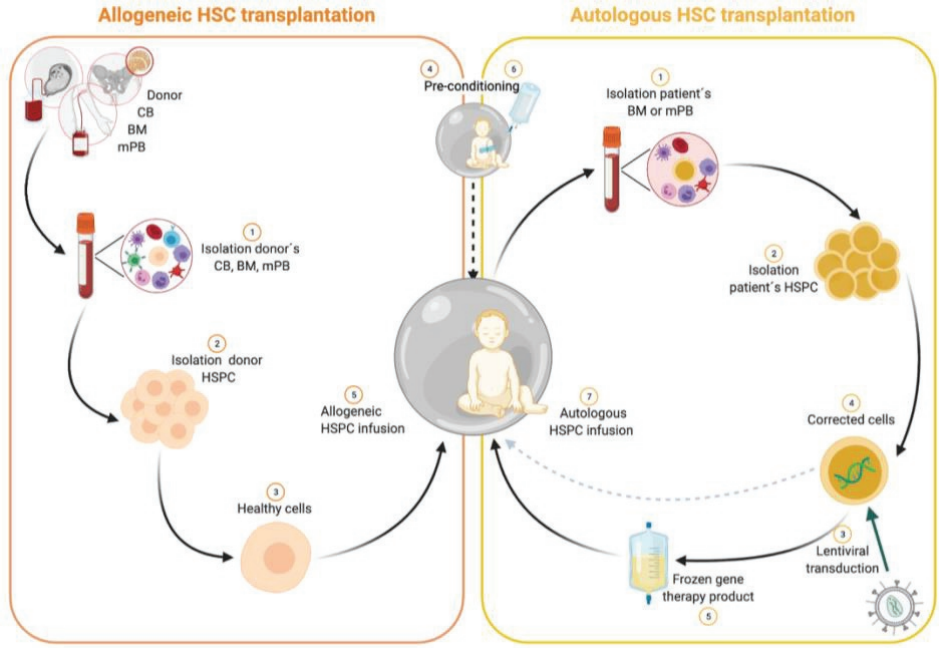


Figure 7: Therapy options for SCID: overview of allogeneic and autologous HSCT. *Conventional allogeneic hematopoietic stem cell (HSC) transplantation relies on the isolation of hematopoietic stem and progenitor cells (HSPCs) from a healthy compatible donor (1). Enriched healthy HPSC (2)(3) can be transplanted back (5) into the (pre-conditioned (4)) patient. Alternatively, autologous HSC-based gene therapy is an emerging approach for come SCID deficiencies. HSPC are isolated from the*

patient BM or mPB itself (1)(2). Cells are transduced ex vivo with a correct version of the defective gene (3). Corrected cells (4), i.e., the therapy product, are frozen down for validation (5) and infused (7) into the (pre-conditioned (6)) patient. CB (cord Blood), BM (Bone Marrow), mPB (mobilized Peripheral Blood). (Created with BioRender.com)

Conditioning regimens

HSCs, both allogeneic or gene-corrected autologous, may result in limited engraftment of progenitors without prior conditioning regimen, due to the occupation of BM and thymic niches by host cells, which results in incomplete graft function, immune reconstitution and cure. Conditioning agents can create space in the BM niches providing space for the donor HSCs to engraft efficiently. In addition, certain conditioning agents can eliminate recipient T cells (leaky SCID patients) and NK cells (NK^+ SCID patients) and thereby prevent graft rejection after transplantation [224]. Conditioning regimens have shown to contribute to an improved HSCT outcome by increasing HSC engraftment, T- and B-cell chimerism and immune function and reducing the risk of graft rejection. Administering conditioning regiment prior transplantation has led to higher T-cell counts and higher B-cell chimerism, IgA recovery and lower rates of ongoing Ig replacement therapy [225-227]. However, conditioning regimens have a significantly negative impact on patient survival, with significant short-term and long-term related mortality and morbidity. The use of irradiation-based regimens and alkylating chemotherapy in infants impacts children's growth, fertility and chance of secondary malignancies [228].

The basic backbone of the conditioning regimen for primary immunodeficiencies was set in WAS patients successfully treated with busulfan, a chemotherapy alkylating agent, combined with cyclophosphamide for T-cell ablation. To avoid transplant related mortality due to this myeloablative conditioning, reduced toxicity regimes have been developed by substitution of cyclophosphamide with fludarabine, the use of lympholytic antibodies and the use of individualized busulfan pharmacokinetics monitoring [229]. Busulfan-containing regimens are known to result in better B-cell reconstitution and function. Reduced toxicity regimens have shown to sustain long-term engraftment and immune reconstitution, importantly even in patients with pre-existing organ damage. Moreover, complete donor chimerism is mostly observed after conditioning [225], although an insufficient conditioning dose can carry the risk of mixed chimerism in the HSC compartment [230] and therefore reducing transplantation success.

Current gene therapy protocols for ADA-SCID and X-linked SCID rely on chemotherapy conditioning based on a low dose of busulfan, approximately 25% of the total dose usually used in totally myeloablative protocols, which has minimal toxicity sufficient for effective engraftment. However, it may be insufficient in other forms of SCID (RAG1/2) where there is greater occupancy of marrow niches. Its benefits should also be weighed against its short and long-term toxicity, especially in Artemis deficiency with inherent radiosensitivity due to impaired DNA repair and in newborn patients [227]. Although current conditioning agents are successfully used, there is a pressing need for alternative, less toxic conditioning regimens to create space in the BM niches for a durable engraftment without adverse effects on extramedullary tissues. Development of effective, nontoxic, non–alkylating-based conditioning regimen is essential to ensure a successful transplantation

and good quality of life for all patients with SCID. Accordingly, antibody-based conditioning regimens are being developed, which may achieve long-term myeloid engraftment without the associated toxicities of current chemotherapy-based regimens. Different variations of antibodies-based conditioning are being both pre-clinically and clinically tested [231-235].

Conventional allogeneic HSC transplantation

As described, SCIDs are caused by genetic defects intrinsic to HSCs (and all other cells of the body), making HSCT from a healthy donor a rational therapeutic approach to replace diseased cells and to provide a life-saving and curative treatment. The discovery of the human major histocompatibility complex in 1967 [236, 237] opened the possibility for HSCT approaches. Shortly after the discovery, the first successful BM transplants with successful engraftment and immune reconstitution was reported in a SCID patient treated with stem cells from a healthy sibling donor [238, 239]. Since then, HSCT has been intensively study as a definitive curative treatment for SCID. The recent advances in HSCT including a more accurate human leukocyte antigen (HLA) typing, development of less-toxic conditioning regimens and pharmacokinetic monitoring, development of more effective T-lymphocyte depletion methods and more effective supportive care have significantly improved the outcome of HSCT [22, 240, 241]. Success rate of HSCT for SCID is generally over 70%, resulting in 90% success in patients with early identification of SCID and HLA-matched family donor [201, 242-244]. Despite this improvement over time, the broad spectrum of clinical and immunological phenotypes associated with SCID makes it difficult to define a universal transplant regimen. The HSCT outcome differs depending on the source of the donor HLA–matched related donor or HLA-mismatched donor, the disease genotype, the use of conditioning, the age at transplantation and the health status of the patient at the time of the treatment. A graft from a genetically full-matched sibling donor has greater likelihood of achieving 5-year survival and freedom from immunoglobulin substitution (90%) than unrelated (66%) or HLA-mismatched donors (54%) [102, 201, 242]. The use of this last source has diminished over time, while the use of unrelated matched donor transplants increases. Successful HLA-matched cord blood transplantation from an unrelated donor have also been reported. [245, 246].

The clinical phenotype of SCID itself influences the transplantation outcome, particularly for unconditioned patients. The quality of immune reconstitution depends on the phenotype, with overall better survival and reconstitution in patients with a B+ SCID phenotype than those with a B- phenotype. Indeed, HSCT for RAG SCID patients is associated with a poorer prognosis. Although T-cell reconstitution is obtained in most patient, total B-cell recovery remains more challenging, with only 17% of the patients recovering B-cell function after transplantation. Patients with unsuccessful immune B-cell reconstitution require immunoglobulin replacement or a boost transplantation [227, 243, 247-249]. Moreover, the use of pre-conditioning regimens is not always desirable due to their toxicity. However, conditioning can contribute to improved HSCT outcome, achieving a better cell engraftment and long-term immune reconstitution by clearing BM space prior transplantation [22, 241]. Therefore, the development of less toxic conditioning approaches like antibody-based condition is a pediatric need. Both the absence of pre-existing and active infections as well as the age of the patient (before 6 months of age) improves the outcome transplantation, leading to excellent results also with donors other than matched

siblings [250]. The early diagnosis needed to treat asymptomatic SCID patients can be achieved with the implementation of the NBS program. Despite all improvements, HSCT is associated with short-term and long-term complications. Graft-versus-Host disease (GvHD) remains a significant complication associated, disfavoring all benefits of the transplantation. Therefore, especially patients without suitable HLA-matched donors (>50% of patients)[251] and those with serious comorbidities would benefit from autologous gene therapy [252].

Autologous HSC-based gene therapy for SCID

An essential feature of HSC-based gene therapy is the persistent long-term correction of the disease, lasting for life with a unique one-time treatment, offering a cure for the disease. Moreover, primary immunodeficiencies can affect one or multiple cell lineages. The bases of gene therapy were established following the scientific advances during the 1960s and early 1970s. Friedmann suggested good exogenous DNA could be used to replace the defective DNA in patients with monogenetic defects who suffer from its associated rare diseases [253]. Gene therapy can be broadly divided into two groups based on the targeted disease and the delivery method: *In vivo* and *ex-vivo* gene therapy. In *in vivo* gene therapy the transgene is administered intravenously into the patient either by a viral or non-viral vector and reaches the target cells inside the body. In contrast, *ex vivo* gene therapy is performed outside the body. For instance, for SCID, HSCs are isolated from the patient's BM or mPB, modified with the therapeutic transgene using a crippled virus for gene delivery, after which corrected cells are transplanted back into the (pre-conditioned) patient (**Figure 7**). Most likely in SCID, gene corrected cells have a selective growth advantage over the non-transduced diseased cells [104].

First gene therapy attempts for SCID

The use of integrating vectors in gene therapy for immunodeficiencies has a long history by now, with over 2 decades of experience since the first clinical trials started for X-linked SCID [254]. Initial trials of gene therapy for ADA and X-linked SCID were accomplished with a retroviral vector derived from Murine Leukemia Virus (MLV_gamma-retrovirus) which drives expression of the transgene by the long-term terminal repeat (LTR). Retroviruses can bind and fuse with the host cell membrane. The viral RNA genome is inserted into the host cell and reverse transcribed into DNA that subsequently integrates into the cell DNA. As the transgene of interest stably integrates into the host DNA, a long-lasting therapeutic effect is most likely achieved, allowing the transmission of the therapeutic material to all progeny of the transduced cells (i.e., all blood lineages developed from transduced HSCs). Although successful correction of the disease with differentiation of all lymphoid cell lineages and improved immune functionality was observed in most of the patients and no problems were observed in the ADA trial [255], safety issues resulted from the X-linked trials. Unfortunately, a total of 5 patients treated with the first generation of γ-retroviral vectors (γ-RV) were reported to develop T cell lymphoblastic leukemia (T-ALL), 4 patients in the X-linked SCID trial conducted in Paris and 1 patient in the London X-linked SCID trial out of the total 20. These leukemias were caused by insertion mutagenesis of the therapeutic vector leading to ectopic expression of oncogenes. It became apparent that there was some preference near transcriptional active sites such as LMO2, LYL1, c-Jun, BMI1 or CCND2 oncogenes [256-259]. Similar adverse effect was detected in gene therapy trials for

other immunodeficiencies like X-linked chronic granulomatous disease [260] and WAS [261], revealing a need to develop a new generation of safer vectors with a decreased risk of insertional mutagenesis [262].

SIN Lentiviral-based system

Self-inactivating (SIN) vectors, lacking potent enhancers in the LTRs were developed, for both γRV and lentiviral vectors (LV). The SIN design eliminates the enhancer activities on neighboring genes by modifying the 3′LTRs regions. The U3 region of the LTRs regions is removed decreasing the transactivational activity it can have on nearby genes [263, 264]. As the SIN system is devoid of LTR activity, an internal promoter needs to be included to drive expression of the therapeutic gene. Although SIN- γRV were developed and no adverse leukemic events were reported so far [265-267], the presence of an integration pattern comparable to the non-SIN γ-retroviral vectors might be of concern [268]. In addition, SIN-γRV reached low transduction efficiency and expression [269].

New vectors were based on human immunodeficiency virus type 1 (HIV- 1) and modified to guarantee vector safety. These SIN-LV are far more effective in transducing non-dividing cells than the MLV-based counterpart allowing an increased transduction efficiency of HSCs [270]. Naldini and colleagues (1998) [271] developed the now well-known 3rd generation LV system resulting in the generation of replication-deficient LV to prevent repackaging (**Figure 8**). The system results in a packaging design of 4 plasmid in which all non-essential viral genes have been removed and the essential viral genes have been separated into several plasmids. Additional improvements have been implemented along the years on the LVs aiming to enhance transgene expression and stability like the addition of insulators, polyadenylation signals [272], the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) [273] and the codon optimization. A more random integration pattern described for the SIN-LVs gives a favorable and safer integration profile by reducing the risk of insertional mutagenesis compared to γ-RV [274, 275]. SIN-LVs have been used widely for preclinical [172, 176] and clinical [133, 276, 277] gene therapy studies with no leukemia development observed; being the safest approach to date with a highly reduced genotoxicity compared to γ-RV [278, 279]. Whereas this risk of severe adverse effects has not surfaced in the clinic to this date as a pathological finding, we must remain aware of its possible occurrence and continue our efforts toward further alleviating its risk.

Gene therapy product for clinical implementation

The gene therapy product, i.e., the medicine, consist of the combination of the HSPCs with the therapeutic transgene integrated. One of the most important release criteria to assess efficacy and safety of the gene therapy product is to determine transduction efficiency by means of the vector copy number (VCN). The VCN indicates the number of integrated transgene copies into the DNA per target cell and it is an important parameter requiring a rigorous control as it can be related to potential product genotoxicity. The therapeutic potency of the transgene correlates positively with the proportion of transduced cells and the vector integrations, which depends on the degree of transduction achieved in the product. The VCN threshold selected for a therapeutic gene therapy product corresponds to the minimal transduction efficiency required to guarantee the

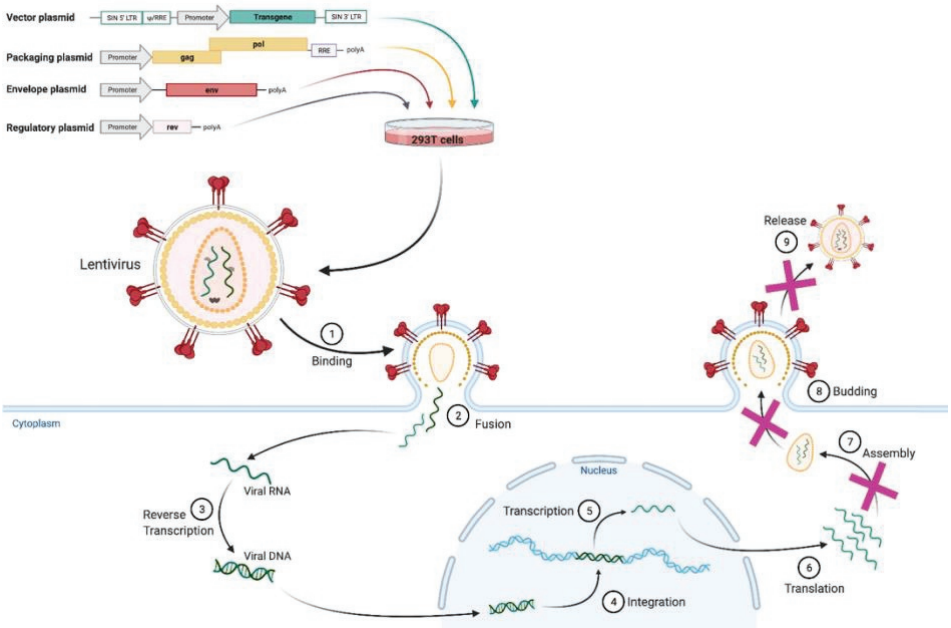


Figure 8: Schematic representation of the 3rd generation lentiviral vector (LV) system. *The 3rd generation LV system consist of a 4-plasmid packaging design in which all essential viral genes have been separated into several plasmids. The 4 plasmids (vector, packaging, envelope and regulatory plasmids) are co-transfected into 293T cells to produce the therapeutic lentivirus harboring the transgene of interest. Deficient hematopoietic stem cells can be transduced with the therapeutic lentivirus generated. In contact with the cells, the lentivirus bind (1) and fuse (2) to the cell membrane allowing to release the viral RNA into cytoplasm. The viral RNA is reverse transcribed into viral DNA (3) that moves into the cell nucleus where it integrates into the cell DNA (4). Using the host cellular mechanisms, the transgene is transcribed (5) and translated (6); however, as all non-essential viral genes have been removed, new lentiviruses cannot be re-assembled (7) and therefore new lentiviruses cannot be produced (8) (9). As shown with the crosses, no virus is formed after transduction with these crippled viruses. (Created with BioRender.com)*

correction of enough HSPCs and the sufficient transgene expression (mostly a codon optimized transgene) to achieve a therapeutic effect. The gold standard technique toreliably assess the VCN and transgene expression is the quantitative Polymerase Chain Reaction (qPCR). Sastry et al. (2002) [280] developed and established qPCR as the method for detecting LV sequences relative to a housekeeping gene and therefore, quantified the number of inserted vectors per cell, allowing the detection of multiple vector copies per cell. In short, transduced cells are kept in culture for 7 to 14 days to avoid the detection of free plasmids and ensure the readout of stable vector integration. Afterwards, DNA from bulk cultured cells is isolated and VCN is determined by qPCR. After transduction only a proportion of the targeted HSPCs carry the therapeutic vector. However, the qPCR method is based upon bulk HSPC population averages. Unfortunately, the current method does not provide a specific indication on the actual initial portion of transduced cells, nor does it allow to assess the integration pattern on the single therapeutic cells. Thus,

determining transgene expression with a multiparametric technology such as single cell-based flow cytometry represents an attractive alternative to bulk methods, allowing to unmask cellular heterogeneity in the gene therapy product.

Rationale for gene therapy for RAG-SCID

First attempts to correct RAG deficiencies started by using γRV driving native expression of RAG2, where pre-clinical studies showed sustained correction of the deficiency in RAG2-/- mice [281]. RAG1 gene therapy development started using the same strategy with γRV and a native RAG1 transgene. Long-term immune T- and B-cell reconstitution was achieved, although the high VCN needed lead to the occurrence of one leukemic event and therefore safety issues were raised [282]. SIN LV was continuously being improved, hence a SIN LV with the native RAG1 transgene was developed, and its efficacy was evaluated. Both the *in vitro* (virus production and transduction) and *in vivo* therapeutic effect were assessed. Unfortunately, RAG1 expression was insufficient and therefore, a codon-optimized version of RAG1 was used (c.o.RAG1). Transduction efficiency, transgene expression and *in vivo* efficacy were improved as shown by Pike-Overzet et al. (2011) [283]. Additional studies of SIN LV with a codon optimized version of RAG2 also suggested an improved immune reconstitution [284]. However not full correction was observed with different vectors using c.o.RAG1 [285], suggesting that successful correction of RAG deficiency strongly depends on the transgene expression levels [286]. Gene therapy to treat RAG-SCID seemed to be possible with SIN LV; however, the vector used for proof-of-concept studies was still inappropriate. Accordingly, the vectors have been updated into clinically applicable vectors.

OUTLINE OF THE THESIS

SCID is an immune disorder affecting predominantly T-cell development for which autologous gene therapy is emerging as a suitable treatment option. The aim of this thesis is to unravel a better insight on the transcriptional network involved in early T cell development as well as to develop a lentiviral-based gene therapy approach and protocols to treat RAG1 and RAG2 SCID. In Chapter 2 we focus on understanding the functional definition of transcription factors regulating T cell lineage commitment process as an approach to get new insight on key factors involved in proper T-cell development. In Chapter 3 we review all steps and challenges to develop HSC gene therapy for immunodeficiencies, from preclinical development to clinical implementation, highlighting the laborious pre-clinical studies, challenging scaling up manufacturing and regulatory hurdles. Successful pre-clinical development of autologous LV-based HSC gene therapy to treat RAG1-SCID is depicted in Chapter 4. Gene therapy was successful to safely restore the immune system in Rag1-/- mice, as well as in patient cells transplanted into NSG mouse model. Same LV gene therapy approach is described for RAG2 SCID treatment in Chapter 5, however immune reconstitution is more challenging as RAG2 levels seems to be more tightly regulated. In Chapter 6 we introduce a novel method to improve the detection of transduction efficiency and better understand the heterogeneity of the gene therapy product at the single cell level. As pre-conditioning regimens can influenced transplantation and gene therapy success, in Chapter 7 we explore the

feasibility to model and develop low toxicity conditioning regimens based on reduce dose chemotherapy combined with novel mobilizing agents. In the general discussion, Chapter 8, the data obtained in this thesis and their implications are discussed together with suggestions for future research.

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Chapter 2

Functional definition of a transcription factor hierarchy regulating T cell lineage commitment

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ABSTRACT

T cell factor 1 (Tcf1) is the first T cell–specific protein induced in multipotent progenitors following Notch signaling in the thymus, leading to the activation of two major target genes, Gata3 and Bcl11b. Tcf1 deficiency results in partial arrests in T cell development, high apoptosis, and increased development of B cells and myeloid cells. Phenotypically, seemingly fully T cell–committed thymocytes with Tcf1 deficiency have promiscuous gene expression, an altered epigenetic profile and can dedifferentiate into more immature thymocytes and non-T cells. Restoring Bcl11b expression in Tcf1-deficient cells rescues T cell development but does not strongly suppress the development of non-T cells; in contrast, expressing Gata3 suppresses the development of non-T cells, but does not rescue T cell development. Thus, T cell development is controlled by a minimal transcription factor network involving Notch signaling, Tcf1, and the subsequent division of labor between Bcl11b and Gata3, thereby ensuring a properly regulated T-cell gene expression program.

INTRODUCTION

T cells are disease-fighting leukocytes that, similar to all blood cells, originate from hematopoietic stem cells (HSCs). However, whereas all other blood cell lineages develop in the bone marrow in specific niches, T cells develop in the thymus, a specialized organ located in the chest where progenitor cells migrate from the bone marrow and definitively commit to the T cell lineage, ultimately forming mature T cells [1]. The development of T cells within the thymus is a highly complex process involving successive stages in which the expression of CD4 and CD8 co-receptors occurs in distinct microenvironments [2]. Via a series of progressive developmental stages, T cell precursors (i.e. thymocytes) differentiate from double-negative (DN; $CD4^-CD8^-$) cells into intermediate immature single-positive (ISP; $CD8^+CD3^-CD4^-$) cells, then into double-positive (DP; $CD4^+CD8^+$) cells, and finally into single-positive (SP; $CD8^+CD4^-CD3^+$ or $CD4^+CD8^-CD3^+$) cells. In the DN stage, developing thymocytes can be further subdivided into four stages of differentiation based on their expression levels of CD44 and CD25: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). Early stages are not committed to the T cell lineage (i.e., fate restricted), allowing other lineages to develop [3]. Indeed, B cells, dendritic cells, myeloid cells, and natural killer (NK) cells can all be generated from $CD44^+CD25^-c\text{-}kit^{hi}$ early thymic progenitors (ETPs) [4, 5], DN1 cells, and—albeit to a lesser extent—DN2 cells [6]. These multipotent cells, which can enter a number of differentiation programs, are directed towards the T cell lineage via a process called specification. The irreversible capacity to develop solely into T cells occurs somewhat later and is referred to as T lineage commitment; this process also involves the active repression of non-T cell lineages [7-9].

The microenvironment of the thymus provides a cellular context that drives T cell development. This process is initially driven by the expression of Notch ligands, particularly delta-like protein 4 (DLL4) [10], and later in the DP stage by providing the signals required to control positive selection (for self-MHC) and negative selection (against autoreactive T cell clones). The various stages in T cell development have been investigated in great detail using flow cytometry and genomic analyses; thus, T cell development serves as a paradigm for the molecular regulation of cell fate [11, 12]. The fact that T cell development occurs in an anatomically separate niche has allowed researchers to study the detailed successive steps that underlie lineage specification and commitment. All of the events that establish the identity of T cell precursors are driven by Notch signaling [13], involving binding of the transcription factor RBP-J (also known as CBF1) to intracellular Notch ligands, thereby forming an active transcription factor complex in ETPs.

The subsequent stages of T cell development are governed by several key transcription factors that form an intricate gene regulatory network [14]. The core set of transcription factors in the early phases of T cell development are Tcf1 (encoded by the gene confusingly termed *Tcf7*), Gata3, Bcl11b, and two members of the E2A family (E2A and HEB), Ikaros and Runx1 [14-17]. Importantly, the *Tcf7* gene is a direct Notch signaling target and the first T cell–specific transcription factor induced by Notch signaling [18]; in contrast, Bcl11b drives T cell commitment by limiting the NK cell fate and activating the T cell developmental gene program at the DN2-DN3 stage [19], leading to expression of the fully rearranged TCR-beta gene at the DN3 stage. Rothenberg and colleagues showed that

four transcription factors — Tcf1, Gata3, Notch/RBP-J, and to a lesser extent Runx1 — are required for the timed expression of Bcl11b [14]. Of these four transcription factors, Tcf1 is the most complex, as it can act as both a transcriptional repressor (e.g., when bound by a co-repressor such as Groucho) or a transcriptional activator by binding β-catenin in order to respond to canonical Wnt signals [20]. Interestingly, Tcf1 also acts as a tumor-suppressor gene [21, 22], and it can be functionally replaced — at least partially — by Lef1, a related transcriptional regulator expressed at approximately 50-fold lower levels than Tcf1 [23]. Additional complexity arises from many alterative splice forms and alternative promoter usage, leading to at least 6 different Tcf1 isoforms that are differentially expressed throughout the T cell lineage.

The precise role that Tcf1 plays in regulating T cell specification and commitment, and its interaction with other core regulatory factors in T cell development, is not fully understood. Therefore, we examined the role of Tcf1 at the earliest stages of T cell development, focusing initially on fully committed DN3 cells. We found that Tcf1 is necessary for driving thymocytes down the T cell developmental path even after the T cell commitment stage, as Tcf1-deficient DN3 thymocytes can dedifferentiate into DN1/2-like cells that can then develop into the myeloid and B cell lineages. In addition, we found that Tcf1 supports this "lineage fidelity" via two direct — and functionally complementary — target genes, *Gata3* and *Bcl11b*. An epistasis analysis using retroviral gene complementation in Tcf1-deficient stem cells revealed that the role of Gata3 in immature T cells is to repress B cell and myeloid fate, whereas Bcl11b establishes the T cell lineage program, and its expression can overcome the defect in T cell development in Tcf1 deficient thymocytes.

MATERIALS AND METHODS

Mice

C57Bl/6 TCF-1 -/- ΔVII/ΔVII mice were originally described by Verbeek et al (1995) and C57Bl/6-Ly5.1 mice were purchased form Charles Rivers Laboratories. Mice were bred and maintained in the animal facility of Leiden University Medical Center. All animal experiments were performed in accordance with legal regulations in The Netherlands and with approved protocols of the Dutch animal ethical committee.

Mice used for transplantation assay were kept in specified pathogen-free section and were fed with special food and antibiotic water. Genotyping assay of newborn Tcf1 mice was performed with DNA samples from earpieces using GoTaq Flexi DNA polymerase kit (Promega) according to manufacturer's instructions.

Flow cytometry and cell sorting

Single cell suspensions from thymus, spleen, BM and blood were stained with monoclonal antibodies against CD3e, CD4, Cd8a/Ly-2, CD11b/Mac-1, CD19, CD25, CD27, CD44/Ly-24, CD45.1/Ly-5.1, CD45.2/Ly-5.2, B220/CD45R, CD90.2/Thy1.2, CD117/c-kit, CD135/Flt3, Gr1/Ly-6G-6C, NK1.1, Sca1/Ly-6A, TCRβ, TCRvβ5.1/5.2, TCRβ6, TCRβ8 and Ter-119/Ly-76 (See **Table S3**). All antibodies used were directly conjugated to biotin, fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin Chlorophyll-a Protein (PerCP), PE-Cy7, allophycocyanin (APC), APC-Cy7 or efluor450. Biotinylated antibodies

were revealed with streptavidin conjugated antibodies (PE, efluor450, APC-Cy7, APC or Pe-Cy7) (all antibodies were purchased from BD, Biolegend or eBioscience).

Cells were blocked with normal mouse serum (NMS, Invitrogen) for 10min at room temperature and subsequently cell surface staining was performed in two steps. Firstly, cells were incubated for 30min at 4°C in the dark with the antibody-mix solution including directly conjugated antibodies at the optimal working solution in FACS buffer (PBS pH7.4, 0.1% azide, 0.2% BSA). After washing with FACS buffer, a second 30min incubation step at 4°C was performed with the streptavidin conjugated antibodies mix.

Cell apoptosis was assessed by AnnexinV and 7AAD staining, which was performed following the PE AnnexinV Apoptosis detection Kit protocol (BD Pharmingen) after the cell surface staining. Proliferation assay was done by intracellular Ki67 staining (mIgG as control) with PE Mouse anti-human Set protocol (BD Pharmingen). For that purpose, cells were initially stained for cell surface markers as described previously and subsequently fixated and permeabilized by using fixation/permeabilization buffer (eBiosience) for an hour at 4°C. Cells were then washed with permeabilization (eBiosience) buffer with 2% NMS and stained with Ki67 or IgG1 solution for 30min at 4°C in the dark. The same procedure was used to assess icTCRβ expression.

Double positive CD4&CD8 cells before DN cell sorting and lineage positive cells before LSK/LK sorting were depleted using magnetic-activated cell sorting, autoMACS (Miltenyi Biotec). For DNs sorting, thymocytes were first stained with anti-CD4 and CD8-biotin, following by Streptavidin microbeads staining according to manufacturer instruction (Miltenyi Biotec). For LSK/LK cell sorting, lineage depletion kit (Miltenyi Biotec) was used according to manufacturer instruction. Subsequently, depleted cells were stained again for DNs or LSKs as described before. Cell sorting was performed on FACSAria II (BD Biosciences) or stained cells were measured with FACS-CantoII and LSR Fortessa x-20 (BD Bioscience). Data was analysed using FlowJO (Tree Star). All different hematopoietic populations were defined as described in **Table S4** and **Fig S7**.

Cell culture

Bone-marrow-derived stromal cell line OP9 and OP9-DL1 cells which ectopically express the Notch ligand Delta-Like 1 (DL1) were used as described by J.C. Zuñiga-Pflucker. Sorted DN cells were cultured on OP9 or OP9 WT/OP9-DL1 (10:1) confluent monolayers in αMEM (Lonza)-10%FCS, 1% P/S (Life Technologies) and GlutaMAX (Life Technologies) medium complemented with 50 ng/ml rmFlt3L, 50 ng/ml rmSCF, 10 to 1 ng/ml rmIL-7, and 50μM β-mercaptoethanol (β-ME; Sigma-Aldrich). (all cytokines purchased from R&D). Cells were harvested after 7 to 14 days of coculture and were analysed by flow cytometry.

Transduced LSK and LK with LZRS-ires-eGFP (control), LZRS-Gata3-eGFP or LZRS-Bcl11b-GFP vector were cultured on OP9-DL1 monolayer for 6 to 14 days in αMEM-10%FCS complemented with rmIL7 (10 ng/ml), rm Flt3L (50 ng/ml), rmSCF (10 ng/ml) and β-ME (50μM). Harvested cells were analysed by flow cytometry or sorted.

Retroviral production

LZRS-Gata3 and Bcl11b plasmids were obtained from Addgene and cloned into LZRS-ires-eGFP vector (Addgene, control vector). Control, Gata3 and Bcl11b retroviruses were generated using Phoenix ecotropic and amphotropic packaging cell line (ATTC). Cells were cultured in IMDM (Lonza)-10%FCS-1% Penicillin/Streptomycin -1%Glutamine and transfected with plasmids using X-treme Gene9 DNA transfection reagent (Roche) protocols. Selection of transfected cells was performed with 1mg/mL puromycin (Sigma-Aldrich) for a week and viral supernatant was harvested at 24h and 48h.

Retroviral transduction

LSK and LK sorted cells were stimulated overnight in StemSpan serum-free expansion medium (StemCell Technologies) supplemented with 10ng/ml rmTPO (R&D), 50ng/ml rmFlt3L (R&D) and 100ng/ml rmSCF (R&D). Hematopoietic progenitors were transduced using RetroNectin (Takara Bio Inc) coated wells according to the manufacturer's instructions. Non-tissue culture plates were coated with RetroNectin overnight at 4ºC and then blocked with 2% bovine serum albumin (BSA) in PBS for 30min. Retroviral supernatant (24h or 48h) was centrifuged at 1500xg for 1h at 32ºC and incubated an extra hour at 37ºC. After coating, viral supernatant was removed and stimulated cells were immediately added on the virus-coated plates. Cells were cultured in StemSpan medium supplemented with rmTPO (10 ng/ml), rmFlt3L (50 ng/ml) and rmSCF (100 ng/ml) and transduced overnight at 37ºC. LZRS-ires-eGFP, LZRS-Gata3-ires-eGFP and LZRS-Bcl11b-ires-eGFP transduced cells were used for *in vitro* and *in vivo* approaches.

Quantitative real time q-PCR

RNA from sorted cells was purified using Micro RNeasy kit (Qiagen) and reverse transcribed into cDNA using Superscript III kit (Invitrogen). RT-PCR was performed using TaqMan Universal Master Mix II in combination with specific probes for indicated genes from Universal Probe Library (Roche). Specific primers for ABL-2, Bcl11a, Bcl11b, Gata3, Pax5, PU.1/Spfi1, IL-7Ra, CD117/c-kit, ID2, Axin-2, Hes1, CD3e, CD3d, pTa and ZAP70 were designed and purchased from Sigma-Aldrich (See specific gene sequences on **Table S5**). Samples were analyzed by StepOnePlus RT-PCR system (Life Technologies). Relative transcript abundance was determined by ΔCt and expression levels were normalized for the endogenous reference gene ABL-1. All samples were run in at least in duplicates.

RNA-Seq

RNA from sorted DN3b cells ($Lin^-CD25^+CD44^-CD27^+$) from Tcf1-/- and wild-type littermates thymi was isolated using the Mini RNeasy Kit (Qiagen) The integrity (scores > 9.0) of the RNA was determined on the Agilent 2100 Bioanalyzer (Agilent). Total RNA enrichment for sequencing poly(A) RNAs was performed with the TruSeq mRNA sample preparation kit (Illumina). 1µg of total RNA for each sample was used for poly(A) RNA selection using magnetic beads coated with poly-dT, followed by thermal fragmentation. The fragmented poly(A) RNA enriched samples were subjected to cDNA synthesis using Illumina TruSeq preparation kit. cDNA was synthesized by reverse transcriptase (Super-Script II) using poly-dT and random hexamer primers. The cDNA fragments were then blunt-ended through an end-repair reaction, followed by dA-tailing. Subsequently, specific

double-stranded bar-coded adapters were ligated and library amplification for 15 cycles was performed. The pooled cDNA library consisted of equal concentration bar-coded samples. The pooled library was sequenced in one lane, 36 bp single read on the HiSeq2500 (Illumina). Raw RNA-seq reads are accessible on SRA by accession number SRP158670.

RNA-seq data processing

FASTQ files were aligned to the mm10 genome using STAR 2.5.1b (Dobin et al, 2013). Transcript counts were quantified and annotated using HTSeq-0.6.1. WT sample 3 was removed due to a low number of aligned reads.

Differential expression and statistical analysis

Differential expression of DN3b wild-type vs TCF1-/- was identified by using DESeq2 (Love et al. 2014), after filtering for genes with a low read count (> 5 reads per sample) resulting in 205 differential expressed genes (97 upregulated in KO and 108 downregulated in KO) at p-value < 0.05 (FDR adjusted) and a Log2 Fold Change of > 1.5.

Geneset Enrichment

RNA-seq results from mouse T-cell precursors in different developmental stages including DN1, DN2a, DN2b, DN3 and DP (GEO accession: GSE89198, Rothenberg et al.) were used to create DN1 and DN2 genesets. Of this RNA-seq dataset log2 transformed FPKM values of 25 DN2 and 8 DN3 wildtype mice were used for differential expression analysis with Limma. Genes that were differentially upregulated (p-value < 0.05 and LogFC > 2) between DN1 vs DN3 (365 genes), DN2a vs DN3 (342 genes), DN2b vs DN3 (120 genes) and DN2a/b combined vs DN3(141 genes) were used as genesets for Geneset Enrichement Analysis. GSEAPreranked (GSEA 4.0.3, Broad) was run on all expressed Wild-type vs TCF -/- RNA-seq genes which were ranked by the p-value and LogFC generated by DESeq2. The DN3b TCF -/- was negatively associated with the DN1 geneset (Normalized Enrichement Score of -1.04) and positively associated with all of the DN2 genesets (DN2a NES 1.23, DN2b NES 1.53, DN2a/b combined NES 1.36).

ATAC-Seq

15,000 sorted DN3a ($Lin^-CD25^+CD44^-CD27^-$) and DN3b ($Lin^-CD25^+CD44^-CD27^+$) cells were washed 1 time with cold PBS. Pellets were spin down at 500 g for 5 min at 4°C, and the supernatant was removed carefully. 20 µl of transposase mix (10µl 2xTD buffer, 1 µl TDE (Nextera DNA Library Prep Kit; Illumina), 0.2 µ digitonin (G9441, Promega), 8.8 µl nuclease-free water) was added to the cells. Reactions were incubated at 37°C for 30 min. Transposed DNA was purified using the MinElute Reaction Cleanup Kit (28204, Qiagen), amplified, and again purified according to published protocols (Buenrostro et al 2015, CurrProtocMolBiol). Size selection was done using Low Range Ultra Agarose (161-3107, Bio-Rad). Fragments between 150-600bp in size were used for further analysis. Quality and quantity of the libraries was assessed by Bioanalyzer High Sensitivity DNA Analysis Kit (Agilent) before sequencing. Libraries were sequenced 50 bp, paired-end, on a HiSeq4000.

The reads were filtered by quality using Trim-galore tool (Krueger, 2015) (default values) and the quality control was driven by FastQC (Andrews, 2010) and MultiQC (Ewels, 2016).

The remained reads were mapped to mm10 using bowtie2 (Langmead et al, 2012) with –very-sensitive parameter. After all, before the peak calling, the read duplicates and multiple mapping reads were removed using Picard tools (http://broadinstitute.github.io/picard). The peaks for 2 wild-type and 2 Tcf1 -/- samples were called using MACS2 (Zhang et al, 2008) with the following parameters: -g mm -B –shift -100 -ext 200 –nomodel –q 0.05 and BigWig-tracks with FPKM were generated by deeptools (Ramirez et al, 2014). Coverage plots and heatmaps were generated with deeptools using the BigWig tracks previously generated with the following parameters: --binSize 100 -m 3000 -b 1000 -a 1000. To find differential open chromatin regions, the differential peaks between wild-type and Tcf1 -/- conditions were calculated by DiffBind R Bioconductor package (Start et al, 2011), only the statistically significant peaks (FDR<0.05) were taken in account for downstream analysis. Motif analysis on the differentially accessible regions was performed using Homer (http://homer.ucsd.edu/homer/) using the parameters: size given. MEME-FIMO (Grant et al. 2011) and Tcf1 position probability matrix (MA07769.1) from JASPAR (http://jaspar.genereg.net/) were used to analyze the distribution of the Tcf1 motif on the differentially accessible regions.

Chromatin immunoprecipitation

DN thymocytes ($CD8^-CD4^-$) from Tcf1-/- and wild-type littermates were sorted and subsequently crosslinked with formaldehyde (Sigma). Crosslinking was quenched with Glycine and after cell lysis chromatin was sonicated into fragments. Sonicated chromatin was precleared and incubated with antibodies. TCF-1 (C46C7; #2206 Cell Signalling Technologies). Immuno precipated chromatin complexes were purified and quantified by real-time PCR using Faststart Universal Sybr Green Master mix (Roche). (See specific gene sequences on **Table S5**).

Stem cell transplantation

Competitive transplantation assay is used to determine HSC development and functionality in vivo by measuring multi-lineage reconstitution of hematopoiesis in irradiated transplanted mice. Competitive transplantation Ly5.2/Ly2.1 was used to assess if in vivo re-expression of Gata3 could rescue T cell development in the thymus. Total 52.500 Ly5.2 Tcf1 (wild-type or -/-) transduced cells (mixed LSK and LK progenitors cells) were transplanted into lethally irradiated (8.07Gy) Ly5.1 recipient mice (8-12 weeks), together with 300.000 splenocytes (Ly5.1) as support cells. Chimerism and peripheral T cell were analysed at week 6 after transplantation in peripheral blood. Mice were sacrificed for analysis 7 weeks after transplantation to evaluate hematopoietic system repopulation. Mice were considered repopulated when ≥1% multi-lineage Ly5.2 Tcf1 cells could be detected. Single cell suspension from the thymus, spleen and bone marrow (BM), as well as lysate blood were analyzed by flow cytometry as described previously.

Statistical methods

All statistics were calculated and all graphs were generated using GraphPad Prism6 (GraphPad Software). Statistical significance was determined by Mann-Whitney U test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$), Multiple t-test or Two-Way ANOVA depending on the experimental setting.

RESULTS

Tcf1 deficiency leads to several arrests in T cell development with increased non-T cells

Tcf1 deficiency results in multiple incomplete blocks in T cell development that vary from mouse to mouse. Besides the well documented block at the ISP stage [24-26], T cell development can be arrested at DN1, DN2 and DN3 stages (**Fig S1A**). In contrast to these partial arrests in developing mice, transplanting Tcf1-deficient stem cells into adult recipient mice led to a complete block in T cell development at the DN1-DN2 transition (**Fig.S1B**), presumably the result of an insufficient compensatory expression of Lef1 in these cells [27]. We also observed increased percentages of non-T cell lineages, most notably B cells and myeloid cells (**Fig.S1C&1D**), consistent with previous reports of *ex vivo* cultured Tcf1 deficient cells.

Phenotypically, fully committed DN3 Tcf1-deficient thymocytes have promiscuous gene expression and altered chromatin

Given the effects of Tcf1 deficiency on sequential stages of T cell development, we initially focused on those stages where thymocytes should be fully T cell committed. Therefore, we compared gene expression profiles between Tcf1-deficient thymocytes and wild-type thymocytes. The T cell commitment process starts at the DN2 stage and continues to the DN3a (CD25+CD44-CD27-) stage, in which a rearranged Tcrb gene is expressed in combination with pTA to form the pre-TCR complex in a process known as β-selection. After β-selection, the cells rapidly proliferate, express CD27, and are fully T cell committed based on expression of a functional, rearranged Tcrb gene [28]. We consider thymocytes αβ T cell committed when they express a fully rearranged TCRβ. We realize that there are definitions where T cell commitment occurs at earlier stages but phenotypically defined DN3(b) cells are here considered as the candidate population for committed T cells. We performed whole-transcriptome RNA-Seq on DN3b cells obtained from Tcf1-deficient and wild-type littermates (**Fig.1A**), reasoning that at DN3b thymocytes should be fully T cell lineage committed (see **Fig.S2B**; CD27 and Ptcra). We found 108 genes with downregulated expression (> 1.5 fold, FDR<0.05) in the Tcf1-/- DN3b thymocytes, but also 97 upregulated genes (**Table S1**). For visualization, the top 100 differentially expressed genes are shown and the absence of Tcf7 expression was confirmed in Tcf1 deficient DN3b cells. Furthermore, the RNA-seq analysis shows fewer rearranged Tcrb genes than in wild-type control DN3b thymocytes, as shown for the Trbj expressed gene segments (data not shown). We used the genes differentially expressed between Tcf1 deficient and wild-type DN3b cells in a Gene Set Enrichment analysis (GSEA) and used published gene sets of T cell developmental stages to establish a DN1 and DN2 signatures[29]. The genes highly expressed in Tcf1-/- DN3b clustered strongly with the DN2-specific gene set (DN2a and DN2b but not DN1), indicating that they share many characteristics of earlier developmental stages that are less T cell committed (**Fig.1A** and **Fig.S2A**). The RNA-seq data also indicated that many of the T cell commitment genes were low or not expressed while genes involved in non-T cell lineages (Pax5, Pu.1, Blc11a) were highly expressed in the Tcf1 deficient cells compared to the control DN3b cells. Based on these data we validated the expression of a number of important T cell developmental genes by q-PCR

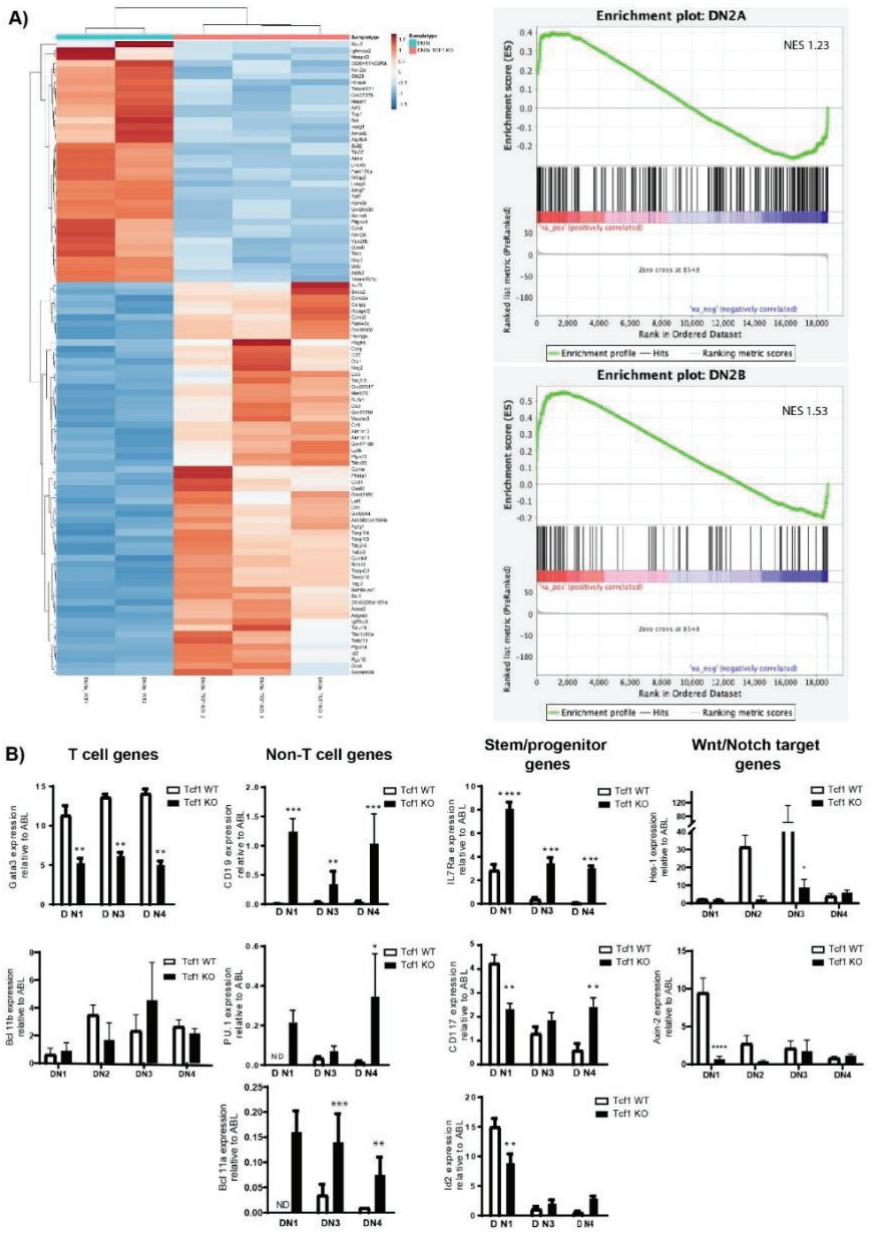

Figure 1: Tcf1-deficient DN3b cells show promiscuous gene expression compared to WT littermate controls. *A) Heat map of the top 100 differentially expressed gene as determined by RNA-seq of sorted DN3b cells from WT and Tcf1-deficient thymi. GSEA of the differentially expressed genes (Tcf1−/− KO over Tcf1 WT for DN3b) is enriched for DN2 genes (DN2a and DN2b with NES +1.23 and + 1.53, respectively). B) qPCR validation of RNA-seq data for selected T cell–specific genes, genes expressed in non-T cells, and legacy genes whose expression is inherited from stem cells/multipotent progenitors. The levels of expression are normalized by ABL-2 expression as*

*housekeeping gene. (Mann-Whitney U test; *P < 0.05, **P < 0.01, and ***P < 0.001. Error bars represent the SD of three pooled mice and from two independent experiments.)*

on sorted DN1, DN2, DN3 and DN4 thymocytes. These results validated the RNA-Seq data and showed lower expression (2-fold change) of the T cell specific transcription factors Gata3 (DN1 to DN4) and Bcl11b (DN2 stage) (with higher expression of its functional counterpart Bcl11a) while the B cell commitment marker CD19 and the myeloid associated factor Pu.1 were significantly higher expressed in the Tcf1 deficient thymocytes (**Fig.1B** and **Fig. S2B**). In addition, genes known to be associated with stem/progenitor cells (sometimes referred to as legacy genes[1]) such as c-kit were also significantly higher expressed (**Fig.1B**), while both Wnt and Notch target genes (HES-1 and Axin2) were decreased. Collectively, these data showed that while in some regards Tcf1-/- DN3b thymocytes were T cell committed (phenotypic markers, expression of some Tcrb genes), they also showed lineage infidelity, with expression of master regulatory genes from non-T cells.

The strongly reduced number of thymocytes due to the lack of Tcf1 is not only explained by the developmental arrests and differentiation into non-T cells, but also by high levels of apoptosis. Compared to wild-type cells, we found increased levels of apoptosis in Tcf1-deficient cells at nearly every stage (**Fig.S3A**), as well as decreased cell proliferation in the DN2 and DN4 stages (**Fig.S3B**).

Gata3 and Bcl11b are direct targets of Tcf1 and downregulated in Tcf1 deficient thymocytes

The downregulated mRNA expression levels of the transcription factors *Gata3* and *Bcl11b* in various DN thymocyte stages in Tcf1 deficient mice, suggested that these factors may be direct target genes of Tcf1. In accordance, the Bcl11b and Gata3 promoter/enhancer sequences contain conserved Tcf/Lef binding sites [30 31]. To check whether in *ex vivo* DN thymocytes these promoters are regulated in a Tcf-dependent manner, we performed chromatin immune precipitation (ChIP) using a monoclonal antibody specific for Tcf1 (**Fig.2A**) followed by q-PCR. This revealed binding of Tcf1 to the Gata3 and Bcl11b promoter sequences in wild-type DN thymocytes but not in Tcf1 deficient thymocytes, consistent with both genes being direct target genes of Tcf1. This supports previous reports on OP9-DL1 cultures [18] and reporter gene assays.

This finding was further substantiated by ATAC-Seq (Assay for Transposase-Accessible Chromatin) data which indicates chromatin accessibility. In general, we found fewer ATAC-Seq peaks in DN3b thymocytes lacking Tcf1 compared to wild-type DN3b cells, 55217 and 50175 peaks were found in wild-type samples and 21142 and 7520 peaks in Tcf1 -/- samples; but in DN3a thymocytes, there is no a clear difference between Tcf1 -/- and wild-type. In total, 68883 and 30357 peaks were found in wild-type samples and for Tcf1 -/- samples, 40716 and 68605 peaks (**Fig.S2C**).

To find regions with differentially chromatin accessibility between Tcf1 -/- and wild-type for DN3a and DN3b thymocytes, we looked for peaks statistically different between the conditions. For this analysis only differential peaks with FDR less than 0.05 were taken in account. In DN3a, 564 accessible sites were lost in Tcf1-/- cells, from which 141 were Tcf1

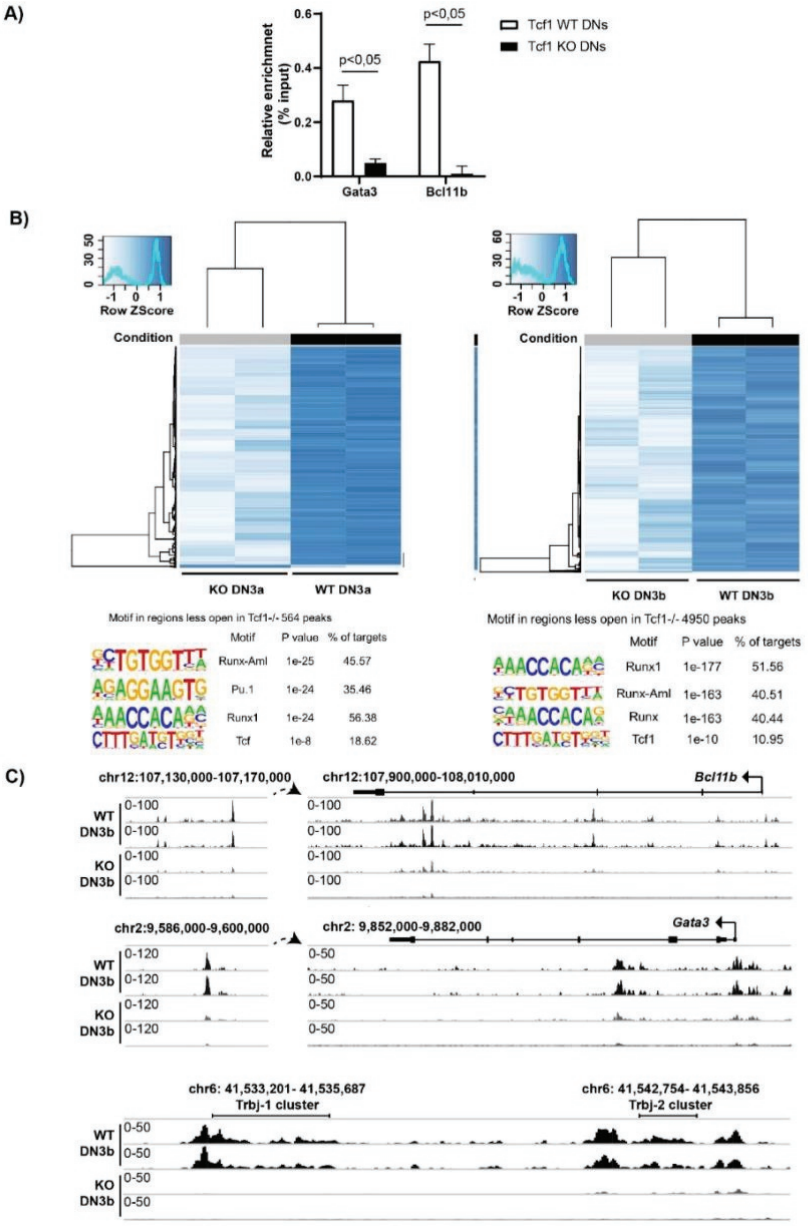

***Figure 2: Chromatin accessibility analysis in Tcf1-deficient versus WT DN thymocytes**. A) Chromatin immunoprecipitation with an antibody specific for Tcf1 revealed that the Gata3 promoter and the Bcl11b enhancer are occupied by Tcf1 in vivo, whereas in Tcf1 KO DN thymocytes, no binding can be detected. Negative controls with IgG instead of anti-Tcf1 showed no enrichment. (Multiple t test. Error bars represent the SD of at least three pooled mice and from two independent experiments.) B) Heat map of DESeq2 normalized read counts of ATAC-seq shows differentially accessible regions between WT and Tcf1−/− in DN3a and DN3b. Motif analysis was performed in the differentially accessible regions using HOMER showing the three highest scores and Tcf1 score. C)*

ATAC-seq data mined for the Bcl11b, Gata3, and Trbj (T cell Receptor Beta) genomic regions. Per locus, the relative abundance of transposase accessible regions is indicated. The individual ATAC-seq profile from each genotype is shown. Data are shown as normalized read density.

binding sites. Only 8 sites were statistically significant higher in Tcf1-/- containing 3 Tcf1 binding sites. In the case of DN3b, extra sites were lost in Tcf1-/- compared to Tcf1 wild-type (4950 in total), including 756 Tcf1 binding sites. 21 sites were more accessible, but no Tcf1 binding sites were found. These results indicate that global chromatin accessibility was higher in wild-type thymocytes than in Tcf1 deficient thymocytes (**Fig.2B**). Interestingly, both DN3a and DN3b share the fact that Runx motifs seem to be abundantly lost upon Tcf1 deficiency (**Fig.2B**), in accordance with the diminished *Runx1* expression shown in the RNA-seq data (**Fig.S2B**).

Focusing on the *Bcl11b* and *Gata3* promoter/enhancer sequences, the chromatin in these promoters was less accessible compared to wild-type littermate control DN3b cells (**Fig.2C**). Similarly, the TCRB loci were much less accessible in accordance with the RNA-Seq data. The full genome-wide data analysis is provided in **Table S2**. Interestingly, no major differences in chromatin accessibility were found at genes involved in alternative lineages (not shown), indicating that expression of these genes was not regulated at the level of chromatin opening. Collectively, these data show profound differences due to the lack of Tcf1 in chromatin accessibility and expression of genes and promoters associated with T cell commitment.

Phenotypically, fully committed DN3 Tcf1-deficient thymocytes dedifferentiate into DN1 thymocytes, B cells, and myeloid cells.

Based on the hypothesis derived from these results, that Tcf1 deficient DN3 thymocytes may not be fully T cell committed, we sought to better investigate the differentiation capacity of Tcf1-/- DN3 thymocytes. Therefore, DN3 cells were sorted and cultured under conditions with strong T cell inducing capacity (OP9-DL1 system). Indeed, the majority of wild-type DN3 thymocytes differentiated further into DN4 cells, with a smaller part remaining DN3 (**Fig.3A&B**). Unexpectedly, most Tcf1-/- DN3 thymocytes dedifferentiated into DN1 and DN2 cells, with extensive B and myeloid development while only a minority of cells remained DN3 without any further development along the T cell lineage (**Fig.3A&B**). Especially development into B cells was extensive, with up to 60% of DN3 thymocytes developing into B cells (**Fig.3A&B**). These dedifferentiated DN1 and DN2 cells were not a contaminating fraction in the sorted DN3 cells that expanded, as intracellular staining for Tcrb revealed high Tcrb expression in these DN1/2 cells at similar levels as cells remaining in DN3 stage and wild-type DN3 and DN4 cells (**Fig.3C**). Therefore, these non-T cells (B and myeloid cells) developing in the assay expressed icTCR indicating that they also derived from the seeded DN3 Tcf1 -/- promiscuous cells (data not shown). We conclude that Tcf1 KO cells dedifferentiate to less committed cells and exhibit lineage DN1 and DN2-like cells were derived from the sorted “fully” committed DN3 cells. Similarly, infidelity with significant development into alternative (non-T) lineages. When ETP cells rather than DN3 cells were seeded on OP9-DL1, as expected, Tcf1 deficient cells were arrested in development at DN1 (**Fig.S4A**), with abundant B and myeloid development,

whereas wild-type stem cells differentiated along the T cell lineage with many fewer non-T cells (**Fig.S4B**).

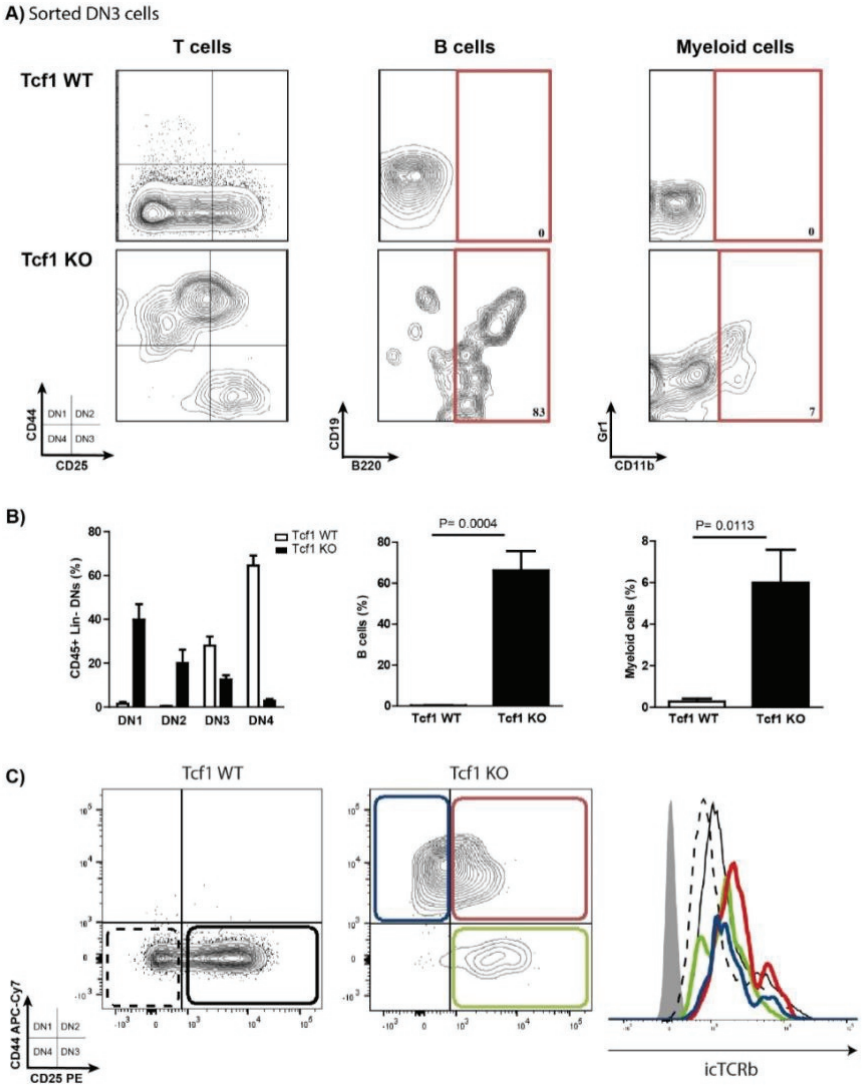

Figure 3: Tcf1-deficient DN3 cells dedifferentiate into DN1/2-like cells with multipotent lineage capacity. *A) WT DN3 cells sorted and seeded on OP9 WT/OP9-DL1 (10:1) cells develop largely further into DN4 or remain DN3 after 7 days in culture, while Tcf1-deficient cells develop into DN1 and DN2 cells (pre-gated Thy1+ Lin− cells) with prominent B cell (B220+ CD19+) and myeloid cell (CD11b+ Gr1+) development. B) Quantification of the developmental plasticity and dedifferentiation effects of DN3 Tcf1-deficient thymocytes into DN1, DN2, myeloid, and B cells. C) Intracellular TCRß staining reveals the dedifferentiated DN1 and DN2 cells to be derived from DN3 cells. (Mann-Whitney U test. Error bars represent the SD of three samples from three independent experiments.)*

Dedifferentiation into alternate lineages can be prevented by expressing Gata3 in Tcf1 deficient thymocytes

Epistasis analysis is a powerful genetic tool, often used in model organisms such as Drosophila to investigate hierarchical relationships between genes [32]. It can be more complex to perform in mammals such as mice, where not only expression per se but also gene dosage is important. For instance, while complete loss of Gata3 blocks T cell development at the earliest stages, transgenic overexpression of Gata3 can lead to development of mast cells in the thymus [33-36]. We therefore expressed Gata3 and Bcl11b using recombinant retroviruses as they have a broad range of expression that would allow different phenotypes to be selected under the strong developmental pressure of the thymic microenvironment. We used retroviruses encoding GFP only, Gata3 together with GFP or Bcl11b together with GFP to investigate complementation of the Tcf1 phenotype by either Gata3 or Bcl11b (**Fig.4A, 5A**). We used retroviruses solely encoding GFP as negative controls. Re-expression of Gata3 could partially rescue the development of Tcf1-/- thymocytes from a DN1 arrest to an apparent CD25+ DN2 stage but not further (**Fig.4B**). However as, Thy1 expression was not increased on the apparent DN2 cells, they cannot be considered real DN2 cells. Similarly key T cell lineage specific (*CD3, PtA*,) gene expression was not induced upon forced Gata3 expression (**Fig.S6A&B**). Strikingly, high Gata3 expression strongly suppressed the enhanced development of B and myeloid cells (granulocytes as well as monocytes) from Tcf1-/- thymocytes. This also occurred to some extend when starting with wild-type cells (**Fig.4C**). Competitive stem cell transplantation (Ly5.2 Tcf1 stem cells / Ly5.1 recipient mice) was used to assess if re-expression of Gata3 could rescue T cell development in the thymus *in vivo.* The suppression of B cell development (Ly5.2 B cells) in the thymus was also observed *in vivo* when Gata3 complemented Tcf1-deficient stem cells were transplanted in irradiated recipient mice (**Fig.4D_right panel**). However, thymic T cell development (Ly5.2 T cells) again was arrested at a DN1/2 transition, barely different than GFP control transduced cells (**Fig.4D_left and middle panel**). Thus, the major role of Gata3 in earliest DN development is the suppression of non-T cell development with only a minor feed forward role into the T cell program.

The T cell lineage-specific defects caused by Tcf1 deficiency can be rescued by expressing Bcl11b

Enforced expression of Bcl11b (**Fig.5A**), in contrast, rescued the T cell developmental defect of Tcf1 deficient cells virtually completely. Bcl11b transduced Tcf1 deficient stem cells developed readily into Thy1 positive (**Fig.5B** and **Fig.S5B**) cells and could develop into DN2 and DN3 thymocytes to a similar degree as wild-type thymocytes (**Fig.5C** and **Fig.S5C**) (while non transduced Tcf1 deficient cells are arrested at the DN1/DN2 stage as the control cells (**Fig.5D**). In addition, expression of TCRβ by intracellular flow cytometry also was restored to wild-type levels in DN3 and DN4 by expressing Bcl11b in the Tcf1 KO background (**Fig.5D**). Accordingly, T cell receptor gene expression was rescued upon Bcl11b overexpression in Tcf1 deficient cells (**Fig.S6B**). In contrast, expression of Bcl11b did not markedly influence B and myeloid development from Tcf1 deficient cells (**Fig.5E** and **Fig.S5C**). Overexpression of Blc11b did suppress the development of NK cells

(**Fig.S5E**), consistent with its described role in promoting T cell fate over NK cell fate at the DN2 stage [19].

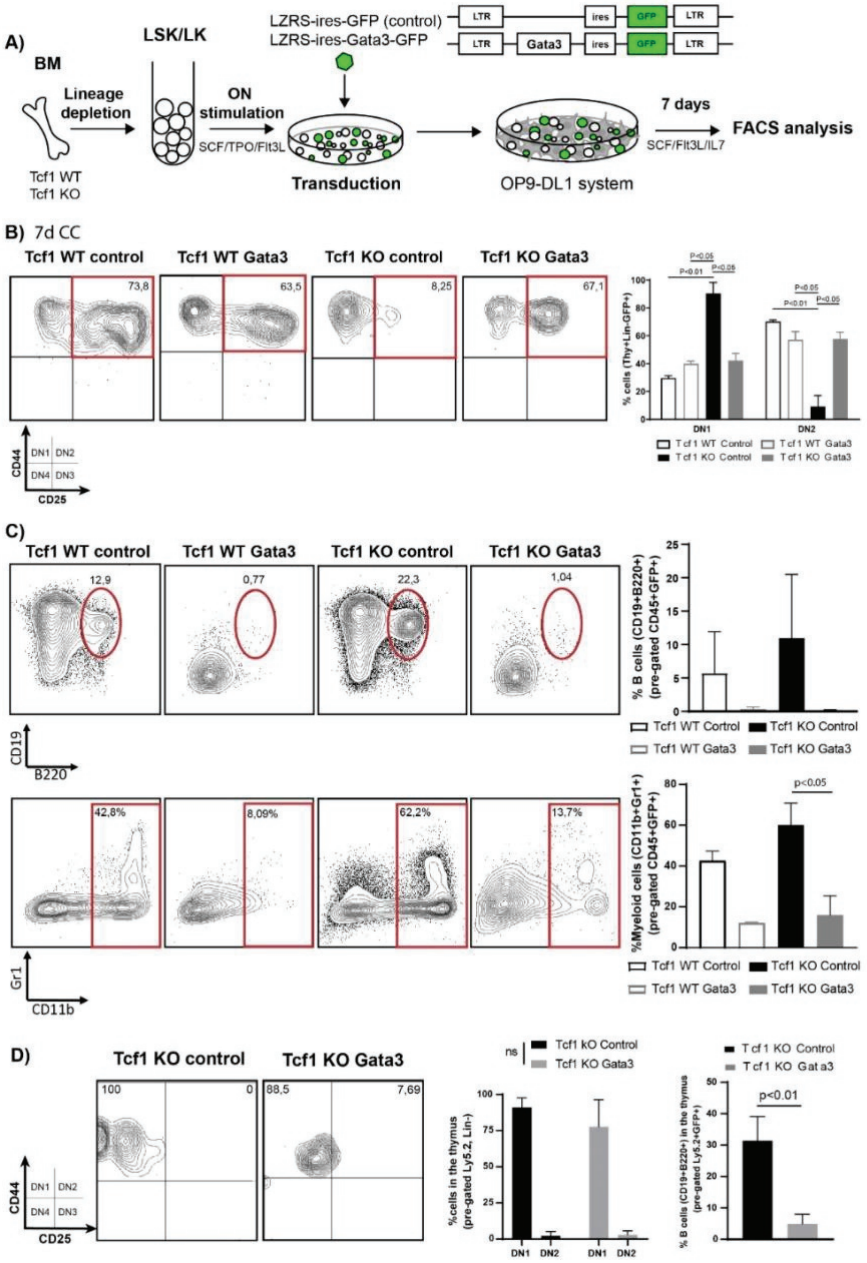

Figure 4: Re-expression of Gata3 suppresses B and myeloid development in Tcf1 deficiency. (A) Layout of retroviral complementation experiments with GFP control and/or Gata3. B) Gata3 expression partially overcomes the DN1 thymocyte block and C) suppressed the enhanced non-T cell

lineages (B and myeloid cell development) after 7 days in the OP9-DL1 culture system. FACS shows representative plots and graphs quantitative data from replicate measurements. (Multiple t test analysis. Error bars represent the SD from two independent experiments.) D) In vivo complementation [Ly5.2 Tcf1 (WT or KO)–transduced stem cells transplanted into Ly5.1 recipient mice] reveals suppression of B cell development also in the thymus (right) 8 weeks after transplantation but minimal and partial rescue of T cell development in the thymus (left and middle). (Middle: Multiple t test analysis. Right: Paired t test. Error bars represent the SD from three individual mice per group.)

DISCUSSION

T cell development has been used as a classic example of a relatively ordered pathway to study cell fate determination [16], thereby giving the impression that transcriptional regulation during T cell development is a well-understood process. Despite this general belief, however, and compared to other developmental processes (for example, B cell development, which has similar requirements in terms of proliferation, lineage restriction, immune receptor rearrangement, and checkpoints for premature and mature immune receptors), the roles of the major transcription factors in T cell development are rather poorly understood. In B cell development, a clearly defined linear hierarchical relationship exists between E2A, EBF1, and Pax5 [37-44]. However, with respect to early T cell development, whether the Notch (RBP-J), Gata3, Bcl11b, Runx1, E2A, Tcf1/Lef1, Ikaros, and/or Hox genes play unique, redundant, or synergistic roles remains unclear and is the subject of intense research that focuses largely on either individual factors or the collective activity of these factors using computational biology. Considering that Notch signaling is required for T cell development, and given that the first T cell–specific target gene is Tcf7 [18], which encodes Tcf1, we investigated the process of T cell lineage commitment in Tcf1-deficient mice.

The study of Tcf1-deficient mice is generally complicated by three factors. First, in the absence of Tcf1, the HMG box transcription factor Lef1 — which is expressed in the thymus, albeit at much lower levels than Tcf1 — plays a compensatory role [23, 27, 45] (**Fig.S2B**). This low-level expression of Lef1 causes incomplete penetrance of the Tcf1-deficient phenotype. However, if adult Tcf1-deficient stem cells are either transplanted into recipient mice or cultured on OP9-DL1 cells to induce T cell differentiation, a complete block occurs at the DN1 stage (see **Fig.1D**), as Lef1 expression is believed to result from reaming fetal expression in the thymus [21,22, 27]. Therefore, in our experiments we used bone marrow-derived cells obtained from Tcf1-deficient mice. Second, Tcf1-deficient mice are prone to developing T cell lymphomas in the thymus [22], which is similar to T-cell acute lymphoblastic leukemia (T-ALL) in patients. As discussed above, this issue can be overcome by using Tcf1-deficient stem cells instead of thymocytes. The third issue associated with studying Tcf1-deficient mice is that Tcf1 functions as both a transcriptional repressor and a transcriptional activator (for example, when bound to the Wnt mediator β-catenin). Indeed, when Tcf1-dependent promoters were tested using in vitro reporter systems, transcription occurred only when β-catenin was also expressed [46, 47]. Consistent with this notion, Tcf1 binds to the promoter/enhancer regions of the target genes Gata3 and Blcl11b, and it seems likely that Tcf1 binds to β-catenin at these promoter regions. Co-chromatin immune precipitation experiments provide initial evidence for this notion, as

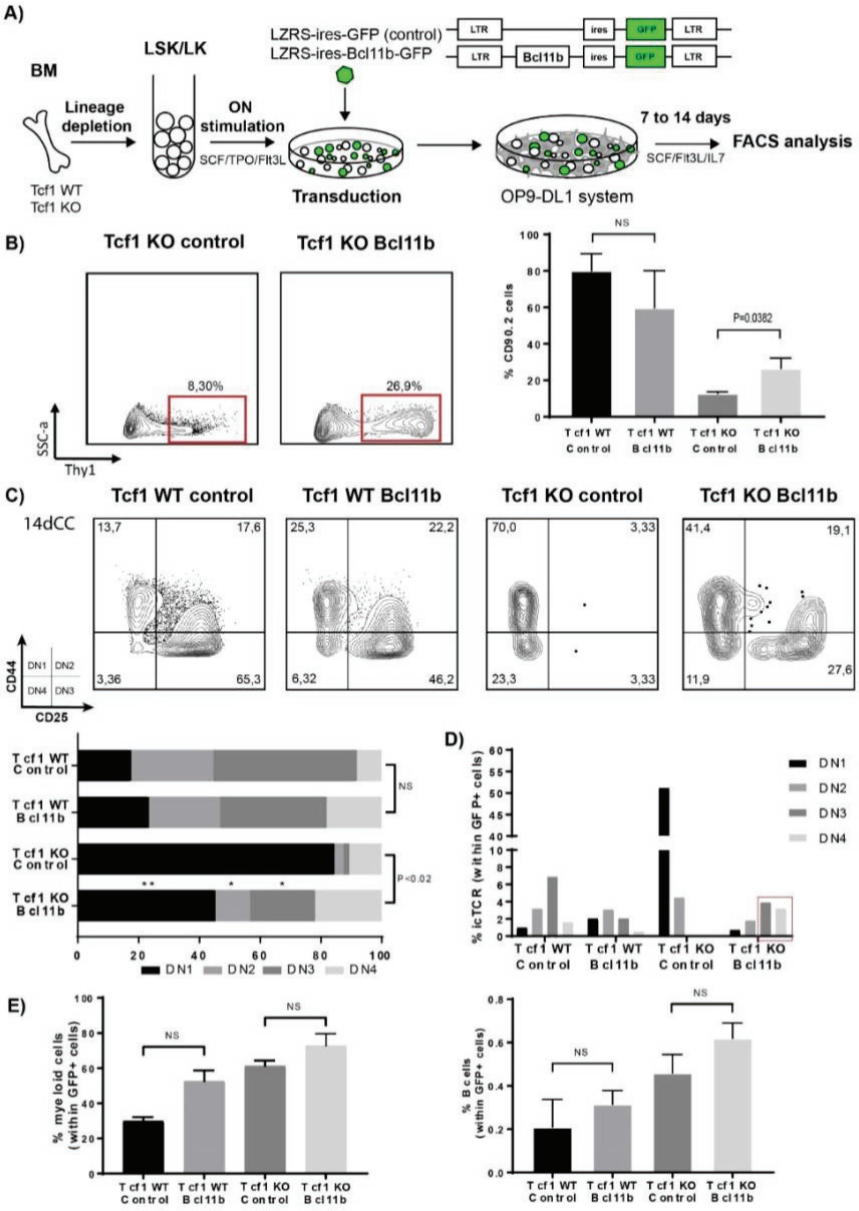

Figure 5: Reexpression of Bcl11b rescues T cell development in Tcf1-deficient stem cells. *A) Layout of retroviral complementation experiments with Bcl11b. B) Thy1 expression is rescued by Tcf1 deficiency by expression of Bcl11b after 14 days in OP9-DL1 culture (pre-gated Lin-GFP+ cells). (C) Bcl11b fully rescues T cell development from Tcf1-/- stem cells that otherwise are arrested in DN1 (pre-gated Thy1+Lin-GFP+). D) Intracellular TCRβ expression can be restored in Tcf1-deficient cells by expression of Bcl11b (pre-gated Thy1+Lin-GFP+ DN subset). E) Bcl11b overexpression does not affect myeloid and B cell development. (Two-way ANOVA. Error bars represent the SD from three independent experiments.)*

β-catenin can also be found at active promoters where Tcf1 binds (data not shown). In addition, DN stages of T cell development show high canonical Wnt signaling, which is driven by β-catenin and Tcf/Lef [48]. Of note, expression of the Wnt target gene Axin2 was markedly reduced in thymocytes lacking Tcf1 (**Fig.1B**). On the other hand, some of Tcf1's functions in the earliest stages of T cell development are independent of β-catenin [18], possibly due to the redundant role of Lef1.

A seminal study by Busslinger and colleagues revealed that Pax5 is a major lineage commitment factor in the development of B lymphocytes [42, 43, 49]. Thus, B cells that lack Pax5 can dedifferentiate into multipotent progenitor cells that can replenish all hematopoietic lineages, even in vivo. In this respect, our findings are somewhat analogous, as Tcf1-deficient DN3 cells —which seemingly are fully committed — have promiscuous gene expression and can dedifferentiate into immature cells that can give rise to non-T cell lineages, including B cells and myeloid cells. In the T cell lineage such de-differentiation has also been shown to occur in E2A or HEB deficient thymocytes [50 51]. Indeed, key transcription factors that drive alternate lineages (e.g., the transcription factors Bcl11a, Pax5, and Pu.1) are robustly expressed in Tcf1-deficient DN3 and DN4 cells, but not in wild-type cells. In contrast with Pax5-deficient cells, however, only a small number of Tcf1-deficient cells survive the dedifferentiation process, which is likely due to the highlevel of apoptosis in Tcf1-deficient thymocytes (**Fig.S3**). Additionally, the assessment of chromatin status by ATAC-seq revealed that in Tcf1 deficient thymocytes, the chromatin is more condensed and several key T cell specific loci (for instance the Tcrb locus) are less accessible and therefore likely not as readily transcribed and expressed (**Fig.2B&C**). Therefore, the mechanisms underlying dedifferentiation in Pax5 deficiency and as reported here in Tcf1 deficiency appear to be mechanistically different. It should also be noted that formal proof of dedifferentiation in Tcf1 deficiency would require use of a conditional knockout model using a Floxed allele with a Cre enzyme under control of a late acting promoter during thymocyte differentiation. As commitment implies loss of plasticity and the capacity to give rise to only one cell type but not to others, Tcf1 deficiency in contrast is associated with lineage infidelity and lack of commitment.

Recent work has investigated the epigenetic status of DP thymocytes in Tcf1 deficiency, similar to our experiments using DN3 thymocytes [52]. In agreement, Tcf1-/- DN thymocytes also display more condensed chromatin (**Fig.2B**). Yet Tcf1 in the context of T cell commitment and immature thymocyte development seems to act mostly as a transcription factor regulating expression of other key T cell specific genes than acting as a chromatin modifying factor per se. Indeed, an intrinsic HDAC activity has been shown for Tcf1 in CD8+ cells [53]. Our analysis in DN3 T cell populations revealed that only a very small number of sites containing a Tcf1 motif (n= 3 in DN3a, n= 0 in DN3b) gained accessibility in Tcf1-/- cells. This supports an activator rather than a suppressor function for Tcf1 in early T cells. Similar observations, i.e. the majority of sites (80%) lost accessibility in Tcf1-/- DP cells, were reported by others in total DP thymocytes [52], again consistent with a function of Tcf1 as a transcriptional activator. One explanation could be that the HDAC activity of Tcf1 is differentially required (e.g. cell type specific, context dependent manner) and would be different in developing T cells in the thymus versus effector cell maturation

in CD8+ peripheral cells. This is consistent with the observation that HDAC-deficient Tcf1 could largely restore differentiation into the CD4+ lineage [53]. Nevertheless, further analyses will be required to fully understand the activator/repressor functions of Tcf1 in immune cell development.

Given that both *Bcl11b* and *Gata3* are key target genes for Tcf1, we expressed these transcription factors in Tcf1-deficient cells in an attempt to rescue the thymic phenotype. Similar analyses of epistasis have been used previously in model organisms (e.g., *Drosophila*) to delineate both hierarchical and functional relationships. The expression of exogenous *Gata3* has been shown to suppress B cell development in the wild-type thymus [35 54 55]; furthermore, we found that Gata3 also suppresses myeloid fate in DN thymocytes. Interestingly, Gata3 does not suppress myeloid fate in the bone marrow, whereas the effect on B cell development also occurs outside of the thymus.

Our finding that the constitutive expression of Bcl11b in Tcf1-deficient cells fully rescued T cell development suggests 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. This is schematically illustrated in figure 6 (**Fig.6**). Taken together, the data from our group and others indicate a gene network in which Notch signaling via RBP-Jκ drives the expression of Tcf1, which in turn activates Gata3 and Bcl11b, most likely in collaboration with Notch signals that can also act directly on these genes' promoters. Importantly, in addition to its requirement for initiating the T cell commitment process, Tcf1 expression is also required to maintain lineage fidelity. In skin stem cells, lineage infidelity increases the likelihood of malignancy [56]. Thus, given that loss of Tcf1 leads to the rapid development of T cell lymphomas [22,23], lineage infidelity may also serve as a previously unrecognized factor in leukemogenesis.

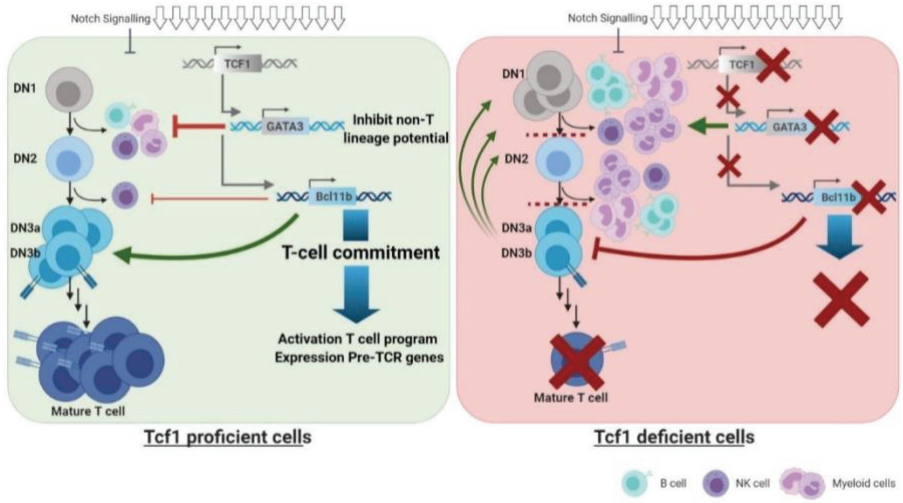

Figure 6: Hierarchy of the core transcription factors in immature T cell development. *On the basis of the proven functional interactions shown in Figs. 4 and 5. Notch signaling (indicated by the open arrow symbols) induces Tcf1 expression that subsequently has two target genes: Gata3 and Bcl11b. Gata3 has a minor role in supporting development along the T cell linage but mainly acts to*

suppress the myeloid and B cell fates. In contrast, Bcl11b induces a T cell–specific program but has minor roles in suppressing alternative lineages with exception of NK cell development that is suppressed by Bcl11b. Collectively, there is a clear functional hierarchy of transcription factors. Potential additional roles for Runx1 and E2A are not shown here.

Supplementary Material

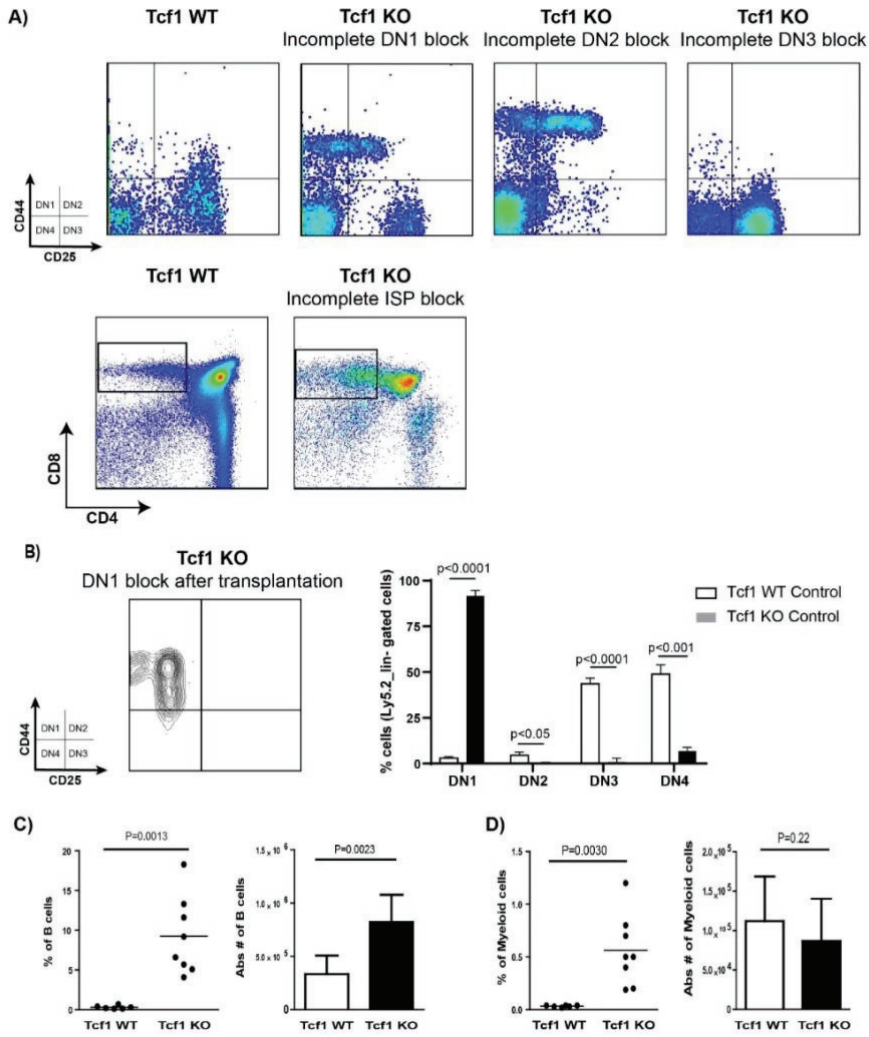

***Figure S1: Tcf1 deficiency leads to several arrests in T cell development with increased non-T cells**. A) Multiple incomplete blocks in T cell development (DN1, DN2, DN3, and ISP) of Tcf1 deficient thymocytes compared to wild-type (pre-gated Thy1+, Lin- cells). B) Transplanted Tcf1 deficient stem cells led to a complete block at the DN1 to DN2 transition in T cell development (pre-gated Thy1+, Lin- cells). C) Increased percentage and absolute number of B cells (B220+CD19+) in Tcf1 deficient thymi compared to wild-type littermates. D) Increased percentage and total number of*

myeloid cells (CD11b+Gr1+) in Tcf1 deficient thymus compared to wild-type littermates. Number of dots indicate number of mice. (Mann-Whitney U test, P<0.05 is statistically significant)

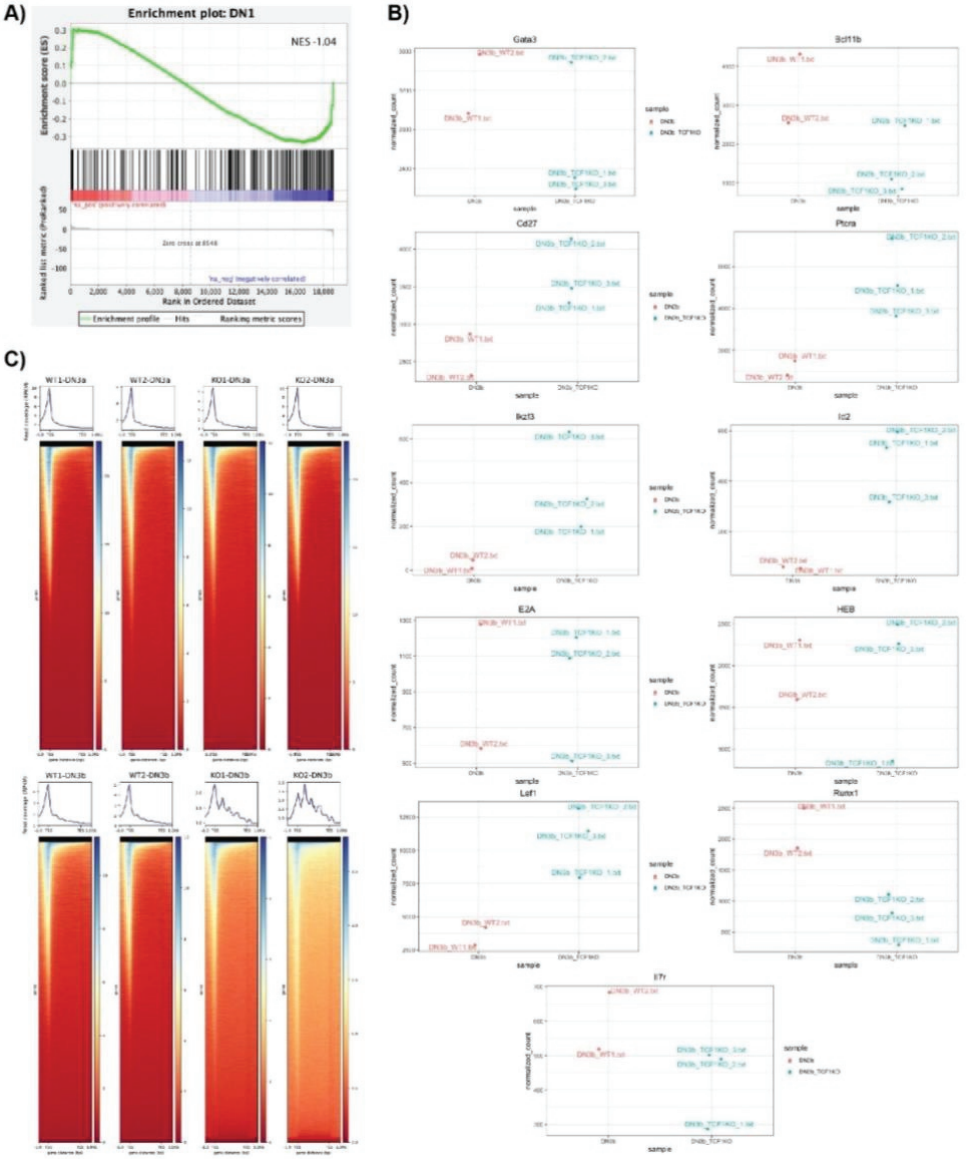

Figure S2: Selected gene expression profile of Tcf1 wt and ko DN3b cells by RNA-seq. *A) GSEA of the differentially expressed genes (Tcf1-/- KO over Tcf1 WT for DN3b) are negatively enriched for DN1 genes (NES -1,04). B) Selected gene expression (normalized count) determined by RNA-seq in DN3b cells from TCF1 wt and deficient cells. C) ATAC-seq read coverage in DN3a and DN3b cells over genes including 1kb downstream and upstream the gene body.*

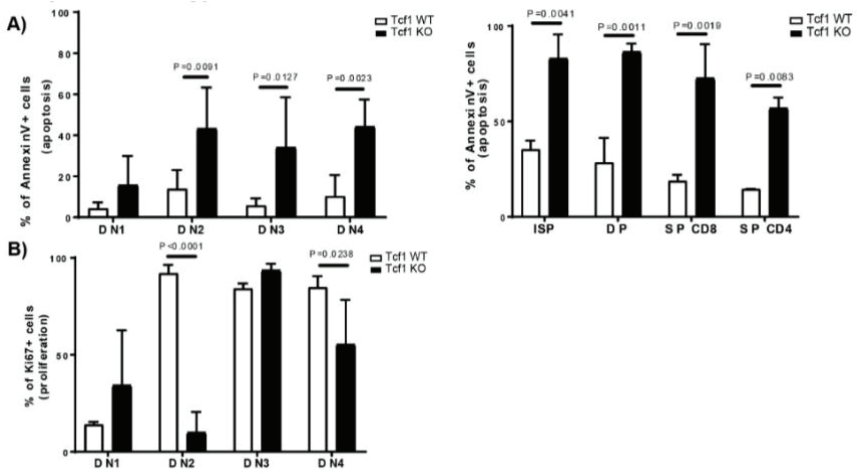

Figure S3: Increased apoptosis and reduced proliferation of Tcf1 deficient thymocytes compared to wild-type cells*. A) Ex vivo wild-type and Tcf1 deficient thymocytes were analysed by flow cytometry for various developmental stages of T cell development in combination with AnnexinV/7AAD. B) Quantification of proliferating cells (Ki67 cells) within the early developmental stage of T cell development of Tcf1 wild-type and Tcf1 deficient thymocytes. Percentage of Annexin V and Ki67 are shown after pre-gating of various subsets *p < 0.05, **p < 0.01 and ***p < 0.001 (Mann-Whitney U test). Error bars represent the SD of three samples from individual mice in two independent experiments.*

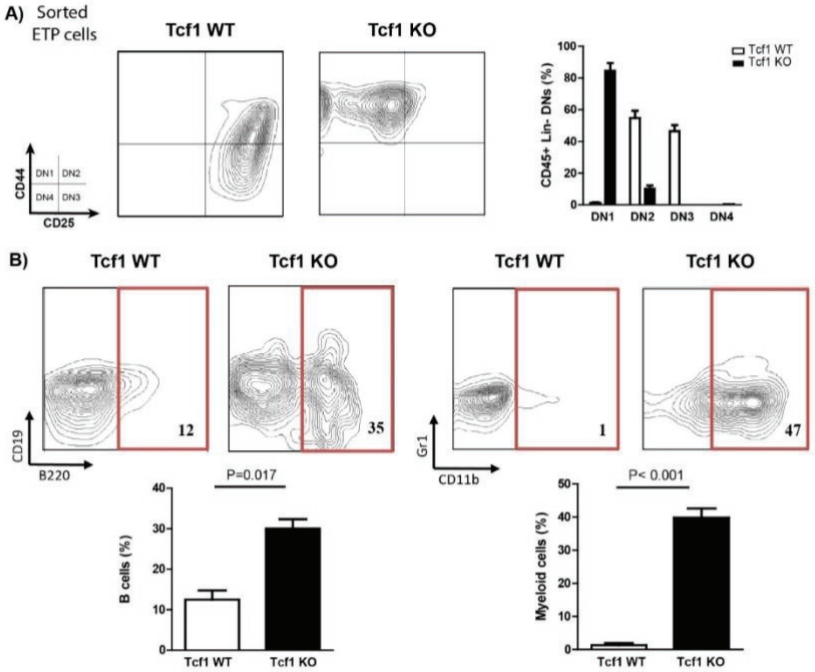

Figure S4: Tcf1 deficient sorted ETP cells are arrested in development at DN1 stage in culture, with prominent B and myeloid development compared to wild-type sorted ETPs*. A) Sorted wild-type ETP cells seeded on OP9-DL1 cells differentiate along the T cell lineage while sorted Tcf1 deficient cells are blocked in development at the DN1 stage. B) Sorted ETPs cells from Tcf1 deficient thymi show abundant B and myeloid development on OP9-DL1 compared to wild-type sorted ETPs. (Mann-Whitney U test. Error bars represent the SD of three samples from three independent experiments.)*

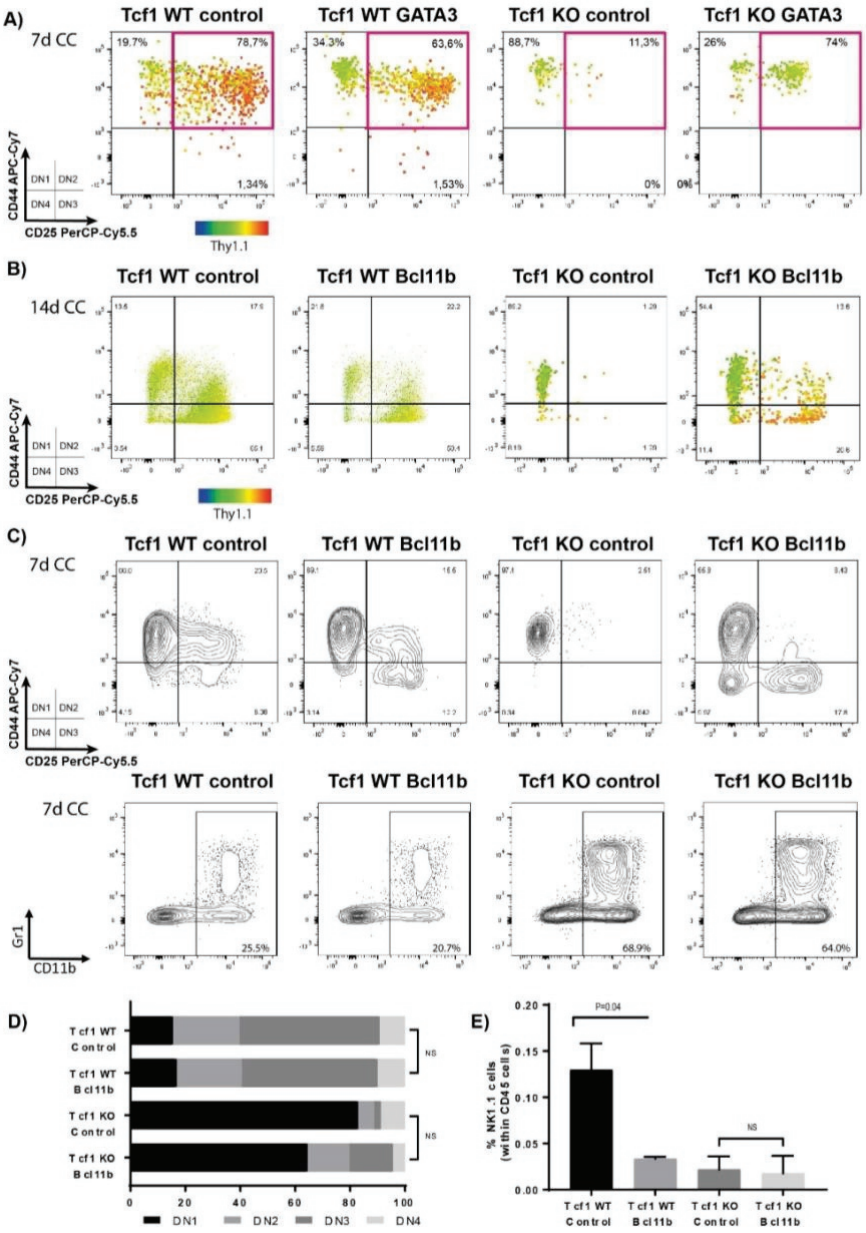

***Figure S5: OP9-DL1 coculture cell development**. A) Gata3 expression partially overcomes the DN1 thymocyte block, but do not induce Thy1 increasing expression through development after 7 days in the OP9-Dl1 culture system. B) Bcl11b fully rescues T cell development from Tcf1 -/- stem cells, with increased in Thy1 expression, that otherwise are arrested in DN1 after 14 days in OP9-Dl1 culture system. (Thy1 is displayed using median color mapping). C) Bcl11b transduced cells are developing through the DN2 stage after 7d in the OP9-Dl1 system. Tcf1-/- cells shows a higher percentage of myeloid cells in culture compared to Tcf1 wt cells, but Bcl11b overexpression does not*

affect that populations. D) Untransduced Tcf1 deficient and wild-type cells (GFP- cells) with Bcl11b preserve control phenotype. Untransduced Tcf1 deficient cells are arrested at DN1/DN2 transition after 14d on OP9-DL1 as the control cells. (pre-gated Thy1+Lin-GFP- cells) E) Bcl11b overexpression does suppress the development of NK cells in wild-type cells after 14d on OP9-DL1. (Two-way ANOVA. Error bars represent the SD from three independent experiments.)

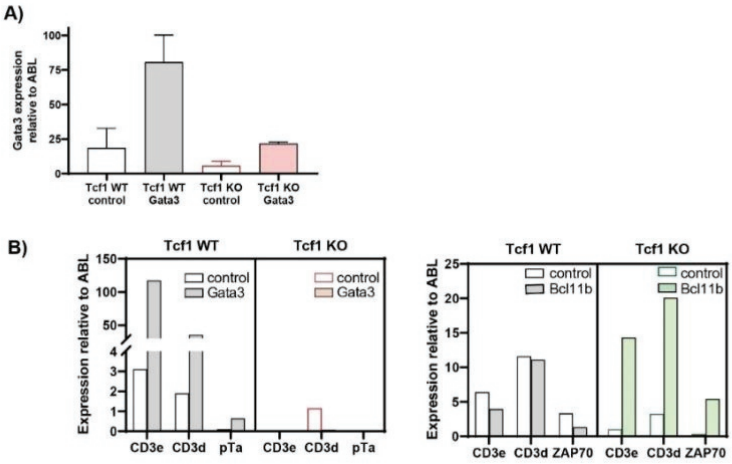

***Figure S6: T cell receptor genes expression in co-culture experiments**. A) Gata3 expression levels relative to ABL determined by qPCR from cells harvested after 7 days in culture. Tcf1-/- reach normal wt levels of Gata3 after transduction. (Error bars represent the SD from two independent experiments.) B) Expression of T cell receptor genes (CD3e, CD3d, pTa, ZAP70) relative to ABL determined by qPCR after 7 days (Gata3) and 14 days (Bcl11b) OP9 DL1 culture. Only Bcl11b overexpression in Tcf1-/- cells (not Gata3) is able to rescue expression of T cell receptor genes.*

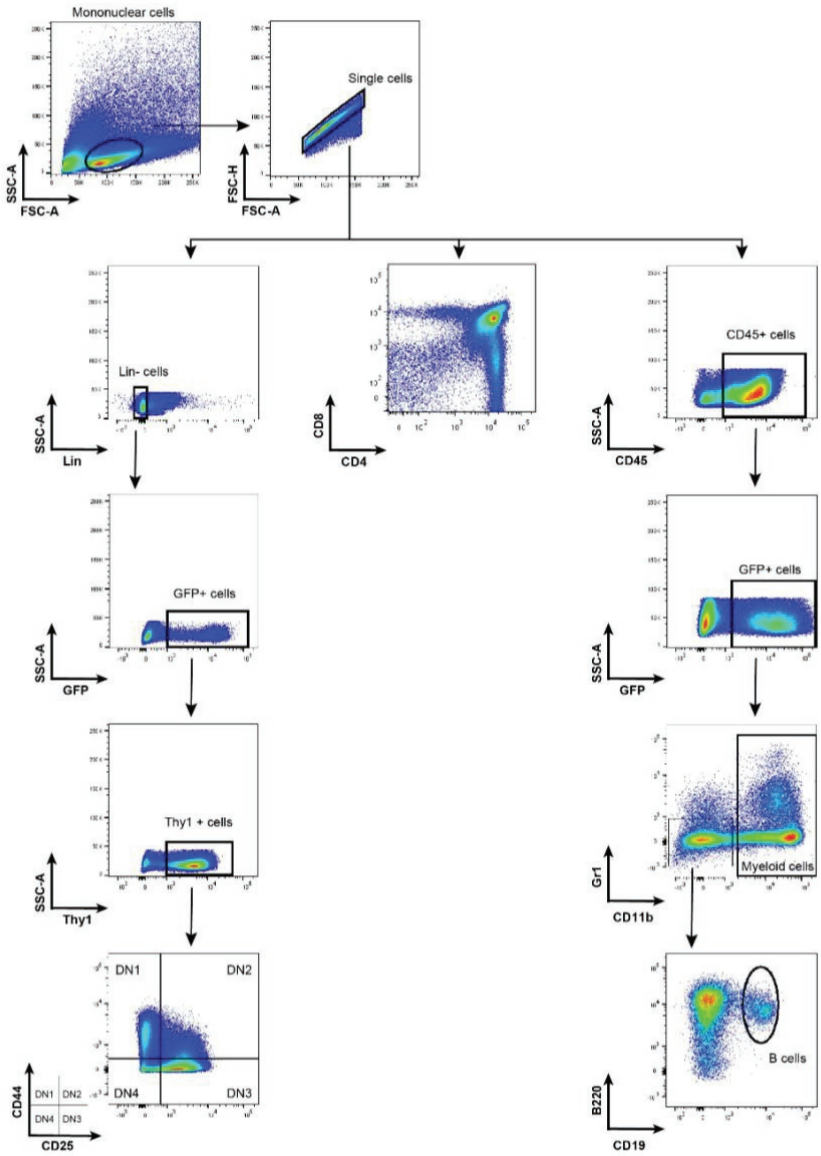

Figure S7: Flow cytometric gating strategy

Table S1. Deseq Differential expression DN3b v.3 (Excel file)

Table S2. The full genome wide ATACseq data analysis of sorted DN3 Tcf1 wt vs ko (Excel file)

Table S 3: List of antibodies used in the study (including manufacturer, clone, catalog number)

Antibodies	SOURCE	IDENTIFIER
Anti-CD3e antibody (clone 145-2C11)	BD Biosciences	Cat# 553060 RRID:AB_394593
Anti-CD3e antibody (clone 145-2C11)	BD Biosciences	Cat# 553062 RRID:AB_394595
Anti-CD4 antibody (clone H129.19)	BD Biosciences	Cat# 553648, RRID:AB_394968
Anti-CD4 antibody (clone RM4-5)	eBioscience	Cat# 25-0042-82, RRID:AB_469578
Anti-CD8a antibody (clone 53-6.7)	BD Biosciences	Cat# 553029, RRID:AB_394567
Anti-CD8a Monoclonal Antibody (Clone 53-6.7)	BD Biosciences	Cat# 553035, RRID:AB_398527
Anti CD11b/MAC1 antibody (clone M1/70)	Biolegend	Cat# 101204, RRID:AB_312787
Anti-CD11b Monoclonal Antibody (Clone M1/70)	BD Biosciences	Cat# 553311, RRID:AB_394775
Anti-CD19 Monoclonal Antibody (Clone 1D3)	BD Biosciences	Cat# 550992, RRID:AB_398483
Anti-CD25 (IL2Ra/p55) antibody (Clone PC61)	BD Biosciences	Cat# 553866, RRID:AB_395101
Anti CD27 Monoclonal Antibody (Clone LG.7F9)	eBioscience	Cat# 11-0271-82, RRID:AB_465001
Anti-CD44 antibody (Clone IM7)	BD Biosciences	Cat# 560568, RRID:AB_1727481
Anti-CD45.1 Monoclonal Antibody (Clone A20)	eBioscience	Cat# 25-0453-82, RRID:AB_469629
Anti-CD45.2 Monoclonal Antibody (Clone 104)	BD Biosciences	Cat# 552950, RRID:AB_394528
Anti-CD45R/B220 antibody (Clone RA3-6B2)	BD Biosciences	Cat# 553085, RRID:AB_394615
Anti-CD45R (B220) antibody (Clone RA3-6B2)	eBioscience	Cat# 25-0452, RRID:AB_2341160
Anti-CD90.2 (Thy-1.2) antibody(Clone 53-2.1)	eBioscience	Cat# 17-0902-81, RRID:AB_469421
Anti-CD117 antibody (Clone 2B8)	BD Biosciences	Cat# 558163, RRID:AB_647250
Anti-CD117 Monoclonal Antibody (Clone 2B8)	BD Biosciences	Cat# 553356, RRID:AB_398536
Anti-Ly-6G, Ly-6C antibody (Clone RB6-8C5)	BD Biosciences	Cat# 553124, RRID:AB_394640
Anti-Ly-6G (Gr-1) Monoclonal Antibody (Clone RB6-8C5)	eBioscience	Cat# 48-5931-80, RRID:AB_1548797
Anti-NK-1.1 antibody (Clone PK136)	BD Biosciences	Cat# 553163, RRID:AB_394675
Anti-TER-119 antibody (Clone TER-119)	BD Biosciences	Cat# 553672, RRID:AB_394985
Anti-TCR beta Monoclonal Antibody (Clone H57-597)	BD Biosciences	Cat# 553174, RRID:AB_398534
Anti-mouse TCR Vb5.1, 5.2 antibody (Clone MR9-4)	Biolegend	Cat# 139504, RRID:AB_10613279
Anti-mouse TCR Vb6 antibody (Clone RR4-7)	Biolegend	Cat# 140003, RRID:AB_10640727
Anti-mouse Vb8 antibody (Clone F23.1)	BD Biosciences	Cat# 555604, RRID:AB_395975
Anti-Ly-6A/E (Sca-1) Monoclonal Antibody (Clone D7)	eBioscience	Cat# 25-5981-82, RRID:AB_469669
TCF1 (C46C7) Rabbit mAb antibody	Cell Signalling Technologies	Cat# 2206S, RRID:AB_2199300

Table S 4: List of markers used to define all different hematopoietic populations in the study.

Subset	Markers
LSK	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $c\text{-}Kit^+$ $Sca1^+$
LK	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $c\text{-}Kit^+$ $Sca1^-$
ETP	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $CD25^-$ $CD44^+$ $c\text{-}Kit^+$
DN1	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $CD25^-$ $CD44^+$ $c\text{-}Kit^-$
DN2	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $CD25^+$ $CD44^+$
DN3a	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $CD25^+$ $CD44^-CD27^-$
DN3b	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $CD25^+$ $CD44^-CD27^+$
DN4	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $CD25^-$ $CD44^-$
ISP	Lin^- ($B220^-$ $CD11b^-$ $NK1.1^-$ $Ter\text{-}119^-$) $CD3^-$ $CD4^-$ $CD8^+$
DP	Lin^- ($B220^-$ $CD11b^-$ $NK1.1^-$ Ter^-119^-) $CD4^+$ $CD8^+$
CD4 SP	Lin^- ($B220^-$ $CD11b^-$ $NK1.1^-$ Ter^-119^-) $CD3^+$ $CD4^+$ $CD8^-$
CD8 SP	Lin^- ($B220^-$ $CD11b^-$ $NK1.1^-$ Ter^-119^-) $CD3^+$ $CD4^-$ $CD8^+$
B cell (Mature)	$B220^+$ $CD19^+$
Granulocytes	$CD11b^+$ $Gr1^+$
Monocytes	$CD11b^+$ $Gr1^-$

Table S 5: Name ad sequences of used primers

	Name	Sequences
Genomic qPCR primers	mGata3	F: CTTATCAAGCCCAAGCGAAG
		R: CCCATTAGCGTTCCTCCTC
	mBcl11a	F: CCCCGCAGGGTATTTGTA
		R: TGAATGGCTGTTTGCAAGTT
	mBcl11b	F: TGTCCCAGAGGGAACTCATC
		R: GGCTGCTTGCATGTTGTG
	mPax5	F: ACGCTGACAGGGATGGTG
		R: GGGGAACCTCCAAGAATCAT
	mPU.1/Spi1	F: GGGATCTGACCAACCTGGA
		R: AACCAAGTCATCCGATGGAG
	mIL-7Ra	F: GATCCATTCCCCATAACGATT
		R: CAGGATCCCATCCTCCTTG
	mCD117/c-kit	F: GGAGCCACAATAGATTGGTAT
		R: CACTGGTGAGACAGGAGTGGT
	mID2	F: GACAGAACCAGGCGTCCA
		R: AGCTCAGAAGGGAATTCAGATG
	mAxin-2	F: AGTCCATCTTCATTCCGCCTAGC
		R: AAGCTGCGTCGGATACTTGAGA
	mHes-1	F: 5'-AAACACTGATTTTGGAGCACT-3'

		R: 5'-TGCTTCACAGTCATTTCCAGA-3'
	Abl-2	F: CAACGTCTTCACCCAGCAC
		R: TCCAGTATTGTCTCCCTCAAA
	CD3e	F: CTTGTACCTGAAAGCTCGAGTG
		R: TGTGATTATGGCTACTGCTGTC
	CD3d	F: TGCTTTGCAGGACATGAGAC
		R: CGATCTCGAAGAGGCTGTAC
	pTa	F: CTGTCAGGGGAATCTTCGAC
		R: GTACCTGCCGCTGTGTCC
	ZAP70	F: AGAAGCACTCATGCTGGTCA
		R: GTTCAGCCACATTGCTCACA
ChIP primers	Gata3-1b promotor:	F: 5' GTACACGGTACTTCGGGGAC 3'
		R: 5' AGGACCTGGGCTTTGATTCG 3'
	Enhancer Bcl11b:	F: 5' CCAACAGCACTGGGGATTCT 3'
		R: 5' ACTTGGGCTGAACTTGCTGA 3'

F: forward primer; R: reverse primer

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Chapter 3

Preclinical Development of Autologous Hematopoietic Stem Cell Based Gene Therapy for Immune Deficiencies: A Journey from Mouse Cage to Bed Side

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ABSTRACT

Recent clinical trials using patient's own corrected hematopoietic stem cells (HSCs), such as for primary immunodeficiencies (Adenosine deaminase (ADA) deficiency, X-linked Severe Combined Immunodeficiency (SCID), X-linked chronic granulomatous disease (CGD), Wiskott–Aldrich Syndrome (WAS)), have yielded promising results in the clinic; endorsing gene therapy to become standard therapy for a number of diseases. However, the journey to achieve such a successful therapy is not easy, and several challenges have to be overcome. In this review, we will address several different challenges in the development of gene therapy for immune deficiencies using our own experience with Recombinase-activating gene 1 (RAG1) SCID as an example. We will discuss product development (targeting of the therapeutic cells and choice of a suitable vector and delivery method), the proof-of-concept (in vitro and in vivo efficacy, toxicology, and safety), and the final release steps to the clinic (scaling up, good manufacturing practice (GMP) procedures/protocols and regulatory hurdles).

Keywords: gene therapy; immunodeficiency; HSC; vector design; animal model; efficacy; safety; scaling up; regulations; GMP complaint

INTRODUCTION

Over the past 5 years, the gene therapy product market has substantially expanded. Several products have been approved by the FDA (U.S. Food and Drug Administration Agency) and the EMA (European Medicines Agency) and have been granted market authorization. Among them, in 2016, the EMA approved the first ex-vivo gene therapy product using autologous hematopoietic stem cells, Strimvelis (GlaxoSmithKline), for the treatment of Adenosine deaminase (ADA) deficiency. Moreover, over 3000 clinical trials have been reported worldwide [1], with the majority addressing human cancer (CAR-T cells) and inherited monogenic diseases like primary immunodeficiencies [2]. Clinical trials with both self-inactivating (SIN) gamma-retroviruses and SIN-lentiviruses (10% of clinical trials) are currently ongoing for various primary immunodeficiencies like ADA Severe Combined Immunodeficiency (SCID) [3–5], X-linked SCID [6–9], Artemis SCID [10–12], Wiskott–Aldrich Syndrome (WAS) [13–15] or X-linked chronic granulomatous disease (CGD) [16,17] (**Table 1**). Severe combined immunodeficiencies (SCIDs) are a group of rare inherited disorders in which both the humoral and cell-mediated immunities fail to function. SCIDs are characterized by the absence of T and often B and/or NK cells, and represent a real pediatric emergency. Indeed, if not properly treated, SCIDs lead to infants' failure to thrive associated with severe and recurrent infections and other metabolic abnormalities that are invariably fatal. Mutations in a number of genes can cause SCID: The IL2R-gamma gene mutations cause X-linked SCID; mutations in Adenosine Deaminase ADA-SCID and mutations in either of the Recombinase Activating Genes RAG-SCID. Other immune disorders include Wiskott Aldrich syndrome that also affects platelets and granulomatous disease, which affects mature granulocyte function with severe and recurrent infections and other metabolic abnormalities that are invariably fatal. Gene therapy provides a life-long cure and has the potential to become a standard clinical procedure for immunodeficiencies and some other diseases when proven safe. However, the journey to accomplish clinical trials has been extensive and laborious.

The bases of gene therapy were established following the scientific advances during the 1960s and early 1970s. Friedmann suggested good exogenous DNA could be used to replace the defective DNA in patients with genetic defects who suffer from its associated rare diseases [18]. Since then, massive efforts from basic science, translational, and clinical research have been made, reaching the first in vivo animal model evidence in 1989 that the procedure could work for primary immunodeficiencies [19]. In parallel, better understanding and characterization of the targeted diseases and improvement of laboratory methods allowed a rapid advance of gene therapy development with remarkable results for several immunodeficiencies. Although allogeneic hematopoietic stem cell (HSC) transplantation remains the prevailing therapeutic treatment for immunodeficiencies, the outcome differs depending on the source of the donor HSC (Human leukocyte antigen (HLA)–matched related donor or HLA-mismatched donor), the disease genotype, the use of conditioning, the age and the health status of the patient at the time of the treatment [20–26]. Despite all improvements, Graft-versus-Host disease (GvHD) remains a significant complication associated with allogeneic HSCT. Therefore, particularly patients without HLA-matched donors and those with serious comorbidities would benefit from autologous gene therapy [27].

Developing successful gene therapy is not easy and several challenges need to be overcome along the journey, starting with the selection of the most suitable target cells and how to isolate them. Next, a suitable clinically applicable vector with the promoter and transgene of interest needs to be designed. Once the vector and the delivery methods have been developed, efficacy is tested both in vitro and in vivo, confirming sufficient transgene transduction, therapeutic expression of the transgene, and immune reconstitution. Furthermore, extensive toxicology and safety studies are essential to minimize potential insertional mutagenesis and clonal outgrowth due to the semirandom integration of the vector into the DNA; potentially causing leukemias or lymphomas.

The FDA [50] and EMA [51] have published guidelines that define scientific principles and provide guidance for the pre-clinical development and evaluation of gene therapy products, focusing on the quality, efficacy, and safety requirements. Extensive pre-clinical data strengthen the proof-of-concept of the potential of the developed gene therapy product, paving the way for the approval of clinical trials. However, the final release steps towards the clinic and patient treatment are lengthy due to the need for adequate scaling-up of the vector production and gene therapy product manufacturing, as well as handling mandatory regulations. From start to finish, all steps and challenges of the gene therapy development procedure (illustrated in **Figure 1**) will be discussed using our own experience in the development of gene therapy for Recombinase-activating gene 1 (RAG1) SCID as an example [52–54].

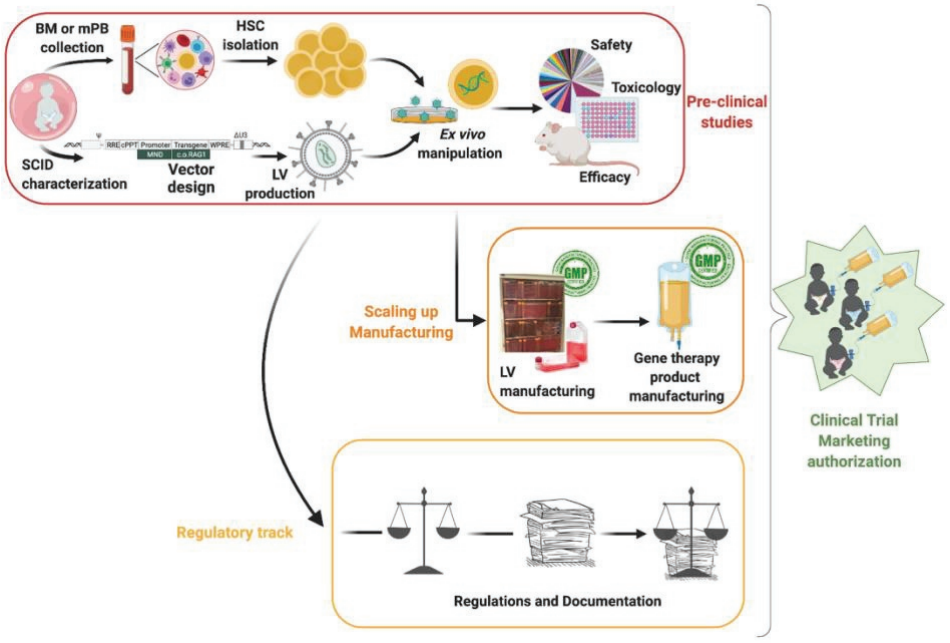

Figure 1: Overview of the pre-clinical assessments of gene therapy treatment: *From disease modeling to clinical application (Bone Marrow (BM); mobilized Peripheral Blood (mPB); Hematopoietic Stem Cell (HSC); Lentiviral Vector (LV); Severe Combined Immunodeficiency (SCID)).*

Table 1: Summary of finished and ongoing clinical trials for primary immunodeficiencies.

Disease	Gene	Vector		Clinical Study	Phase	Participants	Location	Study Type	Status	Outcome/References
ADA-SCID	ADA	RV		NCT01279720	Phase 1/2	8	EU	interventional	completed	Positive benefit-risk profile [28–36]
				NCT00018018	Phase 1	8	USA	interventional	completed	
				NCT00794508	Phase 2	10	USA	interventional	completed	
				NCT00599781	Phase 1/2	8	EU	interventional	completed	
		Strimvelis (RV)		NCT00598481	Phase 2	18	EU	interventional	completed	Immune reconstitution [33,37]
				NCT03232203		10	EU	observational	recruiting	
				NCT03478670		50	EU	observational	enrolling	
		SIN LV		NCT01852071	Phase 1/2	20	USA	interventional	completed	Immune reconstitution, well tolerated [36,38,39]
				NCT02999984	Phase 1/2	10	USA	interventional	completed	
				NCT01380990	Phase 1/2	36	EU	interventional	completed	
				NCT04140539	Phase 2/3	3	USA	interventional	recruiting	
				NCT03645460	n.a.	10	China	interventional	recruiting	
				NCT04049084		70	USA/EU	observational	enrolling	
Artemis-SCID	DCLRE1C	SIN LV		NCT03538899	Phase 1/2	15	USA	interventional	recruiting	Immune reconstitution [40]
Chronic granulomatous disease	Gp91 phox	RV		NCT00927134	Phase 1/2	2	EU	interventional	completed	Sustained engraftment, insertional mutagenesis, [41] [42]
				NCT00564759	Phase 1/2	2	EU	interventional	unknown	
			SIN	NCT01906541	Phase 1/2	5	EU	interventional	unknown	
				NCT00778882	Phase 1/2	2	Korea	interventional	active	
		SIN LV		NCT01855685	Phase 1/2	3	EU	interventional	active	[16]
				NCT02757911	Phase 1/2	3	EU	interventional	active	

				NCT02234934	Phase 1/2	10	USA	interventional	active	
				NCT03645486	n.a.	10	China	Interventional	active	
Leukocyte Adhesion Deficiency-I	CD18	RV		NCT00023010	Phase 1	2	USA	observational	completed	
		SIN LV		NCT03812263	Phase 1/2	9	USA	interventional	recruiting	
				NCT03825783	Phase 1	2	EU	interventional	recruiting	
Wiskott–Aldrich Syndrome	WAS	SIN LV		NCT01347242	Phase 1/2	6	EU	interventional	completed	Successful engraftment, immune reconstitution, no adverse reactions [15,43,44]
				NCT01347346	Phase 1/2	5	EU	interventional	completed	
				NCT02333760	Phase 1/2	10	EU	interventional	active	
				NCT03837483	Phase 2	6	EU	interventional	active	
				NCT01410825	Phase 1/2	5	USA	interventional	active	
				NCT01515462	Phase 1/2	8	EU	interventional	active	
X linked-SCID	IL2RG	RV		NCT00028236	Phase 1	3	USA	interventional	completed	Sustained immune correction, risk acute leukemia [6,45–48]
			SIN	NCT01175239	n.a.	1	EU	interventional	unknown	
				NCT01410019	Phase 1/2	5	EU	interventional	unknown	
			SIN	NCT01129544	Phase 1/2	8	USA	interventional	active	
		SIN LV		NCT03315078	Phase 1/2	13	USA	interventional	recruiting	Multilineage engraftment, immune reconstitution [49]
				NCT03311503	Phase 1/2	10	USA	interventional	recruiting	
				NCT01512888	Phase 1/2	28	USA	interventional	recruiting	
				NCT01306019	Phase 1/2	30	USA	interventional	recruiting	
				NCT03601286	Phase 1	5	EU	interventional	recruiting	
				NCT04286815	n.a.	10	China	interventional	recruiting	
				NCT03217617	Phase 1/2	10	China	interventional	recruiting	

PRODUCT DEVELOPMENT

Gene therapy can be broadly divided into two groups: In vivo and ex-vivo gene therapy depending on the target disease and the delivery method. In in vivo gene therapy, the transgene is administered intravenously into the patient, either by a viral or non-viral vector, and reaches the target cells inside the body. In contrast, ex-vivo gene therapy is performed outside the body. The cells of interest are isolated, modified with the therapeutic transgene, and corrected cells are transplanted back into the patient. In this last approach, the gene therapy product, i.e., the medicine, consists of the combination of the targeted cells and the therapeutic vector target cells of interest.

Target cells of interest

An essential feature of gene therapy is the persistent long-term correction of the disease, lasting for life with a unique one-time treatment, offering a cure for the disease. Moreover, primary immunodeficiencies can affect one or multiple cell lineages. In RAG1 deficient patients, both B and T cells are affected. To achieve the desired correction, a proper understanding of stem cell biology became critical as stem cells have the unique capacity of both self-renewal as well as pluripotency. Regarding immunodeficiencies, hematopoietic stem cells (HSC) are the relevant target cells that differentiate to produce all mature blood cell types for life.

Murine HSCs were first described by Becker et al. (1963) [55], describing the clonal origin of a population of hematopoietic cells. At the beginning of the 1990s, Jordan and Lemischka [56], proposed a model where single stem cell clones are sufficient to maintain lifetime hematopoiesis in an animal model and suggested that the hallmark of the long-term reconstitution system may arise from mono- or oligoclonality. Suitable markers to characterize stem cell subpopulations were identified, allowing the purification of the murine cells of interest [57,58]. The most widely-known murine HSC population is the LSK population, standing for lack of lineage markers (B220, Mac-1, Gr-1, CD3, CD4, CD8, and Ter119), and the presence of Sca1 and c-Kit. LSK comprises a heterogeneous population with different subpopulations distinguished as long-term ($Thy1^{lo}$ Lin^- $Sca1^+$ $cKit^+$ $CD38^+$ $CD34^{-/lo}$ $Slam^+$) and short-term ($Thy1^{lo}$ Lin^- $Sca1^+$ $cKit^+$ $CD38^+$ $CD34^+$ $Slam^-$) populations [59,60]. Mouse bone marrow (BM) HSCs can be isolated and purified by immunomagnetic beads for lineage depletion, from which a lineage negative bulk population including progenitors and long-term stem cells are collected, or by cell sorting from which a purer HSC population can be isolated. Isolated murine HSCs can then be ex vivo cultured and transduced with the therapeutic vector, followed by in vitro or in vivo testing.

In parallel, human HSCs were also identified. Weissman and co-workers (1992) [61] isolated a candidate population in human fetal BM ($Thy1^+$ Lin^- $CD34^+$) that was enriched for the clonogenic activity that established long-term and multilineage capacity. CD34 is the main marker to define human HSCs, consisting of a bulk of populations that represents around 1% of total BM cells. HSCs have been further phenotypically redefined as $CD34^+CD38^-$ cells [62] and further divided into subpopulations based on the expression of CD90/Thy1 and CD45RA [63] and CD49f. Therefore, the first full phenotypic definition of human HSCs proposed by the laboratory of John Dick (2011) [64] was $CD34^+CD38^-CD45RA^-$ $CD90^+CD49f^+$, where single defined HSCs were highly efficient in generating long-term

multilineage grafts in NOD scid gamma (NSG) mouse models. Recently, new HSC markers have been identified like EPCR/CD201, which is also fairly reliable to detect HSCs in culture [65].

In the clinical setting, the $CD34^+$ bulk fraction, also known as hematopoietic stem and progenitor cells (HSPC), with a mix of progenitor and long-term populations (similarly to LSK in the murine setting) is used for transplantation or gene therapy manipulation. The main advantage of using the total $CD34^+$ population is the easy accessibility of these cells [66,67]. HSPCs can be harvested from the bone marrow by direct puncture or nowadays, preferably by leukapheresis. HSPCs are mobilized with two mobilizing agents (G-CSF and Plerixafor) from the bone marrow to the peripheral blood that is then collected, containing an enriched portion of HSCs [68]. HSC mobilization is a less invasive method that is routinely performed, allowing to harvest a high amount of HSPCs, also suitable for babies who are the target population of gene therapy for immunodeficiencies. Moreover, human HSPCs can easily be collected with immunomagnetic beads for CD34 enrichment, also available under Good Manufacturing Practice (GMP) compliance. Finally, HSPCs can efficiently be re-administered by infusion, where HSCs will naturally home to the bone marrow. However, there are two main challenges: Obtain a sufficient number of cells for ex vivo manipulation and successive transplantation, and achieve appropriate gene correction for cell therapy (discussed below). With regards to the need for sufficient therapeutic cells, it should be noted that HSPCs are delicate cells, and, therefore, cell loss needs to be considered during the processing of the cells (enrichment, culture, transduction, freezing, and thawing) [66]. Nowadays, achieving a therapeutic number of $CD34^+$ cells is accessible thanks to the improved protocols for the collection (re-collection if needed) and isolation of HSPCs. Another way to overcome this challenge is by achieving ex vivo expansion of HSCs. Enormous efforts, as reviewed by Tajer et al. (2019) [69] and others [70–73], have been put into improving HSC culture protocols to successfully maintain and even expand the cells of interest ex vivo, and, therefore, help to overcome the shortage of primary material.

The gene therapy field is continuously evolving, offering an alternative approach by further narrowing the isolation of HSPCs to a purer $CD34^+CD38^-$ 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 high recovery [74]. This $CD34^+CD38^-$ population is more enriched with long-term HSCs, decreasing the amount of cells needed to be transduced ex vivo and transplanted back into the patient; reducing the amount of therapeutic virus needed [75]. However, myeloid reconstitution after purified $CD34^+CD38^-$ transplantation was delayed, as the first wave of immune reconstitution is known to be accomplished by progenitor cells present in the bulk $CD34^+$ cells [76]. Therefore, even though a more extensive purification can improve transduction efficiency and reduce the usage of the therapeutic virus (potentially reducing therapy cost), the presence of a mixed HSPC population, including progenitor cells, is actually an advantage for a satisfactory post-gene therapy recovery.

Vector Design: Balancing Insertion Site and Therapeutic Expression

An optimal vector for gene therapy should carry a high DNA load capacity, enable high transduction efficiency, possess favorable cell tropism for the target cell type of interest,

induce low genotoxicity and cytotoxicity, and evoke no or a limited immune response. To achieve these characteristics in the wide number of potential diseases targeted with gene therapy, a variety of vectors have been developed and optimized that can be divided into two main vector categories; non-integrative and integrative vectors. The non-integrative vectors have a safer profile, including both viral vectors such as adenoviral or adeno-associated viral vectors and non-viral vectors, which offer extra advantages on the low induced immunogenicity and the ease to produce [77]. However, as the transgene of interest will not be integrated into the DNA host cell, transgene expression might not always be stable; the expression will be retained for a prolonged period in post-mitotic tissues but diluted progressively in proliferating cells. Therefore, the application of non-integrative vectors in the hematopoietic system is limited. On the other hand, integrative vectors (mainly retroviral and lentiviral vectors) have been used in approximately ¼ of the total gene therapy clinical trials [78]. Retroviruses can enter the host cell and reverse transcribe their RNA genome into DNA that subsequently integrates into the cell DNA. As the transgene of interest stably integrates into the host DNA, a long-lasting therapeutic effect is achieved, allowing the transmission of the therapeutic material to all progeny of the transduced cells (i.e., all blood lineages developed from transduced HSCs). The use of integrating vectors in gene therapy for immunodeficiencies has a long history by now, with over 2 decades of experience since the first clinical trials started for X-linked SCID [6]. The firsts attempts of gene therapy for ADA and X-linked SCID were accomplished with a retroviral vector derived from Murine Leukemia Virus (MLV_gamma-retrovirus). Although successful correction of the disease was observed in most of the patients and no problems were observed in the ADA trial, safety issues resulted from the X-linked trials as leukemia cases appeared in patients treated with the gene therapy product. These leukemias were caused by insertion mutagenesis of the therapeutic vector. Even though retroviral integration across the DNA was thought to be random, it became apparent that there was some preference near transcriptional active sites such as oncogenes [79–81]. These adverse events revealed a need to develop a new generation of safer vectors with a decreased risk of insertional mutagenesis. Self-inactivating (SIN) vectors lacking potent enhancers in the LTRs were developed, for both gamma-retroviral and lentiviral vectors, however, SIN-γRV reached low transduction efficiency and expression [82]. The interest in lentiviral vectors increased thanks to their capacity to also transduce non-dividing cells and therefore allowing an increased transduction efficiency of HSCs [83]. Lentiviral vectors used in gene therapy are HIV derived and modified to guarantee vector safety.

Naldini and colleagues (1998) [84] developed the well-known 3rd generation lentiviral vector (LV) system resulting in the generation of replication-deficient LV to prevent repackaging. SIN-LV are generated based on a 4 plasmid system in which all non-essential viral genes have been removed, and the essential viral genes have been separated into several plasmids. The system consists of a group of separate plasmids: Two packaging plasmids (gag/pol and rev), a plasmid encoding for the envelope (env plasmid), and a minimal transfer plasmid with the LTRs, packaging signals, internal promoter, and the therapeutic transgene. Additionally, 3′LTRs regions were modified, resulting in the deletion of the viral promoter and enhancer activity in 3′LTR [85], and rendering the virus SIN after integration. In addition, insulators can be added into the transfer plasmid blocking the interaction

between the integrating vector and the cell's genome. Additional improvements have been implemented on the LVs aiming to enhance transgene expression and stability, thereby also increasing safety as fewer integrations are needed to achieve the therapeutic effect; fewer integrations reduce the risk as insertion mutagenesis increases with the total amount of integrations. Polyadenylation signals help to improve the correct transcript termination, improving the 3′ processing [86]. Additionally, the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) positioned behind the transgene increases RNA stability and subsequent viral titer and transgene expression [87]. Codon optimization can be an extra modification that leads to further improvement of the titer and expression by depleting secondary RNA structures and improving codon usage. Finally, as the SIN lentiviral transfer vector is devoid of LTR activity, an internal promoter needs to be included. To reduce the risk of integration in non-target tissue, the choice of tissue-specific promoters is advisable when possible. Thanks to all the advances made in vector design, SIN lentiviral vectors are the safest to date with a highly reduced genotoxicity compared to γ-retroviral vectors [85,88,89].

In parallel to the SIN lentiviral vector development, new vectors for immunodeficiencies not yet-treated, have started to be developed, such as for RAG1-SCID. As RAG1-SCID is a primary immunodeficiency, SIN lentiviral vectors were chosen for their ability to transduce HSCs and safety profile. RAG1 gene therapy development started to be developed after γRV safety issues were raised while SIN LV was continuously being improved. Therefore, a SIN LV with the native RAG1 transgene was developed, and its efficacy was evaluated. Both the in vitro (virus production and transduction) and in vivo therapeutic effect were assessed. Unfortunately, RAG1 expression was insufficient, and, therefore, a codon-optimized version of RAG1 was used (c.o.RAG1). Transduction efficiency, transgene expression, and in vivo efficacy were improved, as shown by Pike-Overzet et al. (2011) [52]. Gene therapy to treat RAG1-SCID seemed to be possible with SIN LV; however, the vector used for proof-of-concept studies was still inappropriate. Accordingly, the vector was updated into clinically applicable vectors in which different promoters were tested to achieve optimal transgene expression. Efficacy of these clinically applicable LVs was re-assessed in vitro and in vivo, revealing SIN LV MND-c.o.RAG1 as the most promising vector to correct RAG1 deficiency [54].

PROOF-OF-CONCEPT

Well-conducted clinical trials are essential to establish the benefit/risk profile. To ensure the collection of reliable data in this rapidly expanding field, the FDA and EMA have published guidelines for the development of these complex therapies. The guidelines are multidisciplinary, addressing development, manufacturing, and quality control during non-clinical and clinical development. The main objective is to provide guidance in the structure and the required data to start a clinical trial application, focusing on efficacy (in vitro and in vivo) and safety.

Ex Vivo Manipulation: Transduction Efficiency

One of the most important release criteria for a gene therapy product is to determine transduction efficiency by means of the vector copy number (VCN), which is a measure for the number of transgene copies integrated into the DNA per target cell. The threshold selected for these values in the therapeutic product corresponds to the lowest transduction efficiency required to ensure enough modified cells and a therapeutic effect. The ability to achieve efficient gene delivery has often been described as 'the Achilles heel of gene therapy' [90]. Despite the accomplishment of remarkable improvements and the development of new methods (like transduction enhancers), it is still a bottleneck to translate from pre-clinical murine models to primary human cells and finally to scale up to clinical use. To achieve a reliable VCN in the potential gene therapy product, different developmental steps need to be carefully considered: The method to determine transgene transduction, suitable lentiviral titration in therapeutic cells, and proper adjustment of the viral dose to achieve reliable and sufficient therapeutic effect.

Polymerase Chain Reaction (PCR) has been widely used to determine VCN and transgene expression in the gene therapy field for immunodeficiencies. In short, transduced cells are kept in culture for several days to avoid the detection of free plasmids and ensure the readout of stable vector integration. DNA from cultured cells is isolated to determine VCN by PCR. Sastry et al. (2002) [91] developed and established Real Time-PCR as the method for detecting LV sequences relative to a housekeeping gene. Therefore, the number of vectors inserted in the DNA was quantified, allowing the detection of multiple vector copies per cell (which was not possible with previous techniques like p24-ELISA [92]). No free plasmids were detected 4 days post-transduction by this method, indicating that detection of stable integration in the cell can be measured from that day. Across the literature, analysis of VCN is performed at different timepoints post-transduction form 7 days [93,94, 12], 9 days [52,54], and up to 14 days [9,95,96]. It is important to note that; VCN values may differ when analyzed on different days, and while the differences can be subtle, it can hamper the comparison between trials. Importantly, this PCR-based method was originally generated in a way that could be used for a variety of LV vectors independently of the transgene, allowing to establish a standard method to detect VCN. However, there is no complete consensus between gene therapy studies on the vector region targeted for the PCR, the housekeeping gene, or the standard, leading to potential misinterpretations of the VCN across different laboratories and studies. Recently, the use of the Droplet Digital PCR system (ddPCR) instead of the RT-PCR has added an extra confounder to VCN determination. In principle, ddPCR offers a more accurate and reproducible detection of VCN, with minimum variability for low VCN values. Even though ddPCR is also based on the detection of a vector sequence relative to a housekeeping gene, the detection approach is different in that the VCN is calculated based on a mathematical model by Poison statistics [97–99].

Importantly, a precise estimation of average VCN in the targeted cell is key in defining the therapeutic product. As multi-center clinical trials are getting more attention, there is a need for more standardized protocols to define VCN's in gene therapy products (such as the standard cell culture protocols, vector region, and housekeeping gene used) and to

obtain more reliable and comparable VCN values. This is of high importance in multi-center studies where there might be a need to agree on a release VCN value across countries and different regulatory agencies. Furthermore, it would be interesting to introduce new techniques for further characterization of the therapeutic gene product such as the abundant heterogeneity regarding stem cell subpopulations and the actual percentage of transduced cells (by flow cytometry or colony-forming assays); these are key features for the success of the gene therapy outcome that is currently being assessed differently in different trials [9,12,37,54,96].

A crucial aspect for ex vivo transduction of stem cells with a lentiviral virus is the accurate determination of the viral titer of the produced viral supernatant; notably the functional titer, i.e., the lentivirus' ability to transduce a particular cell type or cell line under specific conditions. Indeed, gene therapy is based on the most suitable amount of virus added to the target cells to obtain sufficient (but not too high) and reliable vector integration into the genome. Therefore, an accurate viral titer assessment on proper, informative cells is needed. Generally, the functional titer for different therapeutic purposes has been determined in various cell lines like HEK293T cells [91,92,95,100], Hela cells [91], HT1080 cells [101], or HT-29 cells [96]. An essential aspect to consider is that different cell lines have different permissiveness to lentiviral transduction, and, therefore, the assessed viral titer can vary depending on the cell line used; one same viral batch may have different viral titers depending on the transduced cell line. Moreover, primary cells, i.e., HSCs, are known to be more challenging to transduce. Therefore, the titer determined with a cell line may not be suitable for primary cells, getting unexpected efficiencies in the primary cells. As changes in target cell type and transduction conditions can have a dramatic effect on transduction efficiency, titration of the virus on primary cells, mainly murine HSPCs and human HSPCs from different sources (cord blood, bone marrow, and/or mobilized peripheral blood) is highly advisable. Thereby, having a specific titer in primary cells will help to achieve more reliable VCN in pre-clinical studies and in the gene therapy products across patients. Not only target cells but also transduction culture conditions should be taken into consideration, giving a more accurate read out for transduction efficiency.

As mentioned above, human primary cells (such as HSCs) can be more challenging to properly transduce and to achieve sufficient therapeutic efficiency. Together with the costly production of a clinical therapeutic lentiviral batch, transduction enhancers (TEs) are valuable compounds in the past years to boost VCN in primary HCSs using less virus if possible. Successful use of TEs will allow treating more patients with one viral batch, which will help to implement gene therapy as a standard protocol. In the past, to get a sufficient proportion of gene-corrected cells in the therapeutic product, high vector doses (2 transduction hits) and prolonged ex vivo culture (3 days in total) were needed [12,96]. Various TE compounds can be added to the culture media to increase lentiviral transduction efficiency, VCN, and transgene expression; they include Cyclosporin and Rapamycin [95], Prostaglandin E2 [93], Staurosporine [94], or LentiBOOST™ [102], [96]. Combinatorial TE application has also been tested, yielding even more potent effects [94–96]. Higher transduction due to TEs was achieved in all HSPCs subpopulation, 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 implementation in clinical protocols. In addition, TEs compounds may allow getting reliable effects to achieve the correct VCN in the gene therapy product across patients and diseases. With this approach, the use of LVs can be maximized, requiring less virus per product and enabling the use of one batch for multiple patients. For example, Schott et al. (2019) [96] showed the combinatorial use of protamine sulfate and LentiBOOST™ that allows to adjust their clinical protocol by reducing the amount of virus needed and shortening the culturing time (from 2 hits strategy to 1 hit), preserving at least similar transduction. Accordingly, gene therapy for Artemis-SCID, for which preclinical studies described a 2 hits approach [12], may benefit from a similar strategy to enable adaptation to a more efficient protocol.

Call for Suitable Models to Test the Efficacy of Gene Therapy

The British statistician George Box stated: “Essentially all models are wrong, but some models are useful”, which can also be generally applied to scientific research models. The therapeutic effect in the gene therapy field has been demonstrated in relevant in vivo studies using a broad range of animal models from mouse to Rhesus Macaques, including dogs and pigs. However, some of these models are still far from humans, which can limit the translatability of the discoveries in non-human animals to clinical applications [103], potentially leading to failure in phase I/II clinical trials. As animal models are essential in the pre-clinical assessment, it is important to choose the most suitable disease-specific model and understand its limitations.

Animal Models

Large animals such as dogs, pigs, and non-human primates, have been used in gene therapy [104] for several neuromuscular disorders such as myopathies, Duchene dystrophy, or Huntington [105–108], lysosome storage disorders [109,110], eye diseases [111,112], or cystic fibrosis [113]. These large models have been used to assess efficacy, dosage, route of administration, and safety. Although large animal models for immunodeficiency have been described [114–116], immunodeficient mice are still the most used preclinical models to study gene therapy for immunodeficiencies like SCID. Due to the broad range of available immunocompromised mouse models, including the “humanized” mouse model, human patient cells can be xenografted and directly tested in vivo. Moreover, different genetic mouse models have been developed to mimic the different forms of SCID described in humans, such as ADA-SCID [19,117,118], X-linked (IL2rg)-SCID [9,119], Artemis-SCID [10–12] or RAG1/2-SCID [120,121]. Importantly, these mice present a similar immunodeficient phenotype as found in humans, such as the Rag1-deficient mouse model, which present a full block at the early stages of T (DN2) and B (pre-B) cell development allowing close monitoring of the effects of the gene therapy in their development. Additionally, RAG1 and RAG2 hypomorphic SCID models are also available [122–125], allowing to study gene therapy in a wider range of immunodeficiencies with one same strategy. For example, analyzing whether the same vector can be used to correct both full RAG1-SCID and hypomorphic RAG1-SCID. 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 [126]. With the development of new editing tools (zinc-finger nucleases, TALENs, CRISPR-Cas9), transgenic mice can be generated to reproduce SCID phenotypes that do not have an established animal model yet.

Even though we can find useful mouse models to study the efficacy of the developed gene therapy for immunodeficiencies, the gap between the mouse and the human physiological and pathological mechanisms is still substantial. The most recent achievement to overcome this gap is the development of "humanized mouse models"; immunodeficient mice such as nude or NSG mice carrying functioning human genes, human cells, or human tissues/organs. Importantly, these immunodeficient mice allow engraftment of functional human immune cells [127,128], enabling refined modeling of many areas of human biology and disease, especially immunology. 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 [129]. Humanized mice are becoming an established model to study different human diseases such as infectious diseases, cancer, autoimmunity, and testing human-specific drugs [130,131]. In the field of primary immunodeficiencies, this xenograft mouse model has allowed to provide previously unattainable insight into human T-cell development and contributes to functionally identify the arrest in thymic development caused by the three major types of SCID, as this data was largely missing due to the non-availability of thymic biopsies [132]. Although the humanized mouse model is suitable to recapitulate most of the human SCID phenotypes, murine enzymes can complement and overcome human deficiency in SCIDs that result from lacking certain metabolic enzymes. Indeed, human T, B, and NK developed from ADA-SCID $CD34^+$ patient cells (T-B-NK- diagnosed patient) transplanted into NSG mice [132], in which the secreted murine ADA complemented the human deficiency, comparable to ADA enzyme replacement therapy.

Accordingly, humanized mice are an appropriate tool to study the therapeutically modified stem cells from SCID patients directly. This model has a big impact on assessing gene therapy potential in pre-clinical studies. It allows us to directly assess gene therapy efficacy and safety in developing functional immune cells. An increasing number of pre-clinical immunodeficiency studies include a proof-of-concept in patient cells transduced with the therapeutic vector and transplanted into NSG mice, to get extra therapeutic evidence closer to the human setting, hoping for a successful subsequent clinical trial. In our example, transplantation of hypomorphic RAG1-SCID patient stem cells into NSG mouse showed that functional human T cells developed after ex vivo gene therapy with our MND-c.o.RAG1 SIN LV, restoring human T cell receptor rearrangements. Such data provides additional robust evidence for starting a phase I/II clinical trial for gene therapy as an alternative curative treatment for RAG1-SCID patients [54].

In Vitro Models

While the humanized mouse model is the best model available for pre-clinical immunodeficiency studies and irreplaceable in the foreseeable future, there is an increased pressure to reduce the number of animals used in experiments in many countries (3 R's concept [133]). To reduce the number of experimental animals, the

development of useful in vitro models is crucial. Available in vitro systems to study gene therapy in immunodeficiencies are mainly focused on T cell development. Fetal Thymus Organ Culture (FTOC) is a powerful 3D system where stroma interactions are maintained to sustain human T cell development; however, progenitor seeding efficiency and cell yield is limited, and the procedure is highly technically challenging [134]. A promising 2-D in vitro system has been developed by Zuñiga-Pflucker and colleagues where B, T and NK cell development can be studied: The OP9 co-culture system [135]. While B and NK cells develop on OP9 stromal cells, T cells need the expression of Delta-Ligand 1 and therefore develop only in the optimized OP9-DL1 system (in which B cell development is hampered) [136]. Several efforts to improve T-cell development in vitro have been performed, allowing to define an optimal medium and cytokine cocktail for optimized T cell development through all the differentiation stages up to complete single positive CD4 and CD8 T cells [137]. The OP9-DL1 system is an efficient tool for pre-clinical validation for gene therapy in cells from γc deficient patient for the correction of T cell development, as was described by Six et al. (2011) [137]. However, it can mainly be applied for T-B+NK+ SCID phenotypes. The in vitro study of more complex SCID phenotypes remains challenging. Moreover, the OP9-DL1 system cannot mediate all aspects of selection because proper positive selection of mature CD4 T cells is absent due to the lack of proper MHC class II expression [138]. Furthermore, this assay is very sensitive to subtle differences in cytokines and labile contents of culture media, making it a delicate assay [132,137]. However, these last hurdles have been overcome by the generation of an artificial thymic organoid 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 [139].

Overall, with these in vitro assays, we still lack crucial information for gene therapy such as homing, long-term stability, biodistribution, or toxicology of the therapy, that can only be assessed in an animal model. Nevertheless, an interesting in vitro platform in development that may overcome the last issues is the body/human-on-a-chip. It is a flexible system that integrates human cell culture with microfluidics in vitro, integrating multiple tissues or organ system surrogates, providing a unique platform for measuring drug response or toxicity. Although still not completed, this system has a promising potential for rare disease research and orphan drug development and could be a good alternative to animal experiments [140,141]. 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 [142]. Such a system can also support the later stages of human T cell differentiation.

Safety and Toxicology Assessment for Gene Therapy

Not only in vitro and in vivo proof-of-concept, but also toxicology studies are requested before starting any gene therapy clinical trial. Accordingly, the EMA requires a risk assessment before the use of any gene therapy medicinal product for which toxicology, biodistribution, and integration studies have primary priority. Genotoxicity assays are not generally required but valuable and often included in pre-clinical work [143,144].

Toxicology and biodistribution are assessed by in vivo studies with the appropriate animal model (discussed above). To reduce the number of animals, all efficacy, pathology, and biodistribution studies can be combined in one same experiment. To assess the toxicology of the therapy, a full necropsy of the animals is performed after long-term reconstitution (over 16 weeks after transplantation) to assess potential long-term effects of the therapy. Organs are collected and subjected to macroscopic and microscopic examination to verify that organs look normal, healthy, and without harmful effects due to gene therapy. To assess toxicology in our RAG1 pre-clinical study, 28 organs were collected (form 14 mice in total, including controls) and analyzed blindly by a European board-certified pathologist. The selection of organs to be examined for gross pathology and histopathology analyses followed the applicable European and international guidelines (EMEA 1995, WHO 2005)[145]. For gross pathology, the external surface of the body, orifices, and the thoracic and abdominal cavities were examined. For histopathological examination, tissues were fixed in paraffin and analyzed by immunohistochemistry.

In parallel, pieces of the same organs were snap frozen to isolate DNA and determine the VCN in each organ. Vector biodistribution can then be assessed after long-term gene therapy; for HSC gene therapy the vector is expected to be present in all immune cells raised from HSC, but not detected in non-immune organs. In these cases, VCN is detected in immune organs like thymus, bone marrow, spleen, lymph nodes, and peripheral blood, but only present in low levels in other organs. However, some positivity may be observed in the intestine or lungs, as these organs have immune cells present. In addition, it is important not to detect VCN in the reproductive organs to avoid transmission to potential offspring. For RAG1, VCN in 16 organs was determined (for 8 gene therapy mice) after mice were perfused with PBS to decrease blood contamination and avoid false positives. Interestingly, if toxicology and biodistribution assays are done in parallel, the data generated can be supportive in explaining unexpected findings. For example, an infection in the lungs and stomach was detected in one of the mice during the necropsy. In parallel, a high VCN was detected in both organs of this mouse, which, thanks to the pathology observations, we could explain by the high abundance of recruited T cells in these organs. Therefore, the potential risks of unintended biodistribution of the vector were very low.

Another minimal requirement by the EMA before the use of gene therapy medicinal products is to perform integration studies. Integration studies examine the insertion sites in which the therapeutic DNA has landed. This assay became important because of the first-generation clinical trial for X-linked SCID. Some of the patients, unfortunately, developed T-lymphocyte acute lymphoblastic leukemia due to retroviral insertions near proto-oncogenes. Since then, continuous progress to develop a robust technique to detect integration patterns have been made. Schmidt et al. (2001) [146] described the first version of a technique (LM-PCR), allowing the characterization of multiple rare integrations in complex DNA samples. This technique was further improved to LAM-PCR [147] and nrLAM-PCR [148] allowing 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.

Finally, although not mandatory, a genotoxicity study is advisable, which can be determined in vivo or in vitro. The in vivo assay is based on oncogenesis onset and follow up on a tumor prone Cdkn2$^{-/-}$ mouse model. The readout for genotoxicity potential is the degree of tumor onset acceleration upon transplanting gene-corrected cells. Cesana et al. (2014) [149] showed that this sensitive method allows the detection of mild existing genotoxicity of SIN lentiviruses, and that insulators used in some vectors slightly reduce tumor growth. However, animal experiments with a tumor end-point as described are not required, as a powerful in vitro assay can be used to detect genotoxicity. In Vitro Immortalization (IVIM) assay [89,150] is based on the findings suggesting that insertional mutagenesis induce competitive growth advantages in vivo [151]. In short, primary bone marrow cells are transduced at high multiplicity of infection with the vector of interest and upon culturing and replating the selective outgrowth advantage of transformed cells is established, reflecting the transforming potential of insertional mutagenesis. The IVIM assay is convenient and shows good sensitivity, without requiring inducing leukemias or tumor growth in an animal model. Currently, an advanced version of the IVIM screening system is developed: "Surrogate Assay for Genotoxicity Assessment" (SAGA). This system integrates a molecular read-out, which enhances reproducibility, sensitivity, and reduces assay duration, paving the way for a better pre-clinical risk assessment of gene therapy vectors [152]. However, a common limitation between both in vivo and in vitro assays is the use of murine cells, from which the relevance in human cells can be questioned. Although the IVIM assay is useful to assess the risk of insertional mutagenesis, the cells are cultured in a myeloid-inducing differentiation medium favoring the readout of selective myeloid mutants (Evi1 and Prdm16) over potentially more relevant B or T cell mutants in the case of SCID therapy [89,153]. Moreover, it is a short-term assay (2 weeks in culture), which is not suitable as a readout for delayed onset genotoxicity that also occurs. Notably, although not required, the assay has been used across multiple pre-clinical studies of different gene therapy development for immunodeficiencies to assess the transforming potential of the newly developed lentiviral vectors such as X-linked SCID [9], Artemis SCID [12] or RAG1 SCID [54].

In the field, the genome editing approach has become an interesting potential alternative tool for gene addition therapy to reduce the risk of random integration and especially for the correction of tightly regulated genes expressed in specific times during development. Plenty of genome editing platforms have been developed (Zinc-finger nucleases, TALENS, Cas9 nucleases) to enable target gene correction under the physiological environment [154]. First attempts targeting the IL2RG gene to correct X-linked-SCID have been successful in pre-clinical studies [155,156]. However, the efficiency of gene correction highly depends on gene accessibility in HSCs, which can lead to insufficient therapeutic effect. Although genome editing represents a promising approach, translation into the clinic is in its infancy compared to gene addition therapies, which are still evolving as well.

PHARMACEUTICAL AND CLINICAL DEVELOPMENT PHASES: FROM MOUSE TO HUMAN TREATMENT

After successful pre-clinical development, the next step in product development is the translation to a gene therapy medicinal product suitable for clinical use. Intensive labor to make the product suitable for the clinic includes scaling up, development of good manufacturing practice (GMP) compliant manufacturing, and complying with other directives and regulations.

Scaling Up: GMP Protocols and Manufacturing

The manufacturing of both the lentiviral starting material and the gene therapy medicinal product ($CD34^+$ cells transduced with the therapeutic transgene) need to be scaled up and translated to a GMP compliant manufacturing process. The first step is to find a manufacturer for the lentiviral vector with appropriate manufacturing facilities and a GMP license; this can be an academic center or a commercial partner. When manufacturing is outsourced, good technology transfer from the research group to the manufacturer is crucial. Lentiviral production of our GMP RAG1 lentiviral clinical batch was outsourced to Batavia Biosciences B.V. Although this company has extensive expertise in virus production for clinical use, it was the first time they produced a lentiviral vector-based product upon adaptation from the research into a large scale GMP protocol. In our example, we will manufacture the gene therapy medicinal product *(*i.e., gene-modified CD34+ cells) in-house, within the academic environment of the Leiden University Medical Center (LUMC) thanks to the availability of proper GMP compliant cleanroom facilities with suitable equipment. To fulfill the gap between research and GMP production, collaboration with knowledgeable departments and qualified personnel has been essential. Furthermore, personnel were encouraged to get familiarized with the relevant regulation and a GMP working environment.

A common hurdle when stepping into GMP manufacturing (viral vector starting material or gene therapy medicinal product) is that all raw materials and disposables should be available in appropriate quality and equipment must be qualified according to GMP guidelines. A GMP compliant quality system should be in place ensuring amongst other the qualification of starting raw materials and suppliers, traceability and the qualification and validation of analytical assays and equipment. Since cell-based medicinal products cannot be sterilized after manufacturing, it is crucial to ensure an aseptic manufacturing process. This can be done by working in qualified cleanrooms (class A in B), making use of closed systems, and applying appropriate measures to avoid cross-contamination [157]. To comply with GMP guidelines is not easy, and research-based protocols may need to be adapted to the new requirements, encountering different challenges that will be discussed below.

GMP Compliant Virus Manufacturing

GMP RAG1 lentiviral vector manufacturing was outsourced and protocols were adapted upon technology transfer from the research laboratory to the Contract Manufacturing Organization. Research protocols needed to be adjusted to produce large volumes, where viral production and concentration systems can differ, moving towards more sophisticated methods to ensure a high-quality product. 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. 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 lentiviral producing stable cell lines are not yet available [158–161]. Therefore, the transient transfection protocol on adherent cells is used. However, it can be challenging to achieve large numbers during the upstream processing (virus production) and to reach high concentration and purity during the downstream processing [162].

The upstream process is costly as high-quality raw materials are expensive and need to be largely used. Adherent cells used for lentiviral production (HEK293T cells), as well as raw materials and plasmids, should ideally be the same as used in pre-clinical studies but at a higher quality grade in most cases. The main problem with adherent cells in the scaling-up is the huge surface area and the laborious manipulation needed. From a simple 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 remains modest as cell density is still “low”. Improvements to allow gene therapy to become a standard therapy and overcome the lack of scalability have been made taking different approaches. Systems that increase cell density by extending the surface to adhere have been developed, such as hollow fiber bioreactors [163] or fixed-bed bioreactors [164]. In addition, the field is moving towards adapting adherent cells to suspension cultures, achieving greater cell density and easier scaling-up.

After overcoming the challenge to produce large volumes of lentiviral supernatant, downstream processing of the sample is crucial to achieve high purity while maintaining high viral titers. Different methods are available with diverse relevant parameters concerning scalability, prices, capacity and throughput, removal of contaminants, maintenance of functional virus, and product losses. The most suitable procedure should be selected to concentrate and remove impurities, i.e., anion exchange chromatography [165,166], size exclusion chromatography [167], affinity absorption chromatography [166], or tangential flow filtration [168]; although a streamlined combination of many techniques is likely to be chosen.

Knowledge transfer when manufacturing is outsourced, and scalability issues are major challenging stages of the lentivirus production stages. The last bottleneck is the extensive LV quality control that needs to be performed on each produced batch. LV production and manufacturing are performed under EU guidelines, which requires rigorous safety controls of the LV starting material. Release tests to assess microbial contamination and purity (free of endotoxin, bacteria, yeast, mycoplasma, toxic agents and residual host cell protein and DNA), safety (Replication competent lentivirus negative and residual plasmid negative) and potency (viral titer and transgene identity assessed) of the LV starting material are recommended for routine batch analysis. Extensive characterization of purified LV GMP grade batches is needed to reduce potentially harmful effects of the therapy in the following steps.

GMP Gene Therapy Product Manufacturing

Protocols to successfully isolate and transduce human $CD34^+$ cells with the produced GMP grade LV needs to be adjusted to be able to manufacture a suitable medicinal product for clinical use under GMP compliance. $CD34^+$ isolation is adjusted to use a close system purification instrument, such as CliniMACS from Milteny Biotec, which is currently a good selection system design for processing a large number of cells [169]. An updated semi-automated system, Prodigy, is also available and can successfully enrich and transduce $CD34^+$ cells with minimal user manipulation [170]. With this GMP compliant equipment and obtaining a comparable yield, purity, and transduction efficiency as current protocols, this semi-automated cell isolation, and transduction equipment has the potential to improve the availability and standardization of HSC gene therapy. Further adaptations in the transduction protocol of the $CD34^+$ cells are needed, both due to the high number of cells that have to be transduced and to the transduction method itself. Spin-oculation is often used in the research setting [52,54] to increase transduction efficiency. However, it was decided that this approach was not suitable to be performed in the cleanroom, due to the high number of cells. The process would be too laborious, which would have a negative impact on the cells and in addition, would enhance the contamination risk as a result of an extensive open production step. Subsequently, this change in the protocol promotes the use of alternative high-quality grade available transduction enhancer methods such as TEs compounds discussed above.

When facilities, personnel, documentation, raw materials, and equipment are ready, validation runs have to be performed, which will show whether the gene therapy medicinal products can be generated successfully, reliably, and aseptically. In the process and release test, such as determining cell numbers, viability, transduction efficiency, and product sterility, should be ready at this point too. It is also very important that the development of analytical assays start early in product development. If the medicinal product is given as a fresh product to the patients, then this constitutes the final product. If the product is cryopreserved, thawing protocols need to be evaluated, as well as post thawing viability of the product. Stability testing of the fresh or frozen product, as well as the lentiviral starting material, is also required and have to be considered during the development process and optimally is combined with the validation runs. Once protocols have been adjusted for clinical use and validation runs have been fully accomplished, one can proceed with the final clinical applicable protocol.

Regulatory Hurdles

Developers of gene therapy products not only face challenges in the scientific and technological fields but also experience additional hurdles in the regulatory trajectory, even though the regulatory environment for ATMPs (Advanced Therapy Medicinal Products) has been globally coevolving with the increasing interest in marketing authorization over the past decade [171,172].

The regulatory requirements may be complex and vary across continents and countries, but their aim is always the same: To ensure the safety and well-being of human beings [173]. The three major regulatory authorities in the European Union (EU), the United States (USA), and Japan have been making great efforts to develop and implement tools that

facilitate ATMP development and enable products to reach the patients as early as possible. They have been making important steps to define appropriate regulatory standards; however, due to the novelty of this field and the complexity of such products, regulators face scientific issues never discussed before. As a result of this, regulatory requirements for approval for market authorization are not standardized or harmonized yet [172,174].

In the EU, the legal framework for ATMPs is laid down in the European Regulation (EC) No. 1394/2007, known as the ATMP regulation, amending Directive 2001/83/ EC and Regulation (EC) No. 726/2004. This regulation is in place since 2009. It defines ATMPs and ensures that such products are subject to appropriate regulatory evaluation before their clinical and commercial use, according to the regulatory framework for human medicinal products [175]. Numerous other directives apply to clinical gene therapy. An overview of the European legislation, legal guidelines, and guidance on various relevant subjects with regards to ATMPs can be found at the website of the EMA [176].

Before an ATMP can be granted with market authorization and be available for patients, clinical trials have to be performed to demonstrate safety and efficacy. To conduct clinical trials, sponsors should follow specific requirements to obtain national authorizations from the regulatory body of the individual EU member states [173]. The review process to assess the benefit-to-risk ratio for patients is currently regulated by Directive 2001/20/EC. This lengthy process, currently 90 days, has been widely criticized by the scientific community. Due to their call for reform, the EC will replace this directive with the new 'Regulation 536/2014 on clinical trials on medical products for human use'. It is expected to come into effect in 2020 [177], [178]. According to this new EU regulation, clinical gene therapy is still considered as a special case, and the review period for gene therapy products can be extended by an additional 50 days [177].

During the process of obtaining regulatory authorization, the sponsor must submit a clinical trial application (CTA), also known as a standard research file, to the national competent authority and an independent ethics committee. The research file includes several essential documents: Clinical trial application form, trial protocol/amendment(s), written informed consent form(s), subject recruitment procedures (e.g., advertisements), written information for subjects, Investigator's Brochure (IB), Investigational Medicinal Product Dossier (IMPD), summary of scientific advice, available safety information, information about payments and compensation available to subjects, the investigator's current curriculum vitae and/or other documentation evidencing qualifications, and any other documents that the regulatory body may need for the review.

Besides, in EU countries, separate legislation has been implemented to assess the environmental risks of genetically modified organisms (GMOs) within clinical gene therapy trials. Thus, in addition to approval by an independent ethics committee and the competent authority, GMO license must also be obtained before trials can commence. The additional GMO legislation is based on two environmental EU directives, the 2009 EU directive entitled 'Contained use 2009/41/EC' and the 2001 EU directive entitled 'Deliberate release in the environment 2001/18/EC'. Unfortunately, there are several issues related to the environmental risk assessment process. First, these directives failed to keep up with the

scientific progress and gene therapy vector development of the past 25 years and still apply to the current clinical gene therapy trials of which the environmental risks can be considered negligible [177]. Second, this process does not only require longer review timelines but are also poorly harmonized within the EU. While in the Netherlands, the deliberate release framework always applies, resulting in a lengthy procedure, in the UK, the length of the procedure depends on the biological characteristics and environmental risk assessment of the GMO. To reduce the review timeline, integration of environmental risk assessment in the EU clinical trial legislation framework was put into the consideration of the EU Parliament's and European Committee. Furthermore, it was suggested that only one organization should be considered responsible and accountable for the review of clinical gene therapy trials similar to the USA. Such improvements would result in a more efficient and transparent review process and reduce the time needed for the product to reach the patients [177]. Overall, harmonization of GMO authorizations across the European would clearly facilitate clinical trials with GMOs.

If a trial has a multinational design involving more EU Member States, there is a possibility to make use of the voluntary harmonized procedure (VHP). In 2009, VHP was introduced by the Clinical Trials Facilitation Group as a pilot of Regulation 536/ 2014. The objectives of the VHP are to establish harmonized assessments and decisions on clinical trials in the EU and identify possible serious issues before the official submission [179]. The possibility of obtaining centralized approval for participating member states could facilitate the approval procedure for the study in a timely fashion. The VHP takes place prior to the official national submissions of the research file. Documents like the protocol, the IB, and IMPD are assessed jointly by one participating member state and the other concerned member states of a VHP. Although this procedure is an efficient tool to achieve harmonized and quick approvals of clinical trials in many EU Member States in one procedure, it currently has no formal status. It is an informal procedure that does not lead to an official decision. The submitting party can, therefore, not derive any rights from the procedure, and sometimes regulatory approval by national agencies can be delayed due to conflicts in the VHP rules with national requirements. However, as there also are clear benefits to having a VHP approval, sponsors should certainly consider pursuing this route.

It should be noted that a large team effort is indispensable to compile a research file that can be granted regulatory approval. Continuous collaboration of preclinical developers, clinicians, pharmaceutical, legal and health technology assessment experts, project managers, patient organizations, and regulatory experts is required [173,180]. Although universities are a major player in the field of ATMP development, unfortunately, researchers face significant hurdles partly due to lack of regulatory expertise and related financial support [180,181]. Thus, next to working with the necessary specialists, early engagement with the national competent authorities and/or EMA is encouraged to succeed in development. University researchers are advised to discuss scientific questions via scientific advice at the national regulators, as this is often easier, cheaper, and can be relatively informal or via the EMA's Innovation Task Force. This way, regulators could provide scientific advice to ensure the development plans are acceptable and in line

with regulatory expectations. Furthermore, with their contribution, the potential for lengthy and costly delays can be reduced [173,182].

The application is approved, and the trial can commence, but that does not mean the work is done. During and after the trial, sponsors must follow relevant regulatory regulations and frequently submit different information to the regulatory authorities (e.g., amendments, SAEs, SUSARs, SADEs and line listings, progress reports, DSU report, a summary on trial results).

When the whole development trajectory is performed successfully, one can apply for market authorization (MA). For ATMPs a centralized marketing authorization is mandatory, which leads to a single marketing authorization that is valid in all EU countries. The EMA, together with its Committee for Advanced Therapies (CAT), Committee for Human Medicinal Products (CHMP), and the network of national agencies, are responsible for the scientific evaluation of the MA applications [183]. CAT offers an ATMP classification and high-level expertise to assess the quality, safety, and efficacy of ATMPs. It reviews whether the clinical development and product manufacturing processes comply with the particular standards and requirements and ensure that the data presented are complete, accurate, and satisfactory [175]. To facilitate the authorization process, EMA provides early access tools and support. The priority medicines (PRIME) scheme is the main tool. PRIME was introduced in 2016 to enhance support for products targeting an unmet medical need and to speed up evaluation, thus, the medicines can reach patients earlier [171]. It is interesting to note that over one-third of the medicines in the PRIME scheme are ATMPs and all of these are gene therapies. In the United States and Japan such support schemes are also actively contributing to the progression of cell and gene therapies [175]. The EU has also released guidelines supporting a risk-based approach to cover quality, safety, efficacy, manufacturing, and biological aspects. The risk-based approach is a strategy to determine the level of data required and to support justification for any deviations made from directives [173].

Unfortunately, ATMPs are often seen as products with a low commercial value and/or a high commercial risk due to the complex manufacturing processes, orphan indications, and tailored production [180]. Currently, all three regulatory authorities show a willingness to accept uncertainty and safety risks with the emphasis of paying accurate attention to post-marketing surveillance and risk-minimization measures [174,180]. As of May 2019, 14 ATMPs have been granted a MA for the European Economic Area, however, 4 of them already have been withdrawn from the market for a variety of reasons [180]. An alternative route to increase access of drugs to patients in Europe is through the hospital exemption, which allows the use of ATMPs under the supervision of a medical practitioner, on a non-routine basis, and in restricted circumstances, in a single member state.

In summary, the development, regulation, and clinical use of most ATMPs are constantly co-evolving, and this path should be further followed. Although the use of gene therapy products and lentiviral vectors, in particular, is still relatively new for developers and regulators, the regulatory authorities can provide guidance and useful information on quality, safety, and efficacy during product development. In the coming years, the number of authorized gene therapy medicinal products is expected to increase significantly. When

more information on such products is available, regulations and guidance are expected to increase and be harmonized, thereby supporting the delivery of more promising new gene therapy medicinal products in the EU and global markets [173].

CONCLUSIONS

Ex-vivo gene therapy using HSCs has extensively progressed over the last three decades, allowing to establish a relevant workflow of the essential steps to develop gene therapy from the pre-clinical stage to clinical trials [184,185]. Obtaining robust pre-clinical data to initiate a successful clinical trial is laborious, time-consuming, and with the risk of becoming a vicious cycle. Every time an improvement in the vector design needs to be implemented or protocols (isolation or transduction) are adapted, in vitro and in vivo efficacy should be tested again. Additional safety tests might also be required, entering successive cycles of adaptation and improvements. Although HSC isolation procedures and used vectors are rather standardized in the field, it is important to keep basic research in parallel to their clinical use, allowing continuous optimization in GMP compliant manufacturing and automated procedures as well as improving vector safety. A key parameter in the generation of the gene therapy product during clinical implementation is the ex-vivo manipulation procedure, which is currently lacking standardized assays—this lack of standardization results in variability between assays and inconsistency between research groups and developing therapies. Standardization of viral titer and VCN determination will help to overcome the variability in one of the most important release criteria of the gene therapy product. Data from in vivo and safety studies are crucial to initiate the way into the clinic. Approved and reliable in vitro safety tests are used regularly. However, a significant amount of the toxicology and safety results are obtained from experiments performed in animal models, revealing the importance of choosing suitable models for the diseases. Once favorable pre-clinical data following the FDA and EMA guidelines is collected, it is time to step out of the pre-clinical development cycle and step towards the pharmaceutical and clinical development phases, with still an extended journey ahead until starting a clinical trial. As gene therapy remains an emerging treatment, GMP manufacturing and regulations have been developed in parallel to the clinical implementation progress made in gene therapy. Communication between researchers, industrial representatives, and regulators is key to learn and grow together in this new field, adapting as the therapy evolves to design solid guidelines for the standard implementation of gene therapy as a medicinal treatment. Although the focus of this review has been on autologous HSC-based gene therapy for immune deficiencies, similar approaches are being used successfully for red blood cell disorders (such as thalassemia) and a wide range of metabolic disorders affecting brain, liver, and muscle (reviewed by Staal, Aiuti and Cavazzana [186]). In all these diseases, a long path of development (**Figure 2**), starting with suitable vectors and disease-specific mouse models, was required to reach clinical implementation.

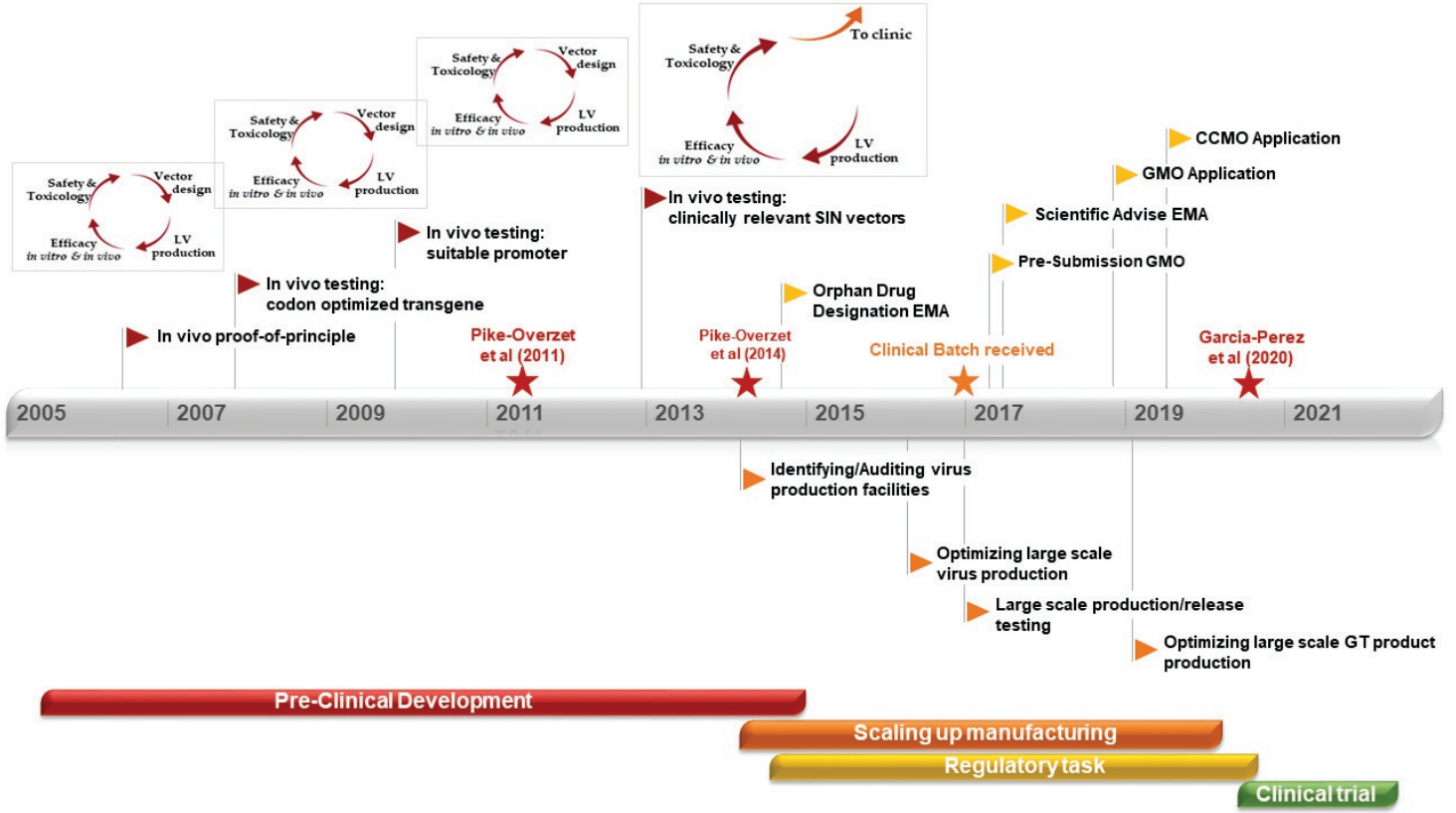

Figure 2: Development of autologous stem cell-based gene therapy for RAG1 severe combined immuodeficiency (SCID): *A journey from mouse house to bed side. (Lentiviral Vector (LV); Centrale Commissie Mensgebonden Onderzoek (CCMO); European Medicines Agency (EMA); Genetically Modified Organism (GMO); Gene Therapy (GT).*

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Chapter 4

Successful preclinical development of gene therapy for Recombinase Activating Gene-1-deficient SCID

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ABSTRACT

Recombinase-activating gene-1 (RAG1) deficient SCID patients lack B and T lymphocytes due to the inability to rearrange immunoglobulin and T-cell receptor genes. Gene therapy is an alternative for those RAG1-SCID patients who lack a suitable bone marrow donor. We designed lentiviral vectors with different internal promoters driving codon optimized RAG1 to ensure optimal expression. We used Rag1-/- mice as preclinical model for RAG1-SCID to assess the efficacy of the various vectors. We observed that B- and T-cell reconstitution directly correlated with RAG1 expression. Mice with low RAG1 expression showed poor immune reconstitution; however higher expression resulted in phenotypic and functional lymphocyte reconstitution comparable to mice receiving wild type stem cells. No signs of genotoxicity were found. Additionally, RAG1-SCID patient CD34+ cells transduced with our clinical RAG1 vector and transplanted into NSG mice led to improved human B- and T-cell development. Together with favorable safety data, these results substantiate a clinical trial for RAG1-SCID.

INTRODUCTION

Severe combined immunodeficiency (SCID) is a life-threatening disorder of the adaptive immune system [1]. In all forms of SCID, the development of T cells in the thymus is arrested due to genetic defects in genes essential for this complex process, while concomitant deficiencies in B lymphocytes and NK cells are depending on the SCID genotype. Affected infants are born with a severe T-lymphocyte deficiency and die within the first year of life unless effective treatment is given. Curative treatment options are limited and confined to allogeneic hematopoietic stem cell transplantation [2, 3] and autologous stem cell gene therapy [4, 5].

Over 20 different genes have been shown to be causative for SCID[1]. Three major types of SCID exist, the common gamma chain cytokine deficiencies, mainly due to defects in the Il2Rγ chain (which is also termed the common γ-chain). The JAK3 and Il7Ra deficiency are much more rare but also fall into this category. The second type of SCID concerns metabolic enzymes that affect highly proliferating cells such as immature thymocytes. Adenosine deaminase (ADA) deficiency is the prototype disease for this subtype, but other deficiencies have also been found, for instance PNP deficiency. The third major type of SCID is formed by recombination deficiencies. In these types of SCID the recombination machinery that is responsible for VDJ recombination of T-cell receptor (TCR) and Immunoglobulin (Ig) genes is affected. Examples are Recombination-activating gene 1 (RAG1), RAG2 deficiency and Artemis mutations. The exact nature of the T-cell developmental arrests in SCID patients has been difficult to elucidate because thymic biopsies cannot be taken, however recent functional experiments using bone marrow stem/progenitor cells from SCID patients has shown that most mutations lead to very early blocks in thymic differentiation [6-8].

Over the last 15 years, clinical trials of gene therapy for two major forms of SCID (SCID-X1 and ADA SCID) have shown significant safety and efficacy in correcting the immunodeficiency and allowing children to live normal functional lives [4, 5, 9-15]. This despite the occurrence of T-cell acute Lymphoblastic Leukemia (T-ALL) as a severe adverse effect in some of these early trials [16-19], which has led to an impetus to further develop safer vectors, the so-called self-inactivating (SIN) vectors [20-22].

For the recombination deficiencies, major steps have been made for correcting RAG1, RAG2 and Artemis deficiency. Artemis gene therapy is closest to clinical implementation and a first clinical trial has started in the US [23]. For RAG1-SCID several attempts to develop gene therapy have been made in the past, first with the now no longer acceptable γ-retroviral vectors [24], later with SIN lentiviral vectors [25, 26]. Our previous work reported successful restoration of Rag1 deficiency using SIN lentiviral vector technology and codon optimized RAG1 (c.o.RAG1) [26], however with a LV vector backbone that is not suitable for large scale GMP production and with a promoter that may lead to genotoxicity (see below). In this previous report, we obtained full restoration of peripheral T-cell numbers after 5 months using spleen focus-forming virus [SFFV]), approximately 35% of normal B-cell numbers, and importantly a polyclonal T-cell receptor (TCR) and B-cell receptor repertoire and full restoration of serum Ig levels, allowing functional responses after immunization with the T-cell–dependent antigens.

However, others have argued that using this approach, it is not possible to fully correct the RAG1 immune deficiency [27], and that oligoclonal T cells could develop, reminiscent of human Omenn syndrome, a disorder known to arise from hypomorphic RAG mutations, resulting in low recombinase activity. We have stated elsewhere [28] that these discrepant results can likely be explained by differences in the expression levels and low transduction efficiencies obtained for the therapeutic gene, *RAG1*. Here we report that successful restoration of the Rag1 deficiency can be obtained using SIN LV vectors that are clinically acceptable and importantly at low vector copy numbers (i.e., ~ 1 copy per cell).

A disadvantage of our previous lentiviral vectors was the use of the so-called RRL backbone, that gives relatively low titers in scaled-up virus productions needed for clinical application. Therefore, we switched to the CCL backbone that has been widely used clinically. In addition, the SFFV promoter sequence that was the most successful in our hands, has become less attractive due to assumed high risk of insertional mutagenesis [29]. Therefore we set out to develop a new set of SIN lentiviral vectors to express *c.o.RAG1* with different types of promoters and to test if they could correct Rag1 deficiency in a preclinical mouse model with low vector copy numbers, as to carry a lower risk of insertional mutagenesis. Through serendipitous effects in the viral production and titration of viral transduction, we obtained a whole range of *RAG1* expression *in vivo* ranging from very low to close to wild type levels. This allowed us to directly address the effects of differences in *RAG1* expression in a gene therapy setting. In addition, it has enabled us to choose a new SIN LV vector that functionally corrects the Rag1 deficiency *in vivo* in mice. The MND-c.o.RAG1 is now the vector of choice capable of high *RAG1* expression that is produced at clinical grade for an international multi-center RAG1-SCID gene therapy trial that is planned in the near future.

MATERIALS AND METHODS

Mice

57BL/6 Rag1$^{-/-}$ mice were originally obtained from The Jackson Laboratory (USA). C57BL/6 wild-type mice and NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were purchased from Charles River (France). Mice were bred and maintained in the animal facility of Leiden University Medical Center (LUMC). All animal experiments were approved by the Dutch Central Commission for Animal experimentation (Centrale Commissie Dierproeven, CCD).

Lentiviral vectors and vector production

The *RAG1* gene sequence was optimized as described by Pike-Overzet et al (2011). Briefly, this resulted in 90% of the codons being adapted to the codon bias of Homo sapiens genes. Furthermore, the GC-content was raised from 48 to 61% and the number of cis-acting motifs was reduced from 21 to 0. The optimized *RAG1* sequence was synthesized by GeneArt (Regensburg, Germany). Codon optimized RAG1 (c.o.RAG1) was cloned into self-inactivating lentiviral pCCL plasmid resulting in pCCL-Cbx3.MND.coRAG1 (hereafter: Cbx3.MND-c.o.RAG1), pCCL-MND-c.o.RAG1 (hereafter: MND-c.o.RAG1), pCCL-PGK-c.o.RAG1 (hereafter: PGK-c.o.RAG1) and pCCL-UCOE-c.o.RAG1 (hereafter: UCOE-c.o.RAG1). DNA sequencing of the transgene was performed

to validate the gene transfer constructs. Helper plasmids pMDLg/pRRE, pRSV-Rev and pMD2.VSVG for lentiviral production were kindly provided by L.Naldini (San Raffaele Telethon Institute for Gene Therapy, Milan, Italy) [30]. Large-scale helper-plasmid preparations were obtained through PlasmidFactory (Bielefeld, Germany).

293T cells were transiently transfected with the transfer and helper plasmids using X-tremeGene HP DNA transfection reagent (Sigma-Aldrich). Lentiviruses were harvested 24h, 30h and 48h after transfection, filtered through 0.22µm pore filters (Whatmann) and stored at -80°C. Pooled lentiviral supernatant was concentrated by ultracentrifugation (Beckman Optima^{TM} LE-80K, rotor SW32Ti) for 16 hours at 10.000 rpm and 4°C under vacuum conditions. Pellets were resuspended in StemSpan Serum-Free expansion medium (SFEM; Stemcell Technologies Inc) and aliquoted to avoid multiple freeze/thaw cycles. Since no suitable anti-RAG1 antibodies were available, we determined the viral titer using qPCR as described later on. A clinical GMP-grade vector was generated by Batavia Biosciences (Leiden, The Netherlands), aliquoted in 200 µL vials and stored at -80 degrees until use. The GMP-grade vector was tested and validated on murine Rag1 deficient bone marrow cells, human CD34+ cells.

Transduction of murine lineage negative bone marrow cells and human CD34+ cells

Murine bone marrow (BM) cells were obtained from femurs and tibias of C57BL/6 wild-type and C57BL/6 Rag1$^{-/-}$ mice. The obtained bones were flushed or crushed, cells were passed through a 0,7 µm cell strainer (Falcon), washed and viable frozen. After thawing, lineage negative cells were isolated using mouse lineage depletion kit and AUTOMacs cell sorter (Miltenyi Biotech). Lineage negative cells were stimulated overnight in StemSpan-SFEM containing Penicilin/Steptamycin (5,000 units/5,000 µg/ml; Gibco) and supplemented with 50 ng/mL recombinant mouse FMS-related tyrosine kinase 3 ligand (rmFLT3L; R&D systems), 100 ng/mL recombinant mouse Stem-Cell Factor (rmSCF; R&D systems) and 10 ng/mL recombinant mouse thrombopoietin (rmTPO; R&D systems). Rag1$^{-/-}$ cells were subsequently transduced with the different lentiviruses using 4 ug/ml proteamine sulphate (Sigma-Aldrich) and by way of spin-occulation at 800xg and 32°C for 1 hour. Cells were cultured at 37°C, 5% CO_2 for 24h in medium supplemented with cytokines.

Human bone marrow from children diagnosed with SCID was obtained according to the Medical Ethical Committee and IRB guidelines at Leiden University Medical Center. The patient in this study was a compound heterozygote with the following confirmed mutations: RAG1 allele 1 C 256-257 deletion AA, allele 2 C 1677 G>T. Mononuclear cells were separated by Ficoll gradient centrifugation, frozen in fetal calf serum (Greiner Bio-one)/10% DMSO (Sigma-Aldrich) and stored in liquid nitrogen. After thawing, human CD34^{+} cells were isolated using CD34 MicroBead UltraPure Kit (Milteny Biotec). Enriched CD34+ cells were stimulated overnight in X-VIVO15 without Gentamycin and phenol red (Lonza) -1% human albumin (200 g/L; Sanquin) - Pen/Strep medium supplemented with 300 ng/ml huSCF (Milteny Biotec), 100 ng/ml huTPO (Milteny Biotec), 300 ng/ml huFlt3L (Milteny Biotec) and 10 ng/ml huIL3 (Milteny Biotec). Cells were transduced in X-VIVO-15 complete medium with 4 µg/mL proteamine sulphate as described previously and cultured for 24h.

Transplantation of Rag1$^{-/-}$ and NSG mice

Control mock-transduced cells (C57BL/6 wild-type cells referred as WT control and Rag1$^{-/-}$ cells referred as KO control) and transduced Rag1$^{-/-}$ murine cells (equal amount of cells per group, up to 5*10^5 cells/mouse depending on the experiment) were mixed with supportive Rag1$^{-/-}$ spleen cells (3*10^6 cells/mouse) in Iscove's Modified Dulbecco's Medium (IMDM) without phenol red (Gibco) and transplanted by tail vein injection into pre-conditioned Rag1$^{-/-}$ recipient mice. Recipient mice (8-12 week old mice) were conditioned with a total body single dose irradiation 24h prior the transplantation using orthovoltage X-rays (8.08Gy) or with two consecutive doses of 25 mg/kg Busulfan (Sigma-Aldrich) (48h and 24h prior transplantation).

After overnight culture, 60.000 to 70.000 human CD34$^+$ cells were resuspended in (IMDM) without phenol red (Gibco) and transplanted intravenously into busulfan pre-conditioned NSG recipient mice (5week old mice, busulfan conditioning as described above).

Mice used for transplantation were kept in a specified pathogen-free section. The first four weeks after transplantation mice were fed with additional DietGel recovery food (Clear H2O) and antibiotic water containing 0.07 mg/mL Polymixin B (Bupha Uitgeest), 0.0875 mg/mL Ciprofloxacin (Bayer b.v.) and 0.1 mg/mL Amfotericine B (Bristol-Myers Squibb) and their welfare was monitored daily. Peripheral blood (PB) from the mice was drawn by tail vein incision every 4 weeks until the end of the experiment. PB, thymus, spleen and BM were obtained from CO_2 euthanized mice.

Immunization

Mice were immunized with synthetic TNP-KLH antigen 4 weeks before the end of the experiment. 100 µg TNP-KLH (Biosearch Technologies Inc.) in 50% Imject Alum (Thermo Scientific) was injected intraperitoneal (i.p.). 3 weeks later, mice were boosted i.p. with 100 µg TNP-KLH in PBS. Serum was collected before immunization and 1 week after the boost injection.

Flow cytometry

Single cell suspensions from thymus and spleen were prepared by squeezing the organs through a 70 µM cell strainer (BD Falcon) and single cell suspension from BM was made as described above. Erythrocytes from PB and spleen were lysed using NH_4Cl (8,4 g/L)/$KHCO_3$ (1 g/L) solution. Single cell suspensions were counted and stained with the antibodies listed in **Table S2**. Briefly, cells were incubated for 30 min at 4°C in the dark with the antibody-mix solution including directly conjugated antibodies at the optimal working solution in FACS buffer (PBS pH 7.4, 0.1% azide, 0.2% BSA). After washing with FACS buffer, a second 30 min incubation step at 4°C was performed with the streptavidin-conjugated antibody solution. When necessary, 7AAD (BD Biosciences) was used as viability dye. Cells were measured on FACS-CantoII and LSR Fortessa X-20 (BD Biosciences) and the data was analysed using FlowJO software (Tree Star).

Determination vector copy number (VCN) and c.o.RAG1 expression by RT-qPCR

qPCR was used for the quantitative analysis of genomic lentiviral RNA, proviral DNA copies and transgene mRNA expression using WPRE, c.o.RAG1, ABL and PTBP2 as targets (Table S3). Total RNA from single cell suspensions was purified using RNeasy

Mini kit (Qiagen) and reverse transcribed into cDNA using Superscript III kit (Invitrogen). Genomic DNA was extracted from single cell suspensions using the GeneElute Mammalian Genomic DNA kit (Sigma-Aldrich). Dneasy Blood and Tissue Kit (Qiagen) was used to isolate genomic DNA from murine organs and tissues. VCN was determined on DNA samples by the detection of WPRE and PTBP2. The levels of transgene expression were determined on cDNA samples, by normalizing *c.o.RAG1* to the expression of the *ABL* gene. qPCR was performed using TaqMan Universal Master Mix II (Thermofisher) in combination with specific probes for indicated genes from Universal Probe Library (Roche). Primers and probes used are listed in Table S3. PCR reactions were performed on the StepOnePlus Real-Time PCR system (Thermofisher). All samples were run in triplicate.

Serum immunoglobulin quantification

Murine IgG, IgM, IgE, TNP-specific IgG and human IgM were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). NUNC Maxisop plates (Thermo Scientific) were coated with unlabeled anti-mouse IgG, IgM (11E10), IgE antibodies (SouthernBiotech) or unlabeled anti-human IgM antibody (Jackson Immuno Research laboratories, kindly provided by Dr. Karahan, LUMC). For detection of TNP-specific IgG, plates were coated with synthetic TNP-KLH (Biosearch Technologies Inc.). Blocking was done with 1% BSA/PBS (mouse) or 2% BSA/0.025 Tween/PBS (human) for 1h at room temperature (RT) and subsequently serial dilutions of the obtained sera were incubated for 3h at RT. After washing, plates were incubated with biotin-conjugated anti-mouse IgG, IgM, IgE (SouthernBiotec) or anti-human IgM (Novex life technologies, kindly provided by Dr. Karahan, LUMC) for 30 min at RT. For detection, plates were incubated for 30 min at RT with streptavidin horseradish peroxidase (Jackson Immuno Research laboratories) and subsequently azino-bis-ethylbenzthiazoline sulfonic acid (ABTS, Sigma-Aldrich) was used as a substrate. Data was acquired at a wavelength of 415 nm using Bio-Rad iMark microplate reader and MPM 6 software (Bio-Rad). Antibody concentration was calculated by using serial dilutions of purified IgG, IgM, IgE proteins (SouthernBiotech) and human reference serum (Bethyl Laboratories, kindly provided by Dr. Karahan, LUMC) as standards.

Repertoire analysis

Total RNA was purified from murine spleen cells and reverse transcribed into cDNA as described previously. GeneScan analysis procedure of the murine T-cell repertoire was adapted from [31]. cDNA was amplified using a FAM-labeled C gene segment-specific primer along with 24 TCR Vβ-specific primers (See **Table S3**). GeneScan™ 500 ROX™ (ThermoFisher) was used as internal size standard. Labeled PCR products were run on the ABI Prism® Genetic Analyzer (Applied Biosystems) for fragment analysis. Raw spectratype data was analyzed, visualized and scored by ImSpectR, a novel spectratype analysis algorithm for estimating immunodiversity [32] ImSpectR identifies and scores individual spectratype peak patterns for overall (Gaussian) peak distribution; shape of individual peaks, while correcting for out-of-frame TCR transcripts. Scores range from 0 when no peaks detected, to 100 for a diverse TCR repertoire.

Human immunoglobulin and T-cell receptor repertoire generated in NSG mice was analyzed on DNA samples from BM and thymus (DNA was extracted as described previously). Rearrangements were analyzed using the EuroClonality/BIOMED-2 multiplex PCR protocol [33]. Amplification of IgH, IgK, TCRβ and TCRγ rearrangements were performed following the *IGH* + *IGK* B-Cell Clonality Assay (InvivoScribe) and *TCRB* + *TCRG* T-Cell Clonality Assay (InvivoScribe) instructions respectively. PCR products were analyzed by differential fluorescence detection using ABI-3730 instrument (Applied Biosystems) for fragment analysis. The output files were visualized and analyzed using ImSpectR.

Non-restrictive Linear Amplification Mediated PCR (nrLAM-PCR)

Lentiviral insertion site was analysed by nrLAM-PCR on murine bone marrow DNA samples as described by Schmidt M. et al [34].

In Vitro Immortalization assay (IVIM)

Genotoxic potential of the viral vectors (Cbx3.MND-c.o.RAG1, MND-c.o.RAG1, PGK-c.o.RAG1, UCOE-c.o.RAG1) was quantified as previously described by Baum et al. [35].

Gross pathology and histopathology

A full necropsy was performed, organs were collected subjected to macroscopic and microscopic examination (Table S1 of collected organs). The selection of organs to be examined for gross pathology and histopathology analyses followed the applicable European and international guidelines (EMEA 1995, WHO 2005) [36]. For gross pathology, the external surface of the body, orifices, the thoracic abdominal and cavities were examined (Analyzed organs are listed in Table S1).

For histopathological examination organs were fixed in 4% neutral buffered formalin for 24 hours and paraffin embedded, 5 µm sections were processed for hematoxylin and eosin (HE) and for immunohistochemistry stainings according to standard procedures [37]. All slides were examined blindly by a European board certified pathologist (ECVP).

Before staining, paraffin sections were deparaffinated. Antigen retrieval was performed for antibody against FOXP3 and Cytokeratin 5/6 by heating during 12 minutes at 98 ^{0}C in citric acid buffer (0,01 Mol/L, pH 6,0). Inhibition of endogenous peroxidase was done in 0.3% H_2O_2 in PBS. After incubation overnight at room temperature with antibody against FOXP3 (1/70, 700914; Thermo Scientific, Waltham, MA, USA) and Cytokeratin 5/6 (1/100, GA780; DAKO, Glostrup, Denmark) the secondary antibody biotinylated Goat anti Rabbit IgG (1/200, BA-1000; Vector Labs, Burlingame, CA USA) and biotinylated Horse anti Mouse (1/200, BA-2000; Vector Labs, Burlingame, CA USA) was incubated for 90 minutes. Visualization was enforced with ABC staining kit (Vectastain ABCkit, HRP, PK6100, Vector Labs, Burlingame, CA USA) for 45 minutes. As substrate for horseradish peroxidase 3,3'-diaminobenzidine tetrahydrochloride (DAB, D5637, Sigma-Aldrich, St Louis, MO USA) was applied for 10 minutes. Mayer's hematoxylin was utilized as nuclear counterstaining.

Statistics

Statistics were calculated and graphs were generated using GraphPad Prism6 (GraphPad Software). Statistical significance was determined by standard one/two-tailed Mann-

Whitney U test, ANOVA test or two-tailed non-parametric Spearmen correlation (*p<0.05, **p < 0.01, ***p < 0.001 and ****p<0.0001).

RESULTS

MND promoter as most optimal vector to correct Rag1 deficiency.

At the onset of this project we constructed four different SIN LV transfer plasmids in the CCL backbone and tested four different promoters: PGK (Human PhosphoGlycerate Kinase (PGK)-1 promoter, nucleotides 5 to 516; GenBank accession no.M11958; [30], MND (myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer binding site substituted promoter; [38], UCOE (the modified chromatin-remodeling element, devoid of unwanted splicing activity and minimized read-through activity; [39],and a tandem combination of UCOE and MND (Cbx3.MND) were used to drive expression of a codon optimized version of the RAG1 (**Fig 1A**).

Recombinant lentiviruses were produced at small and large scale to evaluate virus production and in vitro expression efficiency of the different vectors. The transfer vectors in conjunction with GAG-Pol, REV and VSV-G plasmids were transiently transfected into 293T cells to produce the different lentiviruses. The number of infectious particles of the small and large virus batches was assessed before and after concentration by Q-PCR. Consistently, both the small and large batches of UCOE-c.o.RAG1 lentivirus (both unconcentrated and concentrated) had a significantly lower number of infectious genomes per mL compared to the other vectors (**Fig 1B** and **Fig 1C**), highlighting a difficulty to scale up its production. These lentiviruses were subsequently used to transduce lineage negative bone marrow (BM) cells from Rag1-deficient mice in order to determine their functional characteristics under conditions relevant for in vivo application. We found that UCOE-c.o.RAG1 reached lower VCN (**Fig 1D**) than the other vectors and PGK-c.o.RAG1 was the vector with lowest promoter strength (**Fig 1F**). Unfortunately, both PGK and UCOE-c.o.RAG1 only resulted in low levels of c.o.RAG1 expression (**Fig 1E**) whereas quite high levels are known to be required for immune reconstitution [24, 40, 41]. Indeed, an in vivo pilot experiment where Rag1-deficient mice were transplanted with wild-type (WT) stem cells, mock transduced Rag1-deficient stem cells or gene therapy treated stem cells using the four different promoters, revealed that the promoter strength and essentially the level of c.o.RAG1 is crucial to obtain adequate immune reconstitution (2 independent pilot experiments, total 6 or 7 mice per group). Immune reconstitution of these mice was followed in the peripheral blood every 4 weeks, showing that B-cell and T-cell reconstitution were achieved in the different gene therapy group to different extents (**Fig S1A**). Reflecting the known promoter strengths of these four vectors, a wide range of c.o.RAG1 expression was created by this initial experiment. Interestingly, 16 weeks after transplantation, we observed a clear linear correlation between the expression of c.o.RAG1 achieved in the BM and the number of B cells (B220+IgM+ cells) generated (**Fig 1G,** left and middle panel). For T cells, we observed that there was a threshold of minimal c.o.RAG1 expression to develop an active double positive CD4 and CD8 (DP) population in the thymus, roughly at 10x the house keeping control level (**Fig 1H**, left and middle panel). Mice reconstituted with stem cells having lower c.o.RAG1 expression than this

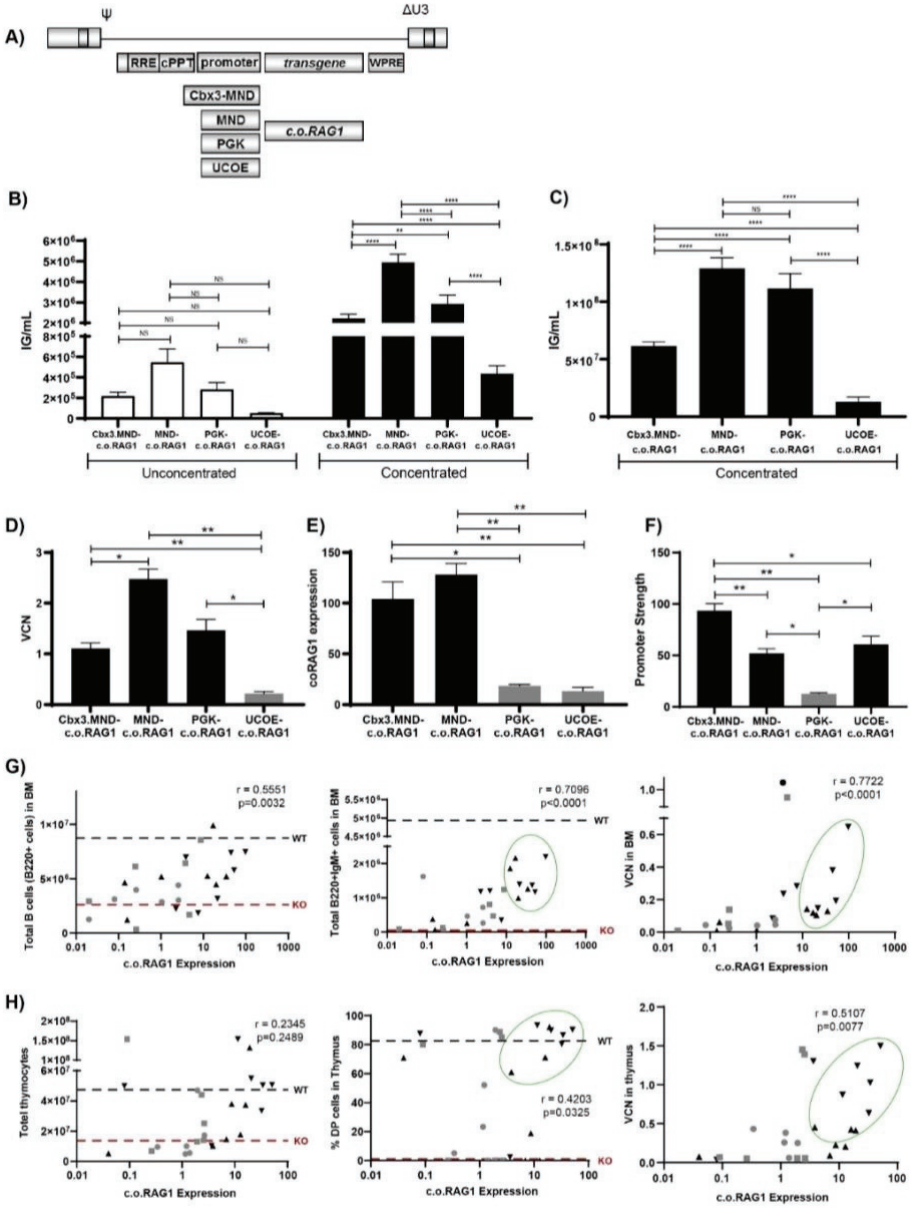


Figure 1: Selecting the Optimal SIN LV Plasmid: Virus Production and In Vitro Efficiency. *(A) Four different SIN LV plasmids in the CCL backbone carrying different promoters (Cbx3.MND, MND, PGK, and UCOE promoter) were tested to drive expression of a codon-optimized version of RAG1. (B) Production of lentivirus batches with the different constructs. The number of infective particles (infectious genomes/mL) from unconcentrated and concentrated small batches was determined. Three independent lentivirus small batches per plasmid were produced and analyzed (two-way ANOVA test; *p < 0.05, **p < 0.01). (C) Production of lentivirus batches on a large scale with the different constructs. The number of infectious genomes/mL after concentration of large lentiviruses*

*batches was determined. (D) Transduction efficiency of the different SIN lentiviruses in murine lineage-negative cells. VCN was determined by WPRE determination on genomic DNA. Three independent lentivirus batches per plasmid were produced and analyzed (one-way ANOVA test; *p < 0.05, **p < 0.01. (E) Determination of transgene expression in the transduced cells by the different constructs. c.o.RAG1 expression relative to ABL1 was determined by qPCR. Three independent lentivirus batches per plasmid were produced and analyzed (one-way ANOVA test; *p < 0.05, **p < 0.01. (F) Determination of the promoter strength (c.o.RAG1 expression/VCN) of the different plasmids. Three independent lentivirus batches per plasmid were produced and analyzed (one-way ANOVA test; *p < 0.05, **p < 0.01). (G) Total number of B220+ cells (left panel) and total number of B220+IgM+ cells (middle panel) correlated with the expression of c.o.RAG1 in BM. The correlation between VCN and c.o.RAG1 expression in BM of immune reconstituted mice is shown (right panel) (:, Cbx3.MND; ;, MND;-, PGK;C, UCOE promoters; gray indicates low-expressing plasmids; black indicates high0expressing plasmids; green circles indicate mice with acceptable immune B and T cell reconstitution). Data shown represent two independent in vivo experiments with in total six or seven mice per group. Each dot represents one mouse. Nonparametric Spearman r correlation, two-tailed; **p < 0.01, ***p < 0.001, ****p < 0.0001. (H) Correlation between total thymocytes (left panel) and DP cells (middle panel) with c.o.RAG1 expression in the thymus. Correlation between VCN and c.o.RAG1 expression in the thymus of immune reconstituted mice (right panel) (:, Cbx3.MND;;, MND;-, PGK;C, UCOE promoters; gray indicates low-expressing plasmids; black indicates high-expressing plasmids; green circles indicate mice with acceptable immune B and T cell reconstitution). Data shown represent two independent in vivo experiments with in total six or seven mice per group. Each dot represents one mouse. Nonparametric Spearman r correlation, two-tailed; **p < 0.01, ***p < 0.001, ****p < 0.0001.*

threshold barely reconstituted thymic T-cell development. Accordingly, B- and T-cell reconstitution was consistently achieved in the BM and in the thymus when c.o.RAG1 expression at 10x the house keeping control level or higher which could be achieved. This expression level was mainly reached with VCN's of 1 and lower (**Fig 1G&H,** right panels) using the high expressing vectors such as Cbx3.MND and MND-c.o.RAG1 (black symbols in **Fig. 1G&H**). We considered mice achieved immune reconstitution when B- and T-cell development was successful (overcoming the early developmental block), the cells were functional, with a diverse TCR Vβ repertoire and without signs of toxicity or adverse side effects (**Fig 1G&H,** green circle). In the low *c.o.RAG1* expression mice (grey dots, mainly PGK and UCOE promoter), we found a number of mice (n=4 out of 9) that developed skin rashes and wasting during the course of the experiments which resulted in the death of some mice (similar to the features due to low RAG1 activity described previously [27]), while the animals in the higher *c.o.RAG1* expression group (black dots,Cbx3.MND and MND promoter) as well as the animals that received wild-type cells or uncorrected Rag1 knock-out cells did not display any health problems. Collectively, our *in vitro* and *in vivo* pilot data highlight the importance of achieving sufficient *c.o.RAG1* expression, at VCN around or below 1, in order to obtain successful immune reconstitution, which was only accomplished using Cbx3.MND-c.o.RAG1 and MND-c.o.RAG1 lentiviruses (**Fig S1B**).

To better compare both vectors, an additional *in vivo* reconstitution experiment was done, with more comparable VCNs. Rag1-deficient mice transplanted with WT stem cells, mock transduced Rag1 KO stem cells, Cbx3.MND-c.o.RAG1 (starting VCN = 0.95) or MND-c.o.RAG1 (starting VCN = 1.1) treated stem cells were extensively analyzed 16 weeks

after transplantation by flow cytometry and qPCR for viral copy number (VCN) measuring WPRE (Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element and expression of the therapeutic gene c.o.*RAG1.* Mice were sacrificed after 4 months and immune organs were analysed by flow cytometry (pilot experiment with a total of 3 mice per group). Restoration of IgM+B220+B cells (**Fig 2A**) in the BM was seen in mice treated with WT stem cells and MND-c.o.RAG1 treated gene therapy mice and occasionally in mice with Cbx3.MND elements, even with comparable VCN. Mock transduced Rag1 KO stem cells did not restore B-cell development, where cells were blocked at the pre B cell stage, as expected. In contrast, in gene therapy treated mice the arrest in B-cell development was alleviated, and immature and mature B cells developed (**Fig 2B,** left panel). MND-c.o.RAG1 gene therapy mice successfully developed all B-cell developmental subsets in the BM, similarly to WT transplanted mice and significantly different from the mock KO transplanted mice. We observed that even though B-cell development in BM was satisfactory, B cells numbers detected in the peripheral blood were significantly lower than in the WT situation (**Fig 2B,** right panel). However, B cell functionality was fully restored to WT degree as the levels of immunoglobulins (IgG and IgM) detected in serum were comparable to WT transplanted mice (**Fig 2G**). We next analysed the thymus for T-cell marker-expression, using (amongst other markers) CD4 and CD8. Proper T-cell development with a full spectrum of DP and single positive CD4 or CD8 (SP) developmental stages was observed with WT and MND-c.o.RAG1 cells, but not with Cbx3.MND-c.o.RAG1 cells where mice showed an exhausted thymus phenotype with mature CD4 and CD8 SP cells but not DP cells anymore 16 weeks after transplantation (**Fig 2C&D**). Similar to B cells, the total number of T cells in the periphery was lower than in mice treated with WT cells; nonetheless mature T cells after gene therapy showed a diverse T cell receptor (TCR) repertoire. We used GeneScan analysis for 24 different Vβgenes and calculated the cumulative complexity score. As shown in the representative plots (**Fig 2E**) as well by the ImSpectR score (**Fig 2F**), the MND promoter performed closer to WT treated mice, revealing an active V(D)J recombination machinery able to successfully rearrange *TCR* genes.

Besides efficacy, safety is an important aspect for clinical use of gene therapy vectors. As an additional selection criterion our research grade lentivirus batches were tested in the IVIM assay, which is the currently accepted (FDA and EMA approved) standard assay for safety of viral vectors. Even though high VCN per cells were achieved in this assay with the test vectors, both vectors were shown to have a frequency of insertional mutagenic events, that were at least 50 fold lower than classical RSF91 gamma-retroviral vectors with known mutagenic potential (**Fig 2H**). In three independent IVIM assays, we did not observe cytotoxicity of the vector supernatants on lineage negative bone marrow cells.

This safety selection criterium, together with the successful *in vivo* immune reconstitution given by the MND-c.oRAG1 treated cells (**Fig S1C**), led us to conclude that the pCCL-MND-c.o.RAG1 LV vector is the best vector of choice and we therefore proceeded to have the vector produced at GMP grade. All following experiments described were conducted with this clinical grade vector for further preclinical testing.

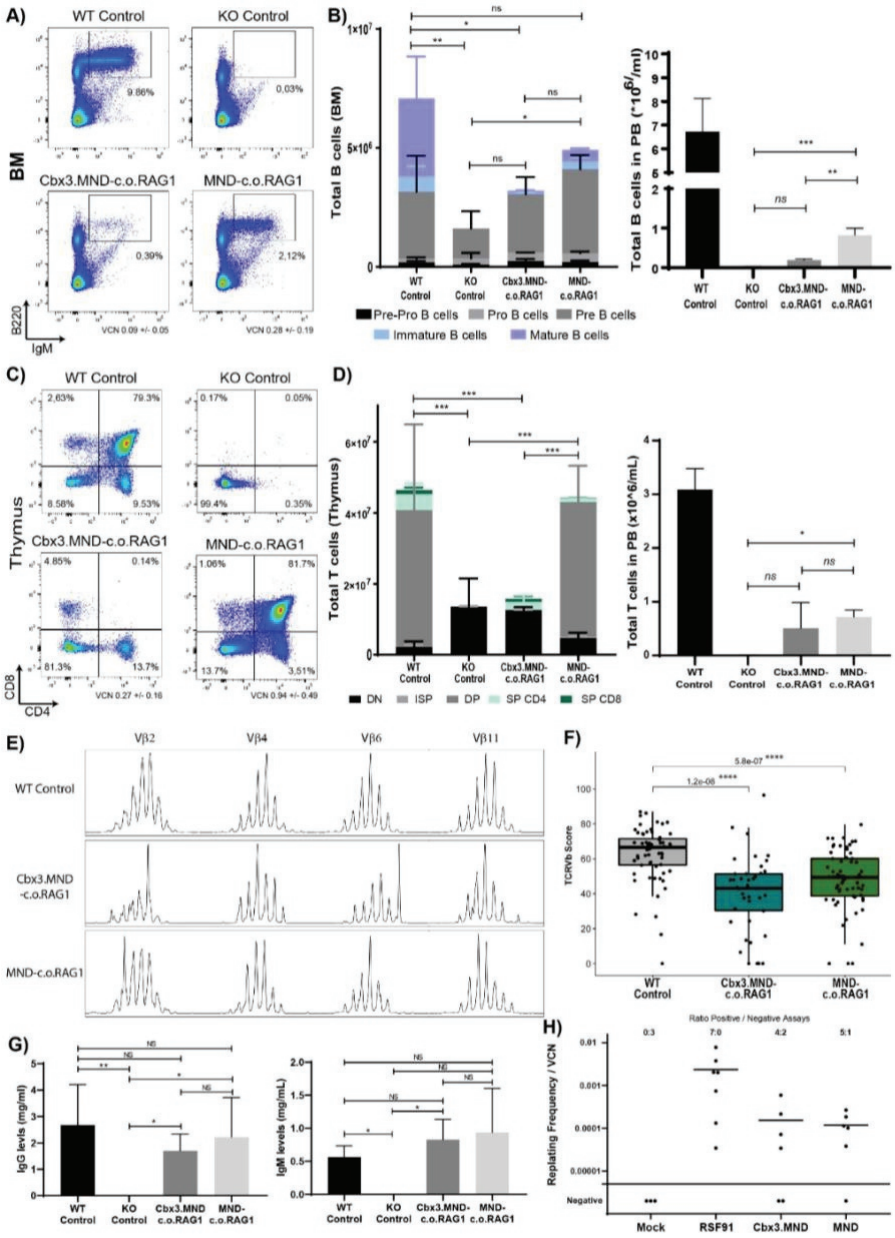


Figure 2: Selecting the Optimal SIN LV Plasmid to Drive an Immune Reconstitution of Rag1 Deficiency. *Rag1-deficient mice (experiment with a total of 3 mice/group) were transplanted with 500,000 stem cells: WT cells, mock Rag1 KO cells, Cbx3.MND-c.o.RAG1-treated KO cells (VCN of 0.95), and MND-c.o.RAG1-treated KO cells (VCN of 1.1). (A) Representative FACS plots showing the restoration of B220high+ B cells in the BM. (B) Total number of the different B cell subsets in the BM (left panel) and total number of B cells (B220high+) in the PB (right panel) 16 weeks after SC*

*transplantation. Graphs represent the means and standard deviation of a pilot experiment with two to three mice per group (Mann-Whitney test, one-tailed; *p%0.05; NS, not significant). (C) Representative FACS plots of the thymus reconstitution (CD4 versus CD8) with the different constructs. (D) Total number of the different T cell subsets in the thymus (left panel) and total number of T cells (CD3+TCRab+) in PB (right panel) 16 weeks after transplantation. Graphs represent the means and standard deviation of a pilot experiment with two to three mice per group (Mann-Whitney test, one-tailed; *p % 0.05; NS, not significant). DN, double negative; ISP, immature single positive; DP, double positive; SP, single positive. (E) Representative samples of GeneScan plots are shown for four different families (x axis indicates CDR3 length; y axis shows the fluorescence intensity of the runoff products). (F) TCR Vb repertoire analysis by GeneScan. A total of 24 Vb families were analyzed on spleen cells from three mice per group. Overall score of all of the families was calculated for the different constructs (Mann-Whitney test; p values are represented on the plot; ****p < 0.0001; NS, not significant). (G) Quantification of total IgG and IgM in mice serum by ELISA (one-way ANOVA test; *p < 0.05, **p < 0.01). (H) IVIM assay was performed on the two constructs to assess their safety (mock cells as negative control; RSF91 g-retroviral vector as a positive control). Data show results from three complete IVIM assays.*

Extensive preclinical testing of the pCCL-MND-c.o.RAG1 LV vector in Rag1-/- mice.

Initial analysis of 8 *Rag1*-/- mice treated with the MND vector (starting VCN = 0,2), positive (WT stem cells; 3 mice) and negative controls (mock transduced Rag1-/- stem cells; 3 mice) 24 weeks after transplantation confirmed good B-cell reconstitution in the periphery (PB) and in BM (**Fig 3A**), although the numbers remained lower than mice treated with WT stem cells (**Fig 3B** and **Fig S2A**), which could be due to partially arrested development from pre-B to immature B-cell stages (**Fig S2B**) originating from cells that were transduced with insufficient levels of c.o.RAG1 to support full Ig rearrangements. Alternatively, residual pro- and pre-B cells could inhibit B-cell development by occupying important developmental niches. However, gene therapy mice showed similar proportion of immature and mature B-cell subsets in the spleen (**Fig 3C**). Concerning T-cell reconstitution, most GT mice showed next to complete thymic T-cell development with thymocyte numbers almost normal (**Fig 3D** and **Fig S2A&B**), although the T-cell numbers in the periphery were restored to ~30% of normal levels (**Fig 3E**), with somewhat lower proportion of naïve CD4 and CD8 T cells and increased effector memory subsets (**Fig 3F**), most likely due to homeostatic proliferation from initial T cell that egressed from the thymus. Indeed, delayed T-cell development can be observed in the GT mice compared to WT controls (**Fig S2A&B**) and therefore the proportions of naïve and memory T cells might still not be entirely balanced after GT. Besides analysing the primary and secondary immunological organs by flow cytometry, we also checked restoration of the immune system by histological analyses. Spleen, lymph nodes and thymus showed remarkably normal architecture after GT (**Fig 3G**), comparable to mice treated with WT stem cells, and quite different from the negative control mice treated with mock transduced Rag1-/- cells. Importantly, restoration of FoxP3 expression which directs T cells into the CD4+ regulatory T cell lineage (Treg) was also observed in mice treated with MND-coRAG1 gene therapy (**Fig 3G**).

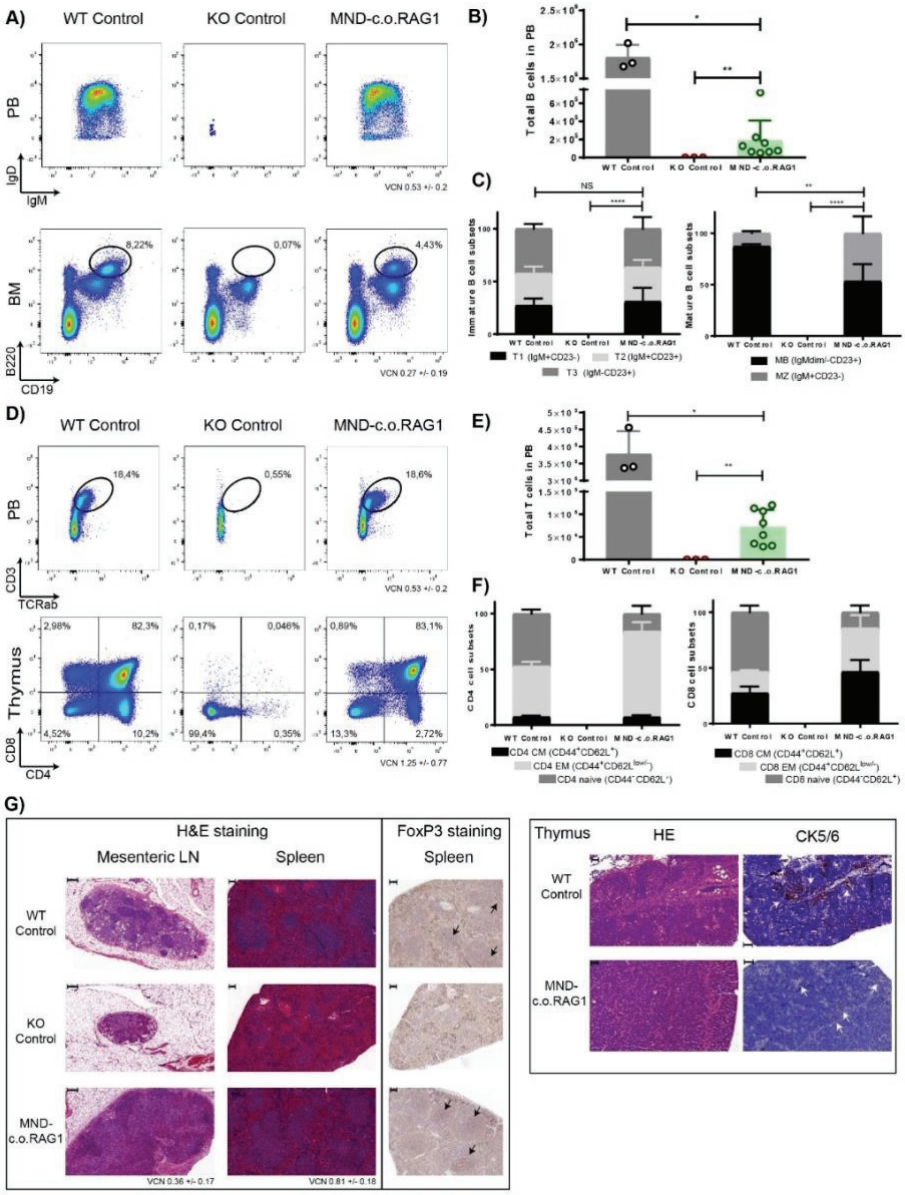


Figure 3: Extensive Immune Reconstitution of Mice Receiving Gene Therapy of Stem Cells with a Clinical-Grade MND-c.o.RAG1 Vector. *Rag1-deficient mice were transplanted with 250,000 stem cells: WT cells (three mice), mock KO cells (three mice), and MND-c.o.RAG1-treated cells (VCN of 0.2; eight mice). (A) Representative plots of B cell reconstitution in the blood (B220+IgM/IgD cells; top panel) and B cell development in the BM (B220+CD19+ cells; bottom panel) 24 weeks after transplantation. (B) Total number of B cells (B220+CD11b/CD43_ cells) in the PB (Mann-Whitney test, one-tailed; *p < 0.05, **p < 0.01). (C) Immature (B220+CD93+ cells; left panel) and mature (B220+CD93- cells; right panel) B cell subsets distribution in spleen. Two-way ANOVA test; ***p <*

*0.001; ****p < 0.0001. (D) Representative plots of T cell reconstitution in the blood (CD3+TCRab+ cells; top panel) and T cell development in the thymus (CD4 versus CD8 cells; bottom panel) 24 weeks after transplantation. (E) Total number of T cells (CD3+TCRab+ cells) in PB at the end of the experiment (24 weeks) (Mann-Whitney test, one-tailed; *p < 0.05, **p < 0.01). (F) Naive, effector memory (EM), and central memory (CM) subset distributions for CD4 (CD3+TCRab+CD4+; left panel) and CD8 (CD3+TCRab+CD8+; right panel). T cell subset distributions in spleen are shown: naive cells (CD44_CD62L+), EM cells (CD44+CD62L-) and CM cells (CD44+CD62L+) 24 weeks after transplantation. (G) Left panel: Hematoxylin and eosin staining of mesenteric lymph nodes (scale bars, 200 mm) and spleen (scale bars, 100 mm; purple indicates germinal centers, and red indicates red pulp). Representative FoxP3 staining in spleen tissue (scale bars, 100 mm) is shown. Arrows indicate positive FoxP3 in germinal centers. Representative images are from WT control, KO control, and MND-c.o.RAG1 gene therapy mice. Right panel: Histological analysis of thymus reconstitution by hematoxylin and eosin staining (scale bars, 50 mm) and cytokeratin 5/6 staining (scale bars, 100 mm) . Representative images from WT control and MND-c.o.RAG1 mice. KO thymus was completely used for phenotyping (FACS, DNA, RNA), but KO thymic histology was previously described by van Til et al.[27]*

Functional reconstitution of immunity after Rag1 gene therapy

Next, we tested if the T and B cells that developed had a diverse repertoire and were capable of mounting an immune response against a T cell dependent neo-antigen. GeneScan analysis (3 WT control mice, 1 KO control mouse and 8 MND-c.o.RAG1 mice) showed a diverse TCR Vβ repertoire, that was slightly less complex before immunization than in mice reconstituted with WT stem cells (**Fig 4A**), but after immunization there was no statistical difference in immune repertoire. Total IgM, IgG and IgE levels were also checked (**Fig 4B** and **Fig S2D**) and reached close to normal levels in GT treated mice. Therefore, although GT mice were lagging behind with regard to B-cell numbers, their functionality in the form of antibody production was restored to WT levels. We used TNP-KLH as T-cell specific antigen and measured the production of TNP specific IgG antibodies, thereby investigating whether the developed T and B cell could collaborate in an active immune response. The TNP-specific IgG levels in serum were similar between mice treated with WT stem cells and GT treated mice (**Fig 4C**), showing the potential of a robust immune response after GT.

Pre-clinical release tests of the vector

As required by regulatory authorities the clinical grade vector was tested by external parties for the presence of replication competent virus (RCL). The vector tested negative in two independent tests (data not shown). Other release tests that are commonly required included biodistribution of the vector *in vivo*, checking of vector insertion sites, especially on possible clonal outgrowth, and tests for insertional mutagenesis such as IVIM.

We checked vector distribution on a large number of perfused organs (**Table S1**) in all GT treated mice (a total of 8 mice; **Fig 5A**). Perfusion was used to remove most of the blood cells, in which the leukocytes should carry the vector. As expected, given the positive selection for c.o.RAG1 transduced cells, high VCN was found in the thymus, followed by other immune organs, spleen, bone marrow, lymph nodes and peripheral blood. All other organs had very low signals, except some incidental positivity in stomach and lungs, possibly due to incomplete perfusion, or an ongoing infection in rare individual mouse.

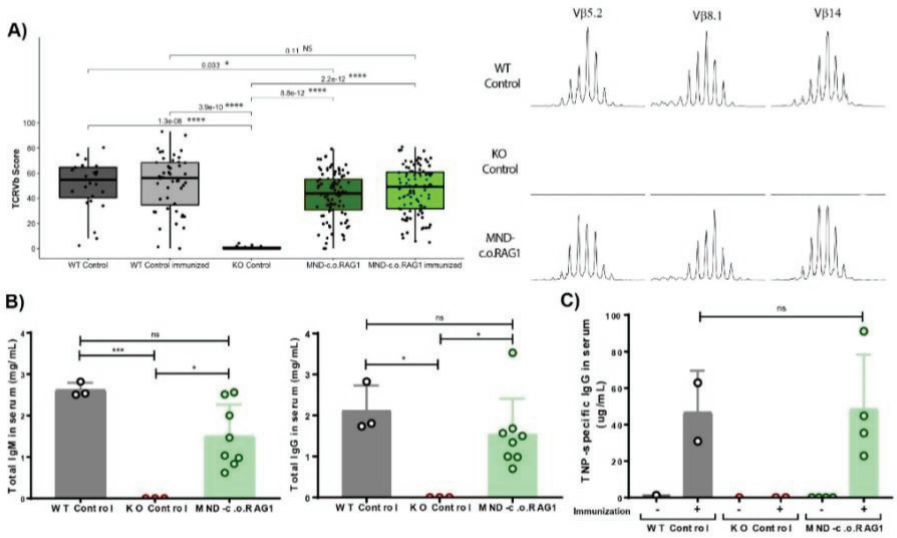


Figure 4: Functional Ig and TCR Rearrangements and Ig Class-Switch after RAG1 Gene Therapy*. (A) TCR Vb repertoire analysis by GeneScan from three WT control mice, one KO control mouse, and eight MND-c.o.RAG1 mice. A total of 24 Vb families were analyzed on spleen cells from three WT control, one KO control, and eight MND-c.o.RAG1 mice (non-immunized and immunized). Overall score of all of the families was calculated by ImSpectR (Mann-Whitney test; p values are represented in the plot; *p < 0.05, ****p < 0.0001; NS, not significant). Representative samples of GeneScan plots are shown for three different families (x axis indicates CDR3 length; y axis shows the fluorescence intensity of the runoff products). (B) Quantification of total IgG and IgM in serum by ELISA (three mice/control group, eight MND-c.o.RAG1 mice) (one-way ANOVA test; *p < 0.05). (C) Quantification of TNP-specific IgG in serum of immunized mice. Each dot represents a value obtained in one mouse (three mice/control group, eight MND-c.o.RAG1 mice) (one-way ANOVA test; *p < 0.05).*

Importantly, pathological examination of histology slides of 29 different organs per mouse (n=14) did not show any abnormalities in mice treated with MND-c.o.RAG1 gene therapy (Examples of 4 organs shown in **Fig S2C**). Indeed, no signs of Omenn syndrome such as skin rashes, high IgE levels, oligloclonal TCR Vβ repertoire or T cell infiltrates in the skin were detected in the immune reconstituted mice.

Next, we checked viral insertion sites using nrLAM-PCR (Fig 5B), a sensitive technique that can detect clonal insertions as discrete bands, which can then be sequenced if needed.[34]. We invariably found a smear of bands indicating polyclonal haematopoiesis with very little indication of oligoclonality, except for a few minor bands from which we could not get extra specific insertion site information by sequencing. We conclude that there was no evidence of vector-induced clonal selection. This is in line with findings by others on using SIN LV vectors in HSCs. Safety of the clinical grade MND-c.o.RAG1 was also tested using the IVIM assay. The clinical vector showed no clonal outgrowth in different independent experiments, close to results from mock-transduced cells (**Fig 5C**). This is better than the research grade vector presumably due to higher purity resulting in a better functional titer leading to fewer side effects after transduction.

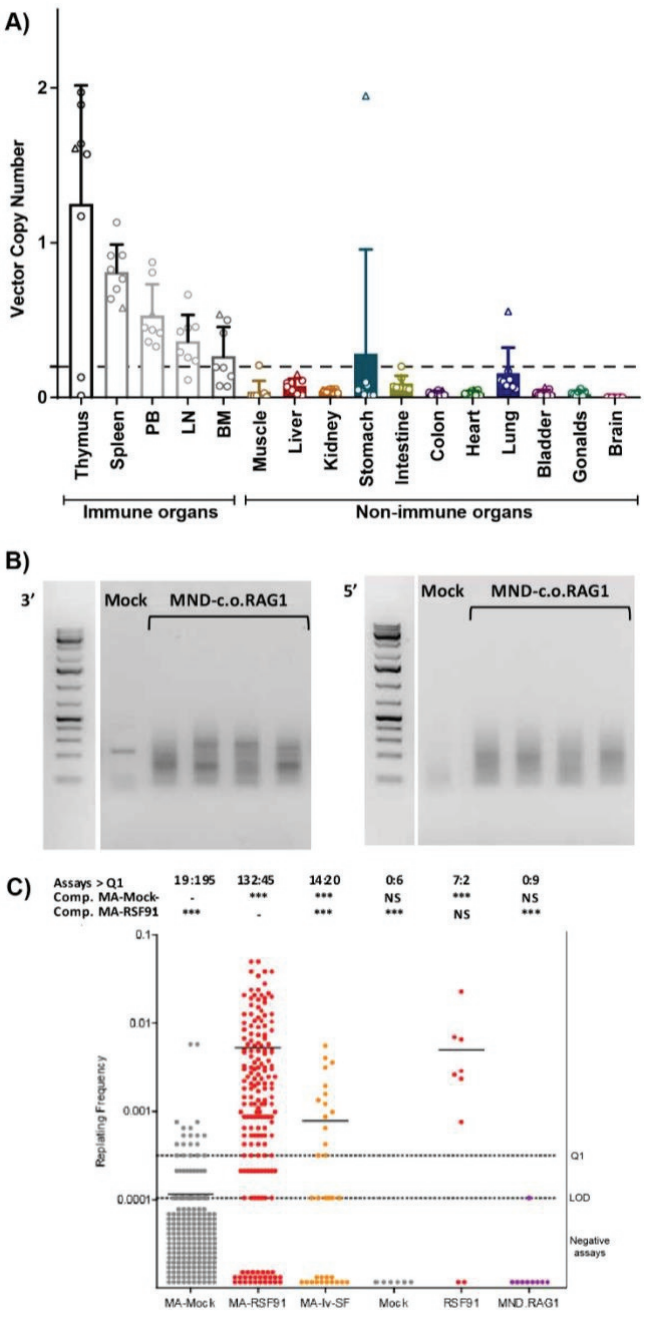


Figure 5: Preclinical Safety Testing of the Clinical-Grade. *(A) Vector biodistribution in immune and non-immune organs assessed by qPCR on DNA samples from 16 organs in total. Each dot represents a value from one mouse (three mice/control group, eight MND-c.o.RAG1 mice). The horizontal dashed line represents the threshold of the VCN of immune organs versus non-immune organs (starting VCN of transplanted cells of 0.2). (B) LV insertion site analysis by nrLAM-PCR of isolated DNA from BM obtained from Rag1-/- untransduced control mouse (mock) and four MND-c.o.RAG1 mice (male non-immunized/immunized, female nonimmunized/immunized). Gels shows results of the linear amplification from the 3₀ long terminal repeat (LTR) and 5₀ LTR, respectively (L = 1 kb plus marker). (C) Replating frequencies (RFs) of the control samples mock or RSF91 and the test vector MND-c.o.RAG1, in comparison to data of a meta-analysis for control samples (Mock-MA, RSF91-MA, lv-SF-MA [a lentiviral vector with an SFFV promoter]). The data points below the limit of detection (LOD; plates with no wells above the MTT threshold) were manually inserted into the graph (due to the logarithmic scale of the y axis). Above the graph, the ratios of positive (left number) and negative plates (right number) according to the MTT assay are shown. Differences in the incidence of positive and negative assays relative to Mock-MA or RSF91-MA were analyzed by Fisher's exact test with a Benjamini-Hochberg correction (*p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant). If above the LOD, bars indicate the mean RF.*

Restored B- and T-cell development in RAG1-SCID patient cells

We have previously shown that transplantation of BM CD34+ cells from SCID patients in NSG mice is informative for identifying where T-cell development is arrested in human SCID [42, 43]. This same model should also be suitable as preclinical efficacy model with patient cells. Hence, we purified CD34+ cells from cryopreserved BM cells from a RAG1-SCID patient. The patient was hypomorphic, with some residual B cells but no T cells. We transplanted busulfan-conditioned mice with either mock transduced or MND-c.o.RAG1 transduced CD34+ cells (1 mouse per group; starting VCN = 0.2) and followed the development of T and B cells over time up to 24 weeks. Human cell engraftment was similar between mice transplanted with gene therapy treated cells and mock transduced cells, indicating that gene therapy did not affect the engraftment of human cells (**Fig S3A**). As expected from the patient phenotype, B cells were observed in the mock transduced humanized mouse, but much higher numbers of B cell were found in the spleen of the GT treated mouse (**Fig 6A** and **Fig S3B**). The B cells that were present also showed polyclonal Ig rearrangement (**Fig S3E**) and produced immunoglobulins, as human IgM could be detected in the sera of the mice (**Fig 6D**), with a tendency towards a more polyclonal repertoire after GT.

Importantly, while no T cells developed in the mouse transplanted with mock transduced RAG1-SCID cells, the gene therapy mouse showed clearly detectable T-cells in PB (**Fig.6B** and **Fig S3C**). After scarifying the mice, we also checked their thymi. As the patient was hypomorphic, we observed that some stages of T-cell development were present, including all double negative (DN; $CD4^-CD8^-$ cells), immature single positive (ISP; $CD4^+CD8^-CD3^-$ cells) and the early CD3- DP stages (**Fig. 6C** and **Fig S3D**). However, there were no cells that were CD3+, so no late CD3+ DP thymocytes, or any SP thymocytes, suggesting that especially the rearrangement of TCRα was affected by this RAG1 mutation. Although immune reconstitution was still not optimal, likely due to the low VCN achieved in that experiment, lentiviral RAG1 GT of CD34+ Rag1-SCID patient cells allows alleviation of the T-cell developmental block and to generate an active thymus. Moreover, human cell engraftment and peripheral B and T cell levels after GT was close to healthy BM CD34+ cell transplantation described in previous work [42]. Finally, we checked *TCRB* and *TCRG* rearrangements by GeneScan analysis. Because of the very limited amount of DNA material, not all possible Vγ and Vβ genes could be analysed, but the selected gene segments showed many more in frame rearrangements in the gene therapy treated group for *TCRG*, while for *TCRB* only in the GT group, rearrangements could be detected (**Fig 6E**). nRLAM_PCR on BM cells revealed a polyclonal pattern with no signs of clonal dominance (**Fig. 6F**).

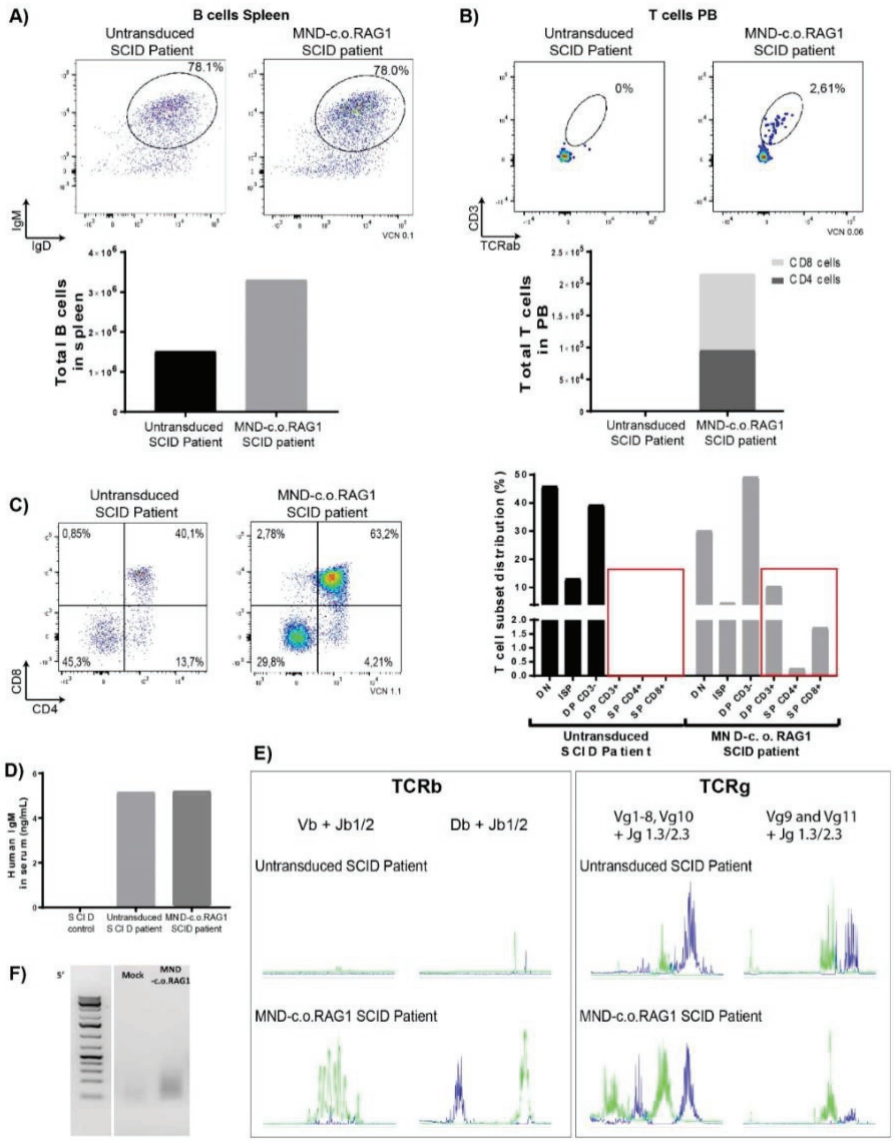


Figure 6: Restored T Cell Development in RAG1 SCID Patient Cells. *65,000 human CD34+ cells were transplanted intravenously into busulfan pre-conditioned NSG recipient mice (one NSG mouse with untreated cells and one NSG mouse with MND-c.o.RAG1 gene therapy cells with a VCN of 0.1). (A) FACS plots of human B cells (CD13/33 CD19+CD20+ cells; top panel) and total number of B cells (CD13/33 CD19+CD20+IgD/IgM cells; bottom panel) in the spleen at week 24 after transplantation. (B) FACS plots of human T cells (CD3+TCRab+; top panel) and total number of T cells, CD4 cells, and CD8 T cells in the PB at week 24 after transplantation (bottom panel). (C) Human T cell development in the thymus: FACS plots (CD4 versus CD8) and distribution of the different T cells subsets in the thymus (24 weeks after transplantation) are shown. (D) Quantification of total human IgM by ELISA of serum from a control NSG mouse transplanted with RAG1-SCID control untreated CD34+ cells*

(non-hypomorphic), our SCID patient CD34+ cells, and our SCID MND-c.o.RAG1 CD34+ cells. (E) Human TCR Vb and Vg repertoire analysis of isolated DNA from NSG thymus (SCID patient and SCID MND-c.o.RAG1) using a TCRB + TCRG T cell clonality assay (x axis indicates fragment sizes; y axis shows the fluorescence intensity of the runoff products). (F) LV insertion site analysis by nrLAM-PCR of isolated DNA from BM obtained from NSG SCID patient untransduced cells (mock) and NSG SCID MND-c.o.RAG1 mice. Gel shows results of the linear amplification from the 5₀ LTR (L = 1 kb plus marker). Data are from an independent experiment with n = 1 per condition.

DISCUSSION

Patients with RAG1-SCID are hampered in the genetic assembly of TCRs and BCRs. Affected children typically experience a wide range of serious, life-threatening infections. Replacing the affected bone marrow with healthy, unmodified, allogeneic stem cells is currently the only therapy for RAG1-SCID. Although overall survival is satisfactory in matched-donor SCT, the outcome in mismatched donor SCT, which represent the majority of cases, is significantly worse. Moreover, approximately 25% of allogeneic SCT-treated patients develop graft vs. host disease, which significantly impairs outcome in terms of morbidity, immune reconstitution, and transplant-related mortality [44]. Additionally, transplant outcome in RAG-SCID (and other recombination-defective forms of T-B-SCID) is significantly worse than for SCID with B cells (i.e. T-B+ SCID) [44, 45]

Transplantation of genetically corrected autologous HSCs, eliminates the risks associated with allogeneic stem cell transplantation (GvHD and rejection) and would therefore provide a valuable alternative particularly for patients lacking a matched donor. Gene therapy for X-SCID with LV or RV SIN vectors has shown to be successful and to lack the xenotoxicity problems previously observed when using γ-retroviral vectors [46, 47, 48]. For ADA-SCID, both RV vectors (currently marketed as approved therapy under the name Strimvelis) and LV vectors have shown excellent clinical results which are comparable to HSCT with matched donors [10, 49, 50.]

Unlike X-linked SCID and ADA-SCID, developing gene therapy for RAG-SCID has been notoriously difficult. Previous attempts [25] used gamma retroviral vectors in a preclinical Rag1$^{-/-}$ model, which carried a high risk of insertional mutagenesis. Although RAG1 gamma retroviral vectors were able to correct the deficiency more readily, SIN lentiviral vectors initially resulted in insufficient expression of the therapeutic *RAG1* gene, leading to 'leaky' SCID or an Omenn-like phenotype. A breakthrough came with the introduction of codon-optimization of the human *RAG1* gene [26].This innovation yielded higher viral titers and much higher levels of *RAG1* expression without the need to introduce multiple copies per cell. Here we have used the same codon optimized *RAG1* therapeutic gene, but in a different lentiviral backbone and under the control of a clinically approved promoter. The first challenge was to develop a vector with a strong promoter driving the high expression of *c.o.RAG1*, to similar levels as native expression. According to Immgen dataset and our previous data in human thymi [40], native *Rag1* expression needed for B- and T-cell development in mouse is at least 10x and 13x the household gene expression (*Abl1*). In accordance, we here show that durable, functional immune reconstitution can be obtained at low VCN (1 or lower) with our MND-c.o.RAG1 vector that is consistently driving sufficient *c.o.RAG1* expression above 10x the household gene. As proper *RAG1*

expression was achieved, gene therapy treated mice survived healthy, without showing representative features of leaky SCID in mice as discussed by Marrella et al. (Rag2 Omenn syndrome mouse model [51]), Khiong et al. (Rag1 Omenn Syndrome [52]), Giblin et al. 2009 (Atypical SCID phenotype [53]) and Ott de Bruin et al. (CID-G/AI phenotype [54]). Our data suggest that the approach using pCCL-MND-c.o.RAG1 transduced HSCP should be able to overcome the broad range of clinical and immunologic phenotypes due to RAG1 deficiency, including hypomorphic RAG1 disease. Experimental proof for correction of hypomorphic RAG1 deficiencies requires extensive experimentation in appropriate mouse models, which are planned in the near future. Moreover, we show that the human RAG1 deficiency can be functionally restored in patient cells, providing important additional efficacy data required for successful clinical implementation.

In some mice, the reconstitution of T- and B-cell development with RAG1 transduced cells lagged behind compared to development observed in wild type stem cells. This indicates that some additional improvements could be made. For example, by optimizing transduction efficiencies which can be achieved by using a non-toxic transduction enhancer [55]; however, VCN numbers should not increase too much as this may increase insertional mutagenic events. Another approach to improve at least the T-cell development may be to co-transplant or the use of CD34+ CD7+ cells prior transplantation [56] [57] from the same patient to support the thymic microenvironment in which the stem/progenitor cells that seed the thymus find their niches. This can be especially important to boost development in the DN compartment.

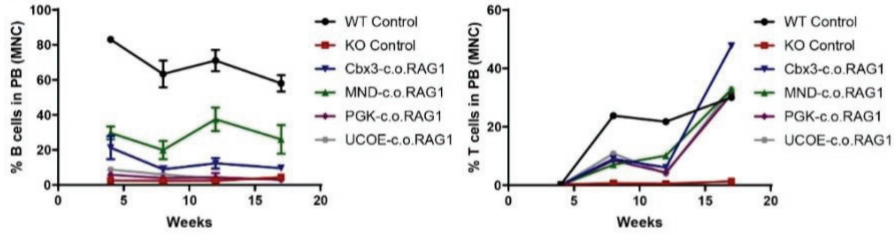
Insertional mutagenesis has been shown to occur in gene therapy trials using γ-retroviral vectors without SIN configuration. In our study a SIN LV vector using the MND promoter was chosen, because this fairly strong promoter is most efficacious in our preclinical models. The MND promoter has previously been used in gene therapy trials for ADA-SCID [58] and Adrenoleukodystrophy (ALD), without any reports of insertional mutagenesis [59] [60]. In the ALD trials there were some clones showing clonal dominance with over-representation of insertion site near SMG6, CCND2 and HMGA2, but this has not led to development of leukemia and may be transient as was reported for a SIN LV vector used for treating β-thalassemia [61]. In addition, our collective preclinical safety data indicate that the MND-c.o.RAG1 vector is relatively safe. Nevertheless, genotoxicity cannot be fully excluded and we therefore favor clinical implementation initially in patients in whom only HLA incompatible donors are available. After clinical efficacy and safety has been demonstrated in this patient group, wider implementation could be considered; potentially, not only for RAG1-SCID, but also for Omenn Syndrome and other RAG1 deficiencies.

Clinical trials have shown that ADA-SCID and X-linked SCID gene therapies result in significant clinical benefit, as well as a significant reduction in healthcare-related costs (reviewed in [58] [62]). We expect similar benefits from our approach to treat patients with RAG1-SCID, as it will reduce the suboptimal outcomes in (mismatched) allogeneic transplants, which are often associated with the need to administer immunoglobulins, and treat infectious and GvHD-related complications. Based on the results reported here, a phase I/II clinical trial is planned to open in 2020. We expect that this trial will provide an

alternative curative treatment for patients with RAG1-SCID, for whom no matched stem cell donor is available.

SUPPLEMENTARY MATERIAL

A)



B)

In vitro	Cbx3-MND	MND	PGK	UCOE
Physical titer	++	++	+	+/-
Functional titer	+	++	+	+/-
Transgene expression	+	+	-	-
Promoter strength	++	+	-	+

C)

In vivo	Cbx3-MND	MND
B cell development BM	-	+
Thymic reconstitution	-	+
Mature immune cells	-	+
Immunoglobulins	-	+
T cell repertoire	+/-	+/-
Safety (IVIM)	+/-	+/-
Omenn-like syndrome in mice (due to low c.o.RAG1 expression)	Observed	Not observed

***Figure S1: Choice of the optimal SIN LV plasmid**. A) Percentage of B cells (CD11b/CD43-B220+ cells; left panel) and T cells (CD3+TCRαβ+ cells; right panel) over time in PB after stem cell transplantation with the different constructs (Cbx3.MND-c.o.RAG1, MND-c.o.RAG1, PGK-c.o.RAG1 and UCOE-c.o.RAG1) (Data from 2 independent experiments, total of 6-7 mice/group). B) Summary of the in vitro decision criteria (from 3 independent lentiviral batches) taken into account for the choice of the most optimal plasmid to correct RAG1 deficiency. C) Summary of the In vivo decision criteria (2 independent experiments with total of 6-7 mice per group) taken into account for the choice of the most optimal plasmid to correct RAG1 deficiency.*

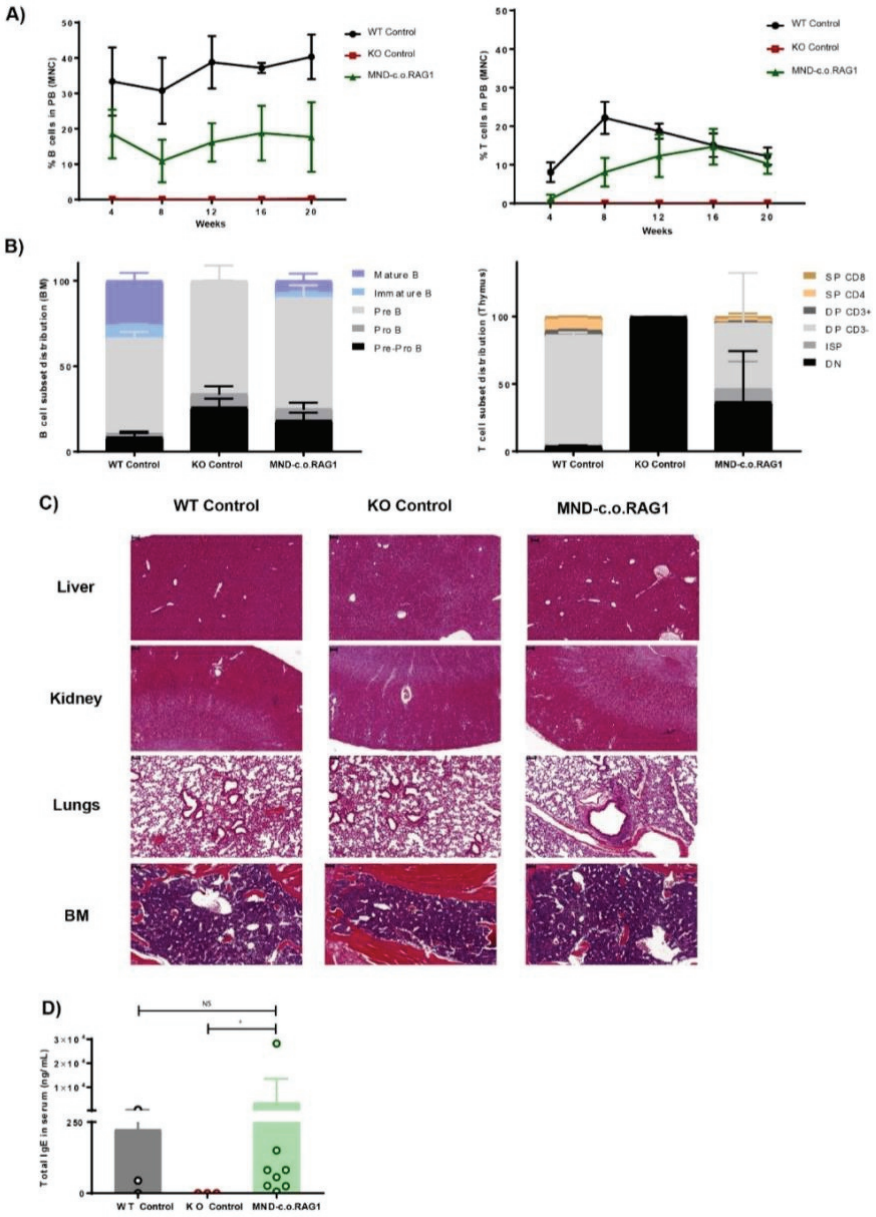


Figure S2: Immune development after MND-c.o.RAG1 gene therapy in Rag1-/- mouse model. *A) Percentage of B cells (CD11b/CD43-B220+ cells; left panel) and T cells (CD3+TCRαβ+ cells; right panel) over time in PB after stem cell transplantation with the clinical MND-c.o.RAG1 batch (3 WT control mice, 3 KO control mice and 8 MND-c.o.RAG1 mice). B) B-cell development subsets distribution in BM (left panel) and T-cell development populations distribution in the thymus (right panel) 24 weeks after SC transplantation. Graphs represent the means and standard deviation of 3 mice for control groups and 8 mice in the gene therapy group. C) Histologic analysis of the liver (scale bar = 100µm), kidney (scale bar=200µm), lungs (scale bar=100µm)* ***and BM*** *(scale=100µm) stained*

*with hematoxylin and eosin. Representative images from WT Control, KO Control and MND-c.o.RAG1 mice. D) Quantification of total IgE in serum by ELISA. Each dot represents a value obtained in one mouse (3 mice/control group, 8 MND-c.o.RAG1 mice). Mann-Whitney test (Two-tailed, *p<0,05; **p<0,01).*

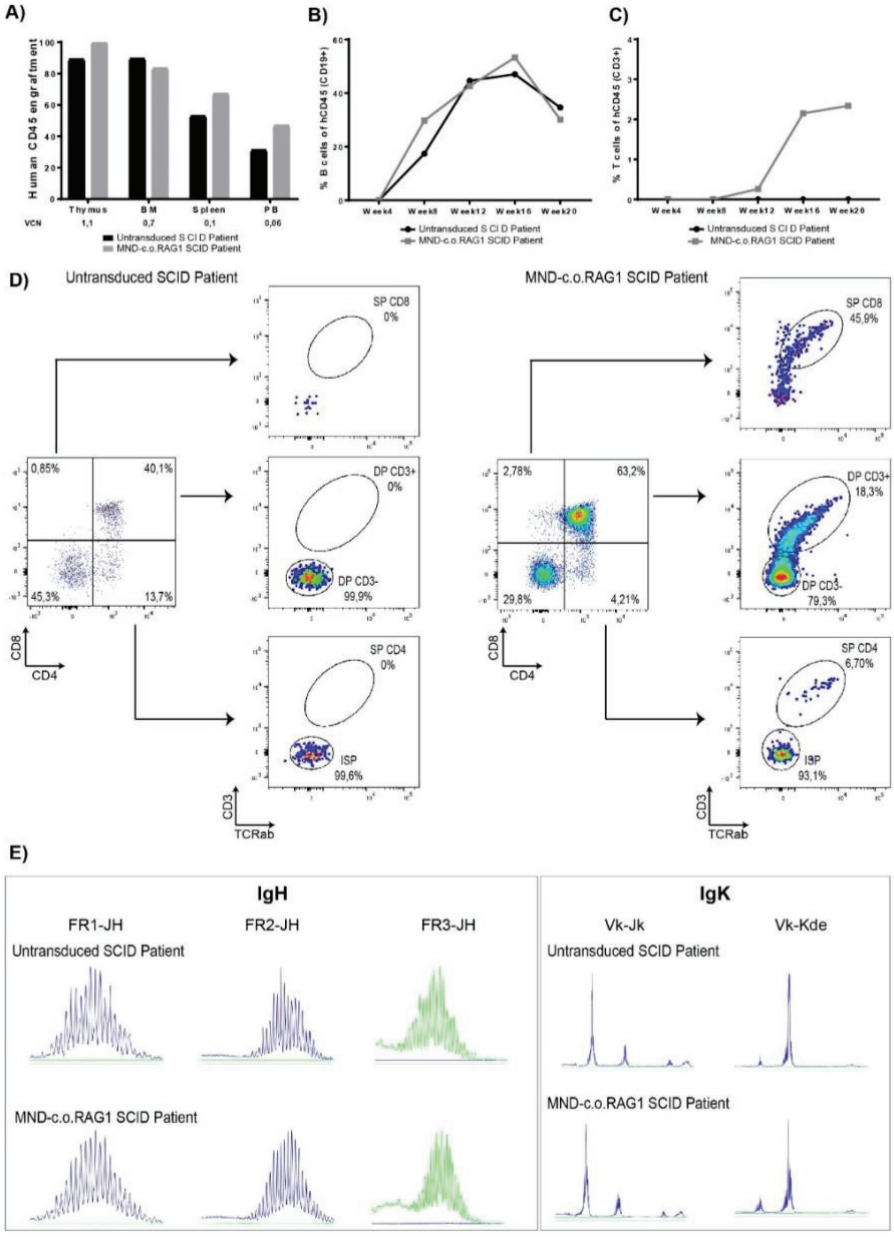


***Figure S3: Human immune reconstitution after CD34+ MND-c.o.RAG1 transplantation**. A) Percentage of human chimerism (hCD45+/(hCD45+mCD45+) in immune organs of NSG mice*

transplanted with CD34+ SCID patient cells and CD34+ SCID patient cells transduced with MND-c.o.RAG1, 24 weeks after transplantation (1 NSG mouse per condition). B) Human B-cell percentage (CD19+ cells per total hCD45+ cells) over time in peripheral blood during transplantation. C)Human T-cell development (CD3+ cells per total hCD45+ cells) over time in PB during transplantation. D) Flow cytometry analysis of thymocytes 24 weeks after transplantation showing T-cell development through the different stages. E) Human IgH and IgK repertoire analysis of isolated DNA from NSG BM (SCID patient and SCID MND-c.o.RAG1) using IgH + IgK B-Cell Clonality Assay. (x-axis indicates fragment sizes; y-axis shows the fluorescence intensity of the runoff products).

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Chapter 5

Developing autologous lentiviral-based gene therapy to correct RAG2 deficiency: challenges and solutions

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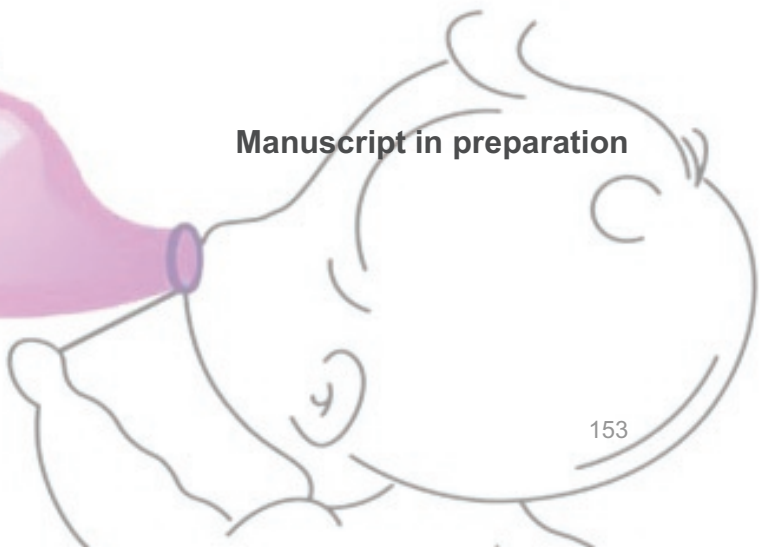

ABSTRACT

Recombinase-activating gene 2 (RAG2) deficient SCID patients lack B and T lymphocytes due to the inability to rearrange immunoglobulin and T-cell receptor genes. RAG2, together with RAG1, is required as a dimer to initiate lymphoid-specific V(D)J recombination. Curative treatment for this form of SCID is limited and confined to allogenic hematopoietic stem-cell transplantation; however, gene therapy might be a valid alternative especially for patients lacking a suitable bone marrow donor. We focused on clinically relevant lentivirus SIN vectors (LV) containing different internal promoters (EFS, MND, PGK, UCOE) driving a codon optimized (c.o.) version of the RAG2 (c.o.RAG2) gene to ensure optimal expression at low vector copy numbers (VCN). Lineage-depleted mouse bone marrow cells were transduced with the novel lentiviral SIN vectors and transplanted into Rag2-/- mice, which were used as a preclinical model to assess efficacy and safety. Immune reconstitution and functional restoration of RAG2 deficiency was obtained with our MND-, PGK- and UCOE c.o.RAG2 LV. However, the UCOE-c.o.RAG2 LV production appeared to be troublesome, and the MND-c.o.RAG2 LV raised genotoxicity and integration concerns in addition to high RAG2 expression that seems to hamper B-cell development. We conclude that functional restoration of RAG2 deficiency at low VCN can be achieved with clinically acceptable vectors using the PGK as suitable promoter, which allows for sufficient but not detrimentally high RAG2 expression.

INTRODUCTION

Severe combined immunodeficiency (SCID) is a rare life-threatening inhered disorder of the immune system, characterized by the absence of functional T cells due to their developmental arrest in the thymus, often accompanied by deficiency in B cells and/or NK cells [1-3]. The lack of a functional adaptive immune response affects infants' failure to thrive, associated with severe and recurrent opportunistic infections and other metabolic abnormalities that are invariably fatal within the first year of life unless effective treatment is provided. SCIDs represent the most severe forms of primary immunodeficiencies and represents a real pediatric emergency. The phenotype of SCID depends on the underlying genetic defect with over 20 different genes being shown to be causative of SCID [4, 5] and persisting cases (around 20%) of affected infants without a known genetic cause [3, 6]. One of the main pathways that can be affected by the molecular defect is the V(D)J recombination. V(D)J recombination is a complex process that occurs in early B- and T-cell development leading to a functional Immunoglobulins (Igs) and T-cell receptors (TCRs) respectively. The deficiency in genes involved in the recombination process like *RAG1 and RAG2* [7] lead to T-B-NK+ form of SCID with an autosomal recessive trait characterized by the absence of functional TCR and Igs. RAG1 and RAG2 proteins form a heterodimer complex at the beginning of the V(D)J recombination process mediating the binding and cleavage of the DNA. Deficiency of one of these proteins is associated with a limited production of T and B cells, associated with the absence of V(D)J recombination causing cell apoptosis. To date, the principal effective treatment is limited and confined to allogeneic hematopoietic stem cell (HSC) transplantation for most of the deficiencies [8, 9], with emerging autologous HSC gene therapy options [10-12] including for RAG1 SCID [13].

Attempts to develop autologous HSC gene therapy to correct RAG2 deficiency have been made by several groups, aiming to relieve the early block in T- and B-cell differentiation by conferring a selective advantage of the gene corrected cells over the uncorrected cells. In these studies the Rag2 knock-out (Rag2-/-) murine model with severe and early block of both B- (pro B stage) and T- (double negative DN stage) cell development, identical to the human phenotype, was used to verify the gene therapy approaches. Murine HSCs were transduced by gammaretroviral transfer-based vector bearing a native human RAG2 transgene. A sustained correction of the immune deficiency without side effects was observed, demonstrating the efficiency of ex vivo RAG2 gene transfer in HSCs [14]. Unfortunately, clonal T-cell proliferation and adverse effects due to transgene insertions near to proto-oncogenes was detected in diverse clinical trials using gammaretroviral vectors [15-18]. RAG2 gene transfer was therefore updated to safer lentiviral vectors (LV) and improved by codon optimization. Codon optimization of RAG2 (c.o.RAG2) lentiviral vector resulted in improved viral production and robust immune reconstitution, driving significant increases in viral titers as well as in B- and T-cell reconstitution. Although immune function was corrected, no safety assessment was reported [19].

Therefore, we set out to develop a new set of SIN lentiviral vectors to express *c.o.RAG2* with different types of promoters (EFS [20], MND [21], PGK [22] and UCOE [23]) and to test their ability and safety to correct Rag2 deficiency in the preclinical RAG2-/- mouse model at low vector copy numbers. This set-up allowed us to directly address the effects of differences

in *RAG2* expression in a gene therapy setting. In addition, it has enabled us to select a new SIN LV vector that functionally corrects the Rag2 deficiency *in vivo* in mice with no adverse effects. The PGK-c.o.RAG2 is our vector of choice capable of providing an appropriate RAG2 expression and therefore a valid gene therapy option for the treatment of RAG2 deficiency and to test their ability and safety to correct Rag2 deficiency in the preclinical RAG2-/- mouse model at low vector copy numbers. This set-up allowed us to directly address the effects of differences in *RAG2* expression in a gene therapy setting. In addition, it has enabled us to select a new SIN LV vector that functionally corrects the Rag2 deficiency *in vivo* in mice with no adverse effects. The PGK-c.o.RAG2 is our vector of choice capable of providing an appropriate RAG2 expression and therefore constituting a valid gene therapy option for the treatment of RAG2 deficiency.

MATERIALS AND METHODS

Mice

Balb/c Rag2/Il2rg double-knockout mice were a kind gift from Dr. E.J.Rombouts from the Department of Hematology at Erasmus MC (University Medical Center Rotterdam, The Netherlands) or were purchased from Taconic Biosciences, Inc (C.129S6(B6)-*Rag2*^{tm1Fwa} N12). Balb/c wild-type mice were purchased from Charles River (France). Mice were bred and maintained in the animal facility of Leiden University Medical Center (LUMC). All animal experiments were approved by the Dutch Central Commission for Animal experimentation (Centrale Commissie Dierproeven, CCD).

Lentiviral vectors and lentiviral production

Optimized RAG2 sequence was synthesized by GenScript USA. Codon optimized RAG2 (c.o.RAG2) was cloned into self-inactivating lentiviral pCCL plasmid harbouring different promoters resulting in pCCL-EFS-c.o.RAG2 (hereafter: EFS-c.o.RAG2; elongation factor 1α short promoter) [20], pCCL-MND-c.o.RAG2 (hereafter: MND-c.o.RAG2; myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer binding site substituted promoter) [21], pCCL-PGK-c.o.RAG2 (hereafter: PGK-c.o.RAG2; human phosphoglycerate kinase-1 promoter) [22] and pCCL-UCOE-c.o.RAG2 (hereafter: UCOE-c.o.RAG2; the modified chromatin-remodeling element, devoid of unwanted splicing activity and minimized read-through activity) [23]. DNA sequencing of the transgene was performed to validate the gene transfer construct. Helper plasmids pMDLg/pRRE, pRSV-Rev and pMD2.VSVG for lentiviral production were kindly provided by L.Naldini (San Raffaele Telethon Institute for Gene Therapy, Milan, Italy) [22]. Large-scale helper-plasmids were obtained from Plasmid Factory (Bielefeld, Germany).

293T cells were transiently transfected with the transfer and helper plasmids using X-tremeGene HP DNA transfection reagent (Sigma-Aldrich). Lentiviruses were harvested 24h, 30h and 48h after transfection, filtered through 0.45µm pore filters (Whatmann) and stored at -80°C. Pooled lentiviral supernatant was concentrated by ultracentrifugation (Beckman Optima^{TM} LE-80K, rotor SW32Ti) for 16 hours at 10.000 rpm and 4°C under vacuum conditions. Pellets were resuspended in StemSpan Serum-Free expansion medium (SFEM; Stemcell Technologies Inc) and aliquoted to avoid multiple freeze/thaw

cycles. Since no suitable anti-c.o.RAG2 antibodies are available, we determined the viral titer using qPCR as described later on.

Murine HSPC isolation and transduction

Murine bone marrow (BM) cells were obtained from femurs and tibias of Balb/c wild-type and Balb/c Rag2-/- mice. The obtained bones were crushed, cells were passed through a 0,7 µm cell strainer (Falcon), washed and viable frozen. Depletion of lineage-positive cells was performed using the Direct Lineage Cell Depletion kit from Miltenyi Biotec, to isolate hematopoietic stem cell from frozen murine bone marrow. In short, cells were magnetically labelled with the Direct Lineage Cell Depletion Cocktail and incubated for 10min at 4ºC. Lineage negative cells were subsequently enriched using the appropriate magnetic columns and the MACS separator (Miltenyi Biotec). Directly enriched HSPC were stimulated overnight in StemSpan™-SFEM (StemCell Technologies Inc) supplemented with Penicilin/Streptamycin (5000 units/5000ug/mL; Gibco), 50ng/mL recombinant mouse FMS-related tyrosine 3 ligand (rmFlt3L; R&D systems), 100ng/mL recombinant mouse Stem-Cell Factor (rmSCF; R&D systems) and 10ng/mL recombinant mouse thrombopoietin (rmTPO; R&D Systems) at 37˚C with 5%CO2. Depletion efficiency and purity of lineage negative population was analysed by flow cytometry with FACSCanto (BD). $Rag2^{-/-}$ cells were subsequently transduced with the different lentiviruses using 4 ug/ml proteamine sulphate (Sigma-Aldrich) and by way of spin-occulation at 800xg and 32°C for 1 hour. Cells were cultured at 37°C, 5% CO_2 for 24h in medium supplemented with cytokines.

Transplantation Rag2-/- mice

Control mock-transduced cells (Balb/c wild-type cells referred as WT control and $Rag2^{-/-}$ cells referred as KO control) and transduced $Rag2^{-/-}$ murine cells (equal number of cells per group, up to $2{,}5*10^5$ cells/mouse depending on the experiment) were mixed with supportive $Rag2^{-/-}$ spleen cells ($3*10^5$ cells/mouse) in Iscove's Modified Dulbecco's Medium (IMDM) without phenol red (Gibco) and transplanted by tail vein injection into pre-conditioned $Rag2^{-/-}$ recipient mice. Recipient mice (8-12 week old mice) were conditioned with a total body single dose irradiation 24h prior the transplantation using orthovoltage X-rays (8.08Gy) or with two consecutive doses of 25 mg/kg Busulfan (Sigma-Aldrich) (48h and 24h prior transplantation). Mice used for transplantation were kept in specified pathogen-free housing. The first four weeks after transplantation mice were fed with additional DietGel recovery food (Clear H2O) and antibiotic water containing 0.07 mg/mL Polymixin B (Bupha Uitgeest), 0.0875 mg/mL Ciprofloxacin (Bayer b.v.) and 0.1 mg/mL Amfotericine B (Bristol-Myers Squibb) and their welfare was monitored daily. Peripheral blood (PB) from the mice was drawn by tail vein puncture every 4 weeks until the end of the experiment. Peripheral Blood, thymus, spleen and bone marrow were obtained from CO2 euthanized mice.

TNP-KLH mice immunization

Mice were immunized with synthetic TNP-KLH antigen 4 weeks before the end of the experiment. 100 µg TNP-KLH (Biosearch Technologies Inc.) in 50% Imject Alum (Thermo Scientific) was injected intraperitoneal (i.p.). 3 weeks later, mice were boosted i.p. with 100

µg TNP-KLH in PBS. Serum was collected before immunization and 1 week after the boost injection.

Flow cytometry

Single cell suspensions from thymus and spleen were prepared by squeezing the organs through a 70µM cell strainer (BD Falcon) and single cell suspension from bone marrow was made as described above. Erythrocytes in peripheral blood and spleen samples were lysed using NH_4Cl (8,4 g/L)/$KHCO_3$ (1 g/L) solution (Pharmacy Leiden Academic Hospital). Single cell suspensions were counted on the NucleoCounter 200 or 3000 (Chemometec) and stained with the following anti mouse antibodies from BD Bioscience, eBioscience or BioLegend: CD3e Biotin (145-2C11; AB_394593), CD4 PE-Cy7 (RM4-5; AB_469578), CD8a PerCP (53-6.7; AB_893423), CD11b Biotin (M1/70; AB_312787), CD19 APC (1D3; AB_398483), CD23 Pe-Cy7 (B3B4; AB_469603), CD43 Biotin or PE (S7; AB_2255226 or AB_394609), CD44 APC-Cy7 (IM7; AB_1727481), CD45 FITC (30-F11; AB_394609), CD45R/B220 PerCP or Pe-Cy7 (RA3-6B2; AB_893355 or AB_2341160), CD62L APC (MEL-14; AB_313098), CD93 APC (AA4.1; AB_469466), CD138 PE (281-2; AB_395000), FoxP3 ef450 (FJK-16s; AB_1518812) IgD ef450 (11-26c; AB_1272239), IgM FITC (II/41; AB_394857), TCRb FITC (H57-597; AB_394683), TCRgd PE (GL3; AB_394689) and Sterptavidin APC-Cy7 and ef450 (AB_10054651 and AB_10359739). Briefly, cells were incubated for 30 min at 4°C in the dark with the antibody-mix solution including directly conjugated antibodies at the optimal working solution in FACS buffer (PBS pH 7.4, 0.1% azide, 0.2% BSA). After washing with FACS buffer, a second 30 min incubation step at 4°C was performed with the streptavidin-conjugated antibody solution. When necessary, 7AAD (BD Biosciences) was used as viability dye. Cells were measured on FACS-CantoII and LSR Fortessa X-20 (BD Biosciences) and the data was analysed using FlowJO software (Tree Star).

Serum immunoglobulin quantification

Murine IgG, IgM, IgE, TNP-specific IgG were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). NUNC Maxisop plates (Thermo Scientific) were coated with unlabeled anti-mouse IgG, IgM (11E10), IgE antibodies (SouthernBiotech). For detection of TNP-specific IgG, plates were coated with synthetic TNP-KLH (Biosearch Technologies Inc.). Blocking was done with 1% BSA/PBS (mouse) for 1h at room temperature (RT) and subsequently serial dilutions of the obtained sera were incubated for 3h at RT. After washing, plates were incubated with biotin-conjugated anti-mouse IgG, IgM, IgE (SouthernBiotec) for 30 min at RT. For detection, plates were incubated for 30 min at RT with streptavidin horseradish peroxidase (Jackson Immuno Research laboratories) and subsequently azino-bis-ethylbenzthiazoline sulfonic acid (ABTS, Sigma-Aldrich) was used as a substrate. Data was acquired at a wavelength of 415 nm using Bio-Rad iMark microplate reader and MPM 6 software (Bio-Rad). Antibody concentration was calculated by using serial dilutions of purified IgG, IgM, IgE proteins (SouthernBiotech) as standards.

Determination vector copy number (VCN) and *c.o.RAG2* expression by RT-qPCR

qPCR was used for the quantitative analysis of genomic lentiviral RNA, proviral DNA copies and transgene mRNA expression using WPRE, c.o.RAG2, ABL1 and PTBP2 as

targets (**Table S1**). Total RNA from single cell suspensions was isolated using RNeasy Mini kit (Qiagen) and reverse transcribed into cDNA using Superscript III kit (Invitrogen). Genomic DNA was extracted from single cell suspensions using the GeneElute Mammalian Genomic DNA kit (Sigma-Aldrich). VCN was determined on DNA samples by the detection of WPRE and PTBP2. The levels of transgene expression were determined on cDNA samples, by normalizing *c.o.RAG2* to the expression of the *ABL1* gene. qPCR was performed using TaqMan Universal Master Mix II (Thermofisher) in combination with specific probes for indicated genes from Universal Probe Library (Roche). Primers and probes used are listed in **Table S1**. PCR reactions were performed on the StepOnePlus Real-Time PCR system (Thermofisher). All samples were run in triplicate.

Repertoire analysis

Total RNA was purified from murine spleen cells and reverse transcribed into cDNA as described previously. GeneScan analysis procedure of the murine T-cell repertoire was adapted from [24]. cDNA was amplified using a FAM-labeled C gene segment-specific primer along with 24 TCR Vβ-specific primers (See **Table S2**). GeneScan™ 500 ROX™ (ThermoFisher) was used as internal size standard. Labelled PCR products were run on the ABI Prism® Genetic Analyzer (Applied Biosystems) for fragment analysis. Raw spectratype data was analyzed, visualized and scored by ImSpectR, a novel spectratype analysis algorithm for estimating immunodiversity [25]. ImSpectR identifies and scores individual spectratype peak patterns for overall (Gaussian) peak distribution; shape of individual peaks, while correcting for out-of-frame TCR transcripts. Scores range from 0 when no peaks detected, to 100 for a diverse TCR repertoire.

***In Vitro* Immortalization assay (IVIM)**

Genotoxic potential of the viral vectors (MND-c.o.RAG2 and PGK-c.o.RAG2) was quantified as previously described by Baum et al. [26, 27].

Insertion site analysis S-EPTS/LM-PCR (shearing extension primer tag selection ligation-mediated PCR)

Lentiviral insertion site was analysed by non-restrictive Linear Amplification Mediated PCR (nrLAM-PCR) on murine BM DNA samples from MND-c.o.RAG2 and PGK-c.o.RAG2 gene therapy mice as described by Schmidt M. et al [28].

S-EPTS/LM-PCR was used to analyse insertion sites and cancer-related gene insertions on murine bone marrow, spleen and thymus DNA samples from PGK-c.o.RAG2 gene therapy mice 24 weeks after transplantation [29-32].

RESULTS

Clinically relevant LV suitable to overcome immune RAG2 deficiency

Various clinically relevant self-inactivating (SIN) lentiviral (LV) pCCL plasmids harbouring a codon optimized version of RAG2 (c.o.RAG2) driven by four different promoters were tested in murine Rag2$^{-/-}$ model: EFS-c.o.RAG2 (Elongation factor 1α short promoter) [20], MND-c.o.RAG2 (myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer binding site substituted promoter) [21], PGK-c.o.RAG2 (human

phosphoglycerate kinase-1 promoter) [22] and UCOE-c.o.RAG2 (the modified chromatin-remodelling element, devoid of unwanted splicing activity and minimized read-through activity) [23] (**Fig 1A**).

Rag2-/- mice were transplanted with wild-type stem cells (WT control), mock transduced Rag2-deficient stem cells (KO Control) or gene therapy treated stem cells using the mentioned four different plasmids (EFS VCN=0.4, MND VCN=0.4, PGK VCN=0.3 and UCOE VCN=0.2). Unfortunately, mice transplanted with stem cells transduced with the EFS-c.o.RAG2 plasmid did not survive the length of the experiment (**Fig 1B**), and no immune reconstitution was observed compared to the mice transplanted with cells transduced with the other constructs (**Fig.S1A**). Twenty weeks after transplantation, mice were sacrificed and immune organs were analysed for immune cell reconstitution. B-cell development in the bone marrow of mice transplanted with the remaining constructs was rescued similarly to WT control mice, alleviating the block in the pre-B cell stage in the KO control group, and developing further into immature and mature B cells (**Fig. 1C** and **Fig S1B**). Mature B cells were detected in the periphery, blood and spleen, in mice transplanted with the gene therapy cells, albeit in more moderate amounts than WT group (**Fig 1D**). Similarly, T-cell development was recovered at different extend with the different transplanted gene therapy cells, developing beyond the initial KO block at the double negative (DN; CD4-CD8-) stage (**Fig 1E** and **Fig S1C**). Thymocytes in gene therapy-treated mice developed into the immature single positive (ISP; CD8+CD3-), double positive (DP; CD4+CD8+) and single positive (SP; CD3+CD4+ or CD3+CD8+) stages within the thymus. Mature CD4 and CD8 T cells successful migrate to the periphery, detecting equal or even higher numbers of T cells than WT control group (**Fig 1F**). Nicely, all immature and mature B-cell subsets (transitional B cells, marginal zone B cells, memory B cells) as well as CD4 and CD8 T-cell subsets (naïve, effector memory, central memory subsets) were detectable in comparable proportions to WT control mice with the three different constructs (**Fig S1D** and **Fig S1E**).

In addition, RAG2 functionality was restored, with successful V(D)J recombination in B cells after gene therapy. Both IgG and IgM were detected in the serum of all gene therapy mice, with significantly increased levels for PGK-c.o.RAG2 and UCOE-c.o.RAG2 compared to the KO serum (**Fig 1G**). Therefore, although gene therapy mice were lagging behind with regard to B-cell numbers, their functionality in the form of antibody production was restored to WT IgM levels. Genescan analysis of up to 24 different T cell receptor Vb genes also revealed the restoration of T-cell receptor rearrangement. The cumulative complexity score of the Vβ repertoire calculated by ImSpectR [25] showed an active V(D)J recombination machinery able to successfully rearrange TCR genes and provide a diverse TCR Vb repertoire after gene therapy (**Fig 1H**). Overall, gene therapy to treat RAG2 deficiency seems feasible with some of the tested clinically relevant SIN LV such pCCl c.o.RAG2 carrying the MND, PGK or UCOE promoters.

LV production and safety concerns for specific c.o.RAG2

The different lentiviruses were produced by transient transfection of the transfer vectors, GAG-pol, REV and VSV-G plasmids into 293T cells [22, 33]. Virus production and expression

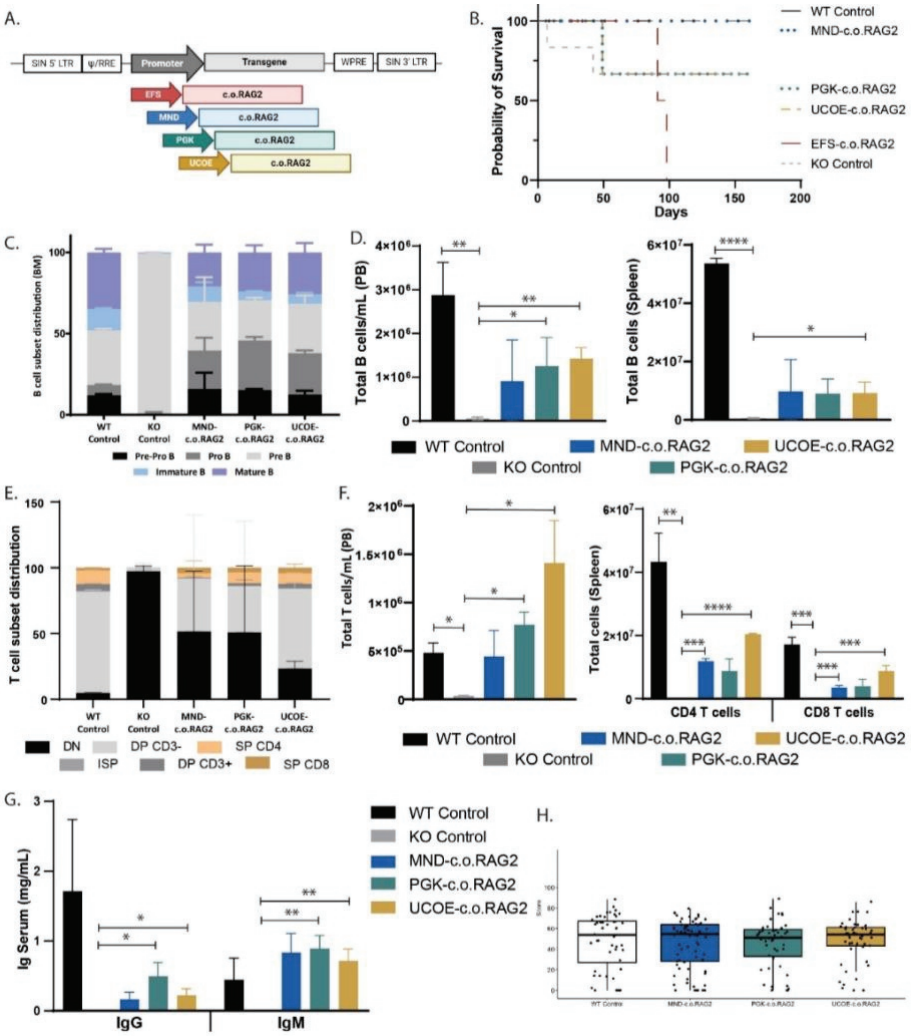

Figure 1: Relevant clinically applicable lentiviral vectors drive immune reconstitution in Rag2-/- mice. *A) Four different SIN LV plasmids in the CCL backbone carrying different promoters (EFS, MND, PGK and UCOE promoter) were tested driving the expression of a codon optimized version of RAG2. Rag2 deficient mice (experiment with total 3 mice/group) were transplanted with 100.000-200.000 stem cells: WT cells, mock Rag2 KO cells, EFS-c.o.RAG2 treated KO cells (VCN=0.4), MND-c.o.RAG2 treated KO cells (VCN=0,4), PGK-c.o.RAG2 treated KO cells (VCN=0,3) or UCOE-c.o.RAG2 treated KO cells (VCN=0,2). B) Survival analysis of the different groups of mice: WT control transplanted mice, KO control transplanted mice and EFS-c.o.RAG2, MND-co.o.RAG2 , PGK-c.o.RAG2, UCOE-c.o.RAG2 gene therapy mice. C) Proportion of the different B-cell developmental subsets in the BM. D) Total number of B cells (B220hi+) in the PB (left panel) and spleen (right panel) 20 weeks after SC transplantation. Graphs represent the means and standard deviation of a pilot experiment with 2-3 mice per group. (Multiple t-test, two-tailed, *p≤0,05, **p≤0,01). E) Proportion of the different T-cell developmental subsets in the thymus. F) Total number of T cells (CD3+TCRαβ+) in PB (left panel) and in the spleen (right panel) 20 weeks after transplantation. Graphs represent the means and standard deviation of a pilot experiment with 2-3 mice per group. (Multiple t-test, two-*

*tailed, *p≤0,05, **p≤0,01, ***p≤0,001) (DN=Double Negative, ISP=Immature Single Positive, DP=Double Positive, SP=Single Positive cells). G) Quantification of total IgG and IgM in mice serum by ELISA. (Multiple t-test *p<0,05, **p<0.01.) H) TCR Vβ repertoire analysis by GeneScan. A total of 24 Vβ families was analysed on spleen cells from 3 mice per group. Overall score of all the families was calculated for the different constructs using ImSpectR [25]. (Mann-Whitney test; p values represented on the plot; NS = not significant).*

efficiency were evaluated for the different lentiviral vectors produced at small and large scale. Only the vectors showing immune efficacy *in vivo* previously described (MND-c.o.RAG2, PGK-c.o.RAG2 and UCOE-c.o.RAG2) were produced at large scale. The number of viral particles and infectious genomes of the small and large concentrated batches was assessed by qPCR. Consistently, both the small and large batches of UCOE-c.o.RAG2 lentivirus had a significantly lower number of infectious genomes per mL compared to the other MND and PGK vectors (**Fig 2A**), highlighting a difficulty to scale up its production. Their promoter strength and their ability to correct the Rag2-/- murine model was determined using transduced lineage negative bone marrow cells from Rag2-/- mice. EFS and PGK-c.o.RAG2 were the vectors with lowest promoter strength while MND-c.o.RAG2 is by far the vector leading to higher c.o.RAG2 expression under relevant conditions for *in vivo* applications (**Fig 2B**). This high expression driven by the MND promoter was also detected in the BM of gene therapy transplanted mice; high c.o.RAG2 expression was obtained in BM of MND-c.o.RAG2 transplanted mice (transplanted cells VCN=0.4) but not with PGK-c.o.RAG2 (transplanted cells VCN=0.3) or UCOE-c.o.RAG2 (transplanted cells VCN=0.2), which reached expression levels within the native RAG2 expression in BM (native RAG2 expression relative to ABL1 calculated from Immgen.com; marked as a grey bar). Importantly, where high c.o.RAG2 was achieved, the number of mature B cell in the BM of these mice was reduced, indicating a potential detrimental effect of high RAG2 expression in B cell maturation (**Fig 2C, left panel**). In contrast, c.o.RAG2 expression in the thymus remained overall lower than in BM and within the range of native RAG2 in the specific organ, allowing for the development of a proper DP population (**Fig 2C, central pane**l). Notably, the promoter strength of the MND-c.o.RAG2 vector in the BM was significantly higher than in spleen or thymus, in accordance with the expression and the B cell development observed in these mice (**Fig 2C, right panel**). In contrast, the PGK and UCOE promoter showed comparable promoter strength across the immune organs analysed.

As safety is an important aspect for clinical use of gene therapy vectors, the remaining candidates for clinical application, MND-c.o.RAG2 and PGK- c.o.RAG2, lentivirus batches were tested in the *in vitro* immortalization (IVIM) assay, which is the currently accepted (FDA and EMA approved) standard assay for safety of viral vectors. VCN per cell higher than 2 were achieved in this assay with the test vectors (MND and PGK). In three independent IVIM assays, no cytotoxicity was observed of the PGK vector supernatants on lineage negative mouse bone marrow cells, compared to classical RSF91 gamma-retroviral vectors with known mutagenic potential. The frequency of insertional mutagenic events was comparable to mock transduced cells instead suggesting a relatively low risk to elicit insertional transformation in hematopoietic stem and progenitor cells. However,

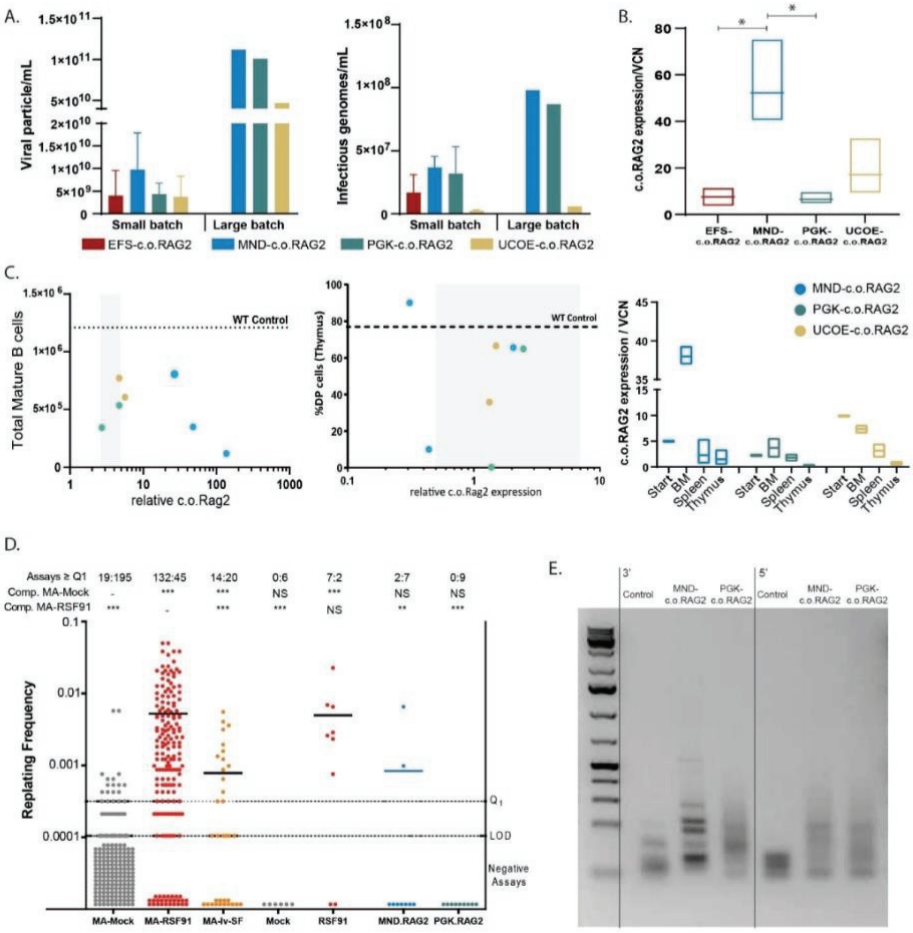

Figure 2: Lentiviral production and safety evaluation of different vectors. *A) Production of lentivirus batches in small (two independent batches) and large (one batch) scale with the different constructs. Determination of the number of viral particles (left panel) and of infectious genomes (right panel) per mL after concentration of the lentiviral batches. B) Determination of the promoter strength (c.o.RAG2 expression / VCN) of the different plasmids. Three independent lentivirus batches per plasmid were produced and analysed (One-way ANOVA test *p<0,05; **p<0.01). C) Total number of mature B cells in the BM (left panel) and total percentage of double positive cells in the thymus (middle panel) correlation with the expression of c.o.RAG2 in BM and thymus respectively. (• MND, • PGK, • UCOE promoters, grey window = native RAG2 expression calculated from Immgen). Data shown represents 1 pilot in vivo experiments. Each dot represents one mouse. Non-parametric Spearman r correlation, non-significant). Determination of the promoter strength (c.o.RAG2 expression / VCN) of the different plasmids at the start of the experiment and in the different immune organs of transplanted MND-c.o.RAG2, PGK-c.o.RAG2 and UCOE-c.o.RAG2 mice. D) Data shows results from 3 complete IVIM assays. Replating Frequencies (RF) of the control samples Mock or RSF91 and the test vectors MND-c.o.RAG2 and PGK-c.o.RAG2, in comparison to data of a meta-analysis for control samples (Mock-MA, RSF91-MA, lv-SF-MA [a lentiviral vector with SFFV promoter]). The data points below the limit of detection (LOD; plates with no wells above the MTT-threshold) were manually inserted into the graph (due to the logarithmic scale of the y-axis). Above the graph, the ratio of positive (left*

*number) and negative plates (right number) according to the MTT-assay are shown. Differences in the incidence of positive and negative assays relative to Mock-MA or RSF91-MA were analysed by Fisher's exact test with Benjamini-Hochberg correction (*P < 0.05; **P < 0.01; ***P < 0.001; NS = not significant). If above LOD, bars indicate mean RF. E) LV insertion site analysis by nrLAM-PCR of isolated DNA from BM obtained from Rag2-/- untransduced control mouse (Mock), MND-c.o.RAG2 and PGK-c.o.RAG2 gene therapy mice. Gels shows results of the linear amplification from the 3'LTR and 5'LTR respectively (L=1kb plus marker).*

mutagenic potential was detected for the MND-c.o.RAG2, with some clones getting immortalized (**Fig 2D**). In agreement, insertion site analysis by rLAM-PCR of BM samples from gene therapy transplanted mice showed an oligoclonal insertion pattern for MND-c.o.RAG2 vector with discrete insertion bands and therefore preferential insertion sites, while PGK-c.o.RAG2 presented a more polyclonal insertion pattern.

Knowing the importance of a precise RAG2 expression level for successful B cell development, our MND-c.o.RAG2 vector leads to RAG2 expression that is too high as well as insertion site safety concerns. Altogether, and taking the challenging production of UCOE-c.o.RAG2 vector into account, we choose our PGK-c.o.RAG2 as a potential LV candidate to correct RAG2 deficiency.

PGK-c.o.RAG2 LV as potential candidate to correct RAG2 deficiency

Our PGK-c.o.RAG2 vector showed overall satisfactory lentiviral production, correct RAG2 expression levels, safe insertion site profiles and successful immune reconstitution. Therefore, an extensive analysis of ten transplanted gene therapy mice with HSC transduced with the PGK-c.o.RAG2 cells (VCN=0,55) was performed. B-cell development was rescued in BM after gene therapy, overcoming the Rag2-/- block at the pro/pre-B stage and developing into high B220 expressing population with IgD and IgM expressing cells (**Fig 3A**). The number of B cells in the periphery was recovered, with the total B cells in spleen reaching similar numbers to WT transplanted mice, and significantly different to KO mice (**Fig 3B, left graph** and **Fig S2B**). All immature B cell subsets, transitional T1 ($IgM^{hi}CD23^{-}$), T2 (IgM^{hi} $CD23^{+}$) and T3 (IgM^{low} $CD23^{+}$) cells, and mature B cell subsets _ MB ($IgM^{dim/-}$ $CD23^{+}$) and MZ ($IgM^{+}CD23^{-}$), were present after gene therapy (**Fig 3B, center and right**) in normal proportions. Likewise, the thymocyte developmental block at immature stages was surpassed, although an incomplete block at the DN stage persisted after gene therapy (**Fig 3C** and **Fig S2B**). Mature CD4 and CD8 T cells developed and detected in spleen after gene therapy, as seen after transplantation of WT cells. Different subsets of T cells such as naïve, effector memory and central memory T cells were detected in the spleen, to the same extent as in the WT control group (**Fig 3D**). Importantly, both Tγδ cells (**Fig 3E**) and FoxP3 CD4 regulatory T cells (**Fig 3F**) also were restored after gene therapy with PGK-c.o.RAG2 reaching normal WT levels in spleen. T- and B-cell functionality were also restored by PGK-c.o.RAG2 gene therapy. V(D)J recombination in the thymus was achieved allowing a T-cell receptor rearrangement and diversity score as high as in the WT control (**Fig 4A**). Similarly, IgG and IgM were detected in the serum of the gene therapy mice as well as in WT transplanted mice, indicating successful V(D)J recombination in the BM (**Fig 4B**).

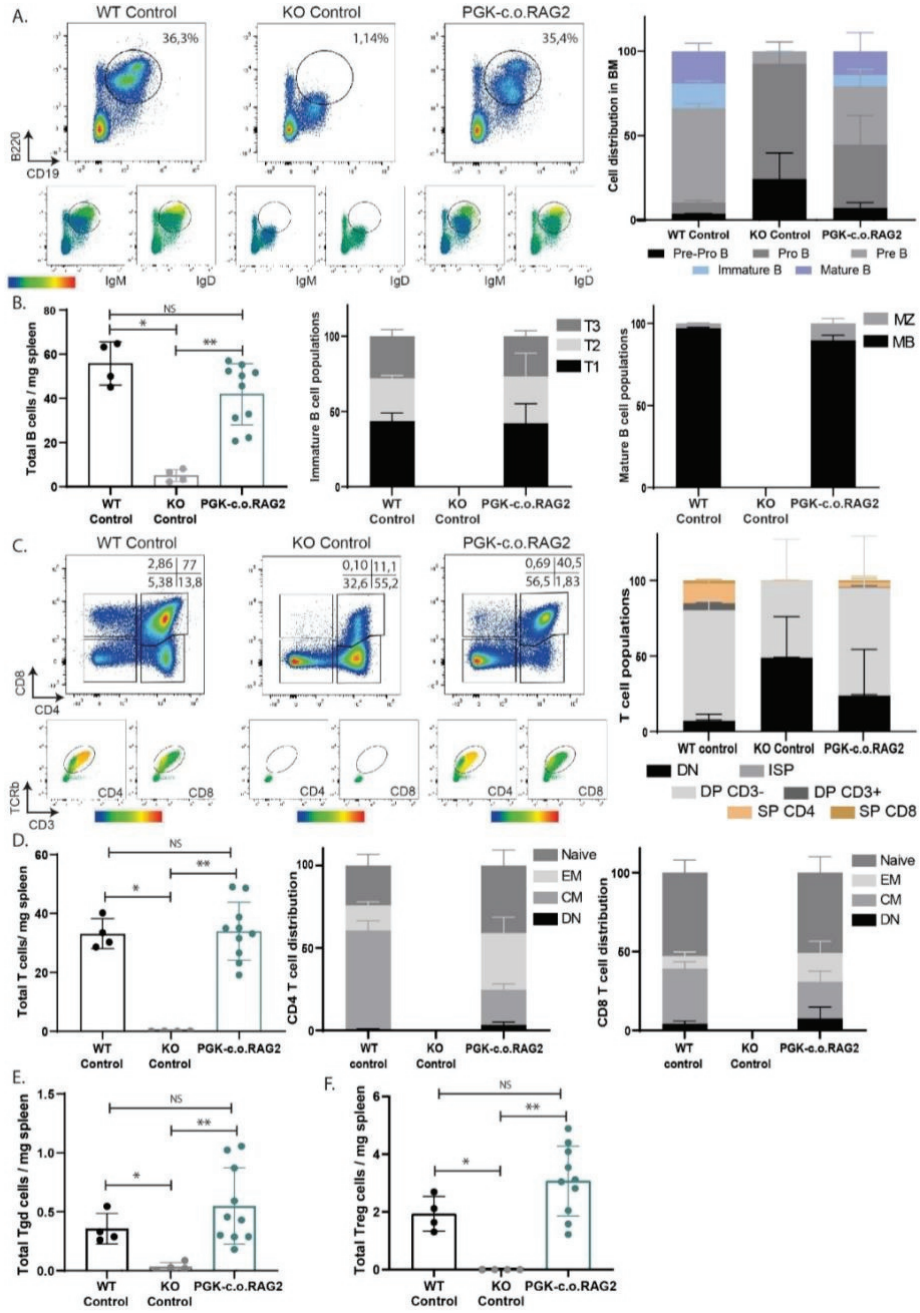

Figure 3: Immune cell reconstitution after gene therapy with the PGK-c.o.RAG2 vector. *Rag2 deficient mice were transplanted with 200.000 stem cells: WT cells (4 mice), mock KO cells (4 mice) and PGK-c.o.RAG2 treated cells (VCN=0,55; 10 mice). A. Representative FACS plots showing the restoration of expressing IgD and IgM B220^{hi+} B cells in the BM. Graph representing the proportion of the different B-cell developmental subsets in the BM. B. Total number of B cells (B220^{hi+}) in the spleen*

24 weeks *after SC transplantation (left panel). Graphs represent the means and standard deviation (Mann-Whitney test, two-tailed, *p≤0,05, **p≤0,01, NS=non-significant). Immature ($B220^+CD93^+$ cells; middle panel) and mature ($B220^+CD93^-$ cells; right panel) B-cell subsets distribution in spleen. (Two-way ANOVA test). C. Representative plots of T-cell development in the thymus (CD4 vs CD8 cells) and T-cell reconstitution in peripheral blood ($CD3^+TCRab^+$ cells) 24 weeks after transplantation. Graph represents the proportion of the different T-cell developmental subsets in the thymus. (DN=Double Negative, ISP=Immature Single Positive, DP=Double Positive, SP=Single Positive cells). D. Total number of T cells (CD3+TCRαβ+) in the spleen (left panel) 42 weeks after transplantation. Graphs represent the means and standard deviation (Mann-Whitney test, two-tailed, *p≤0,05, **p≤0,01, NS=non-significant). CD4 (middle panel)and CD8 (right panel) T cell subset distribution in the spleen (naïve, effector memory, central memory cells) (Two-way ANOVA test; *p≤0,05, **p≤0,01, ***p≤0,001). E. Total number of Tgd cells in spleen 24 weeks after transplantation (Mann-Whitney test, two-tailed, *p≤0,05, **p≤0,01, NS=non-significant). F. Total number of Tregs cells in spleen 24 weeks after transplantation (Mann-Whitney test, two-tailed, *p≤0,05, **p≤0,01, NS=non-significant).*

Importantly, IgE was only detected in low amounts (**Fig S2B**) showing proper functioning of the recombination machinery without causing Omen Syndrome-like features. We used TNP-KLH as T-cell specific antigen and measured the production of TNP specific IgG antibodies, thereby investigating whether the developed T and B cell could collaborate in an active immune response. The TNP-specific IgG level was detected in both mice treated with WT stem cells and gene therapy cells (**Fig 4C**), showing the potential of a robust immune response after PGK-c.o.RAG2 gene therapy. Considering the importance of c.o.RAG2 expression levels in the BM and the thymus after gene therapy for proper immune cell development, this was analyzed in all animals. Most PGK-c.o.RAG2 gene therapy mice clustered within the native RAG2 expression range in BM and thymus (**Fig 4D**). Furthermore, insertion site analysis by S-EPTS/LM-PCR on BM, spleen and thymus revealed that all gene therapy mice grouped together within the polyclonal and oligoclonal landscape showing a predominantly polyclonal integration profile composed of low frequency integration events, except for one mouse (**Fig 4E**). Overall, no particular targeting of cancer genes could be identified.

DISCUSSION

Successful immune B- and T-cell reconstitution and functional V(D)J recombination was achieved after transplantation of HSCs transduced with various clinically relevant lentiviral vectors conferring c.o.RAG2 expression in the Rag2-/- murine model. Although the number of immune cells remained lower than after transplantation with WT HSCs, the recombination machinery function was corrected to WT levels. Various potential applicable promoters led to successful correction of RAG2 deficiency, including the UCOE promoter in accordance with previously data described by Van Til et al. (2012) [19]. However, lentiviral production using the UCOE-c.o.RAG2 plasmid seems to be severely diminished compared to the other tested plasmids, yielding significantly lower infectious genomes in the produced virus supernatant. Although indeed the UCOE-c.o.RAG2 lentiviral vector is a valid option to correct the block in B- and T-cell development due to RAG2 deficiency,

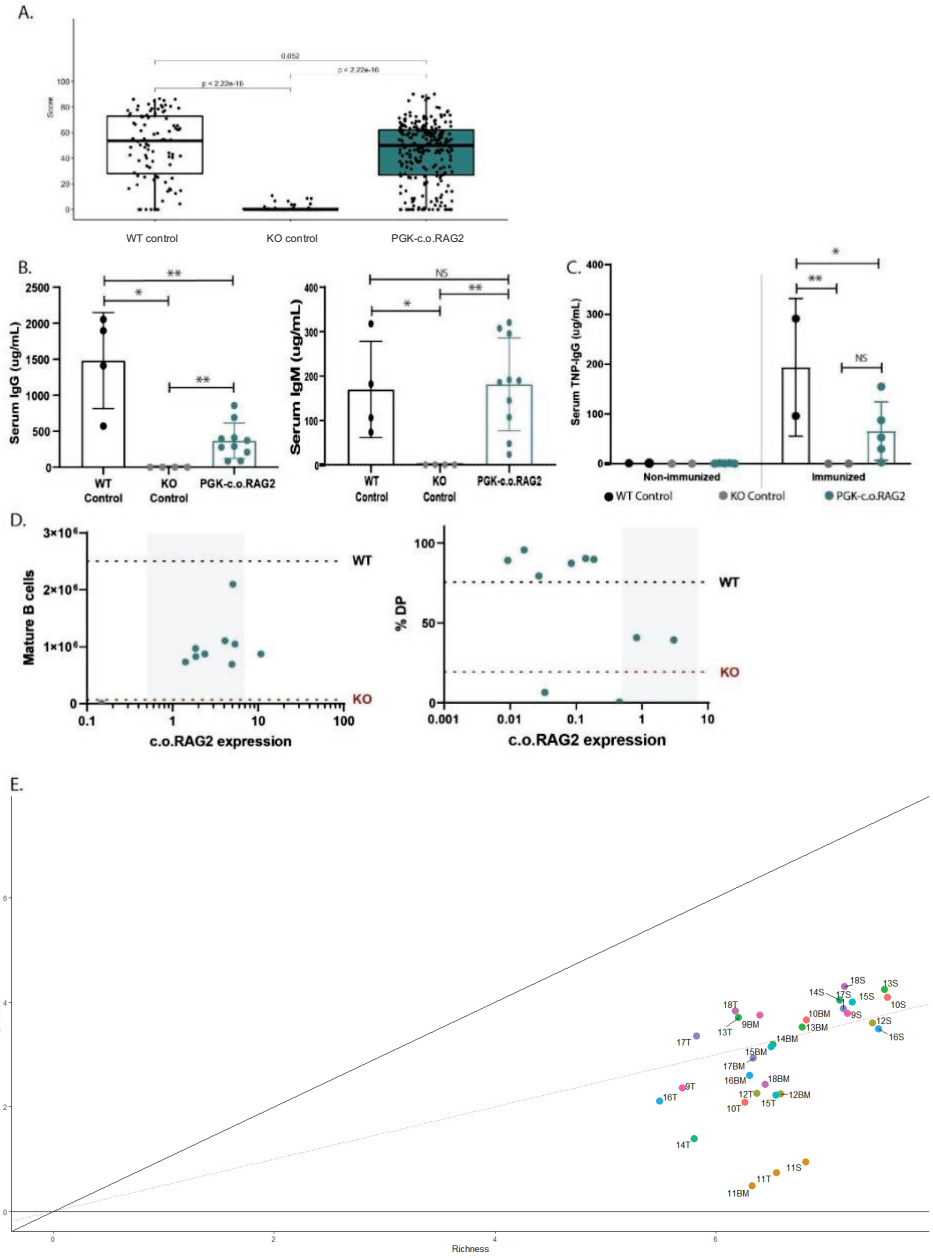

Figure 4: Functional restoration of the immune system after gene therapy with the PGK-c.o.RAG2 vector*. A) TCR Vβ repertoire analysis by GeneScan. A total of 24 Vβ families was analysed on spleen cells from all mice (4 WT control, 4 KO control and 10 gene therapy mice). Overall score of all the families was calculated for the different constructs using ImSpectR. (Mann-Whitney test; p values represented on the plot). B) Quantification of total IgG and IgM in serum by ELISA (4 mice/control group, 10 PGK-c.o.RAG2 mice) (Mann-Whitney test *p<0,05, **p≤0,01, NS=non-*

*significant) C) Quantification of TNP-specific IgG in serum of non-immunized and immunized mice. Each dot represents a value obtained in one mouse. (4 mice/control group, 10 PGK-c.o.RAG2 mice) (Two-way ANOVA test *p<0,05, **p≤0,01, NS=non-significant). D) Total number of mature B cells in the BM (left panel) and total percentage of double positive cells in the thymus (right panel) correlation with the expression of c.o.RAG2 in BM and thymus respectively. Each dot represents one mouse. (Non-parametric Spearman r correlation, non-significant). E) Lentiviral insertion site was analysed by S-EPTS/LM-PCR on murine bone marrow, spleen and thymus DNA samples from 10 PGK-c.o.RAG2 gene therapy mice 24 weeks after transplantation. The samples are placed within the clonal plane regarding richness and evenness of the diversity in the sample. Samples below the red dotted line are considered to be in the mono/oligoclonal area and samples above the red dotted line are in the polyclonal area.*

other lentiviral vectors share the same potential and importantly, capable of more efficient viral production which is beneficial for prospective clinical implementation.

A range of c.o.RAG2 expression was detected *in vitro* and *in vivo*, depending on the promoter used, with MND carrying the highest promoter strength while other promoter strength remained modest. Interestingly, the highest strength with the MND-c.o.RAG2 vector was specially detected in BM where high c.o.RAG2 expression is driven selectively by this promoter and led to a detrimental effect on mature B-cell numbers unlike previous observations described for the counterpart RAG1 [13, 34]. Indeed, higher c.o.RAG1 expression in both BM and thymus led to higher and better immune B and T cells reconstitution respectively, only consistently achieved by the strong MND promoter. While this observation was also true for RAG2 gene therapy reconstituted thymi with an improved active DP population at higher c.o.RAG2 expression levels, the overall c.o.RAG2 expression in immune organs was lower than the c.o.RAG1 expression needed for successful immune reconstitution by HSC-based gene therapy in murine models. The different c.o.RAG1 and c.o.RAG2 expression level requirement in immune organs is in accordance with the measured levels of native RAG1 and RAG2 expressions in the mentioned organs, with a higher requirement of RAG1 than RAG2 through the different B and T developmental stages [35].

The undesired effect observed within B cells due to high c.o.RAG2 expression highlighted the importance of the need of a tight RAG2 regulation, especially within the BM where the window of native RAG2 expression is narrow. The exact mechanism underlying this feature need to be further investigated in this *in vivo* model. In accordance with the research from Zheng X. & Schwarz K. (2006) [36] *in vitro*, an excess of RAG2 may inhibit the V(D)J recombination efficiency due to the dysregulation of the phosphorylation and degradation process inherent to the RAG2 protein, which is essential to regulate RAG1/2 activity within the G1 phase. Dysregulation of this process may lead to V(D)J recombination activity within the other cell cycle phases (S/G2/M) where DNA double-strand breaks created by the RAG1/2 complex may be detrimental for cell survival, explaining the lower number of total mature B cells in the BM of mice expressing high c.o.RAG2 in that organ. Further analysis of the cell survival and V(D)J recombination efficiency with our clinically relevant vectors *in vitro* and *in vivo* might help elucidate the importance of tight RAG2 expression.

Furthermore, the MND-c.o.RAG2 vector raises mutagenic and distinct oligoclonal insertion safety concerns resulting in a high toxicity risk. Further insertion site retrieval of the vector *in vivo* and study of the preferential landing sites will reveal if there might be preferential insertion sited located in the vicinity of the transcriptional start site of cancer related genes. Of note, the MND-c.o.RAG2 vector shows clonal expansion capabilities, while the MND vector driving c.o.RAG1 performed as a safer vector [13]. Therefore, although retrieved data from the IVIM assay was thought to be vector backbone and promoter driven, IVIM results can additionally be a readout of transgene genotoxicity potential.

The clinically relevant PGK-c.o.RAG2, with modest c.o.RAG2 expression within the native RAG2 range in immune organs, emerges as a potential vector for clinical implementation of HSC-based gene therapy to correct RAG2 deficiency. Successful immune reconstitution, with the presence of all different B and T cell subsets, was achieved at acceptable vector copies per cell (VCN=0,55). Functional restoration of the TCR and Ig rearrangements as well as functional T-B cell cooperation was obtained after transplantation, supporting a strong immune response against antigens (TNP-KLH). Phenotypic and functional correction might further improve in the clinical setting, as RAG2 interaction with human RAG1 instead of the murine version might be stronger. In addition, this vector exhibits a safe genotoxicity profile and an oligoclonal/polyclonal skewed safe insertion landscape, without particular targeting of cancer related genes. Gene therapy to correct RAG2 deficiency by gene addition with our PGK-c.o.RAG2 provides a suitable approach; however, clinical implementation can remain challenging due to the tight regulation of RAG2 expression. A more suitable approach to treat RAG2 deficiency, would be gene editing in order to achieve native regulation of RAG2 expression in immune organs.

SUPPLEMENTARY MATERIAL

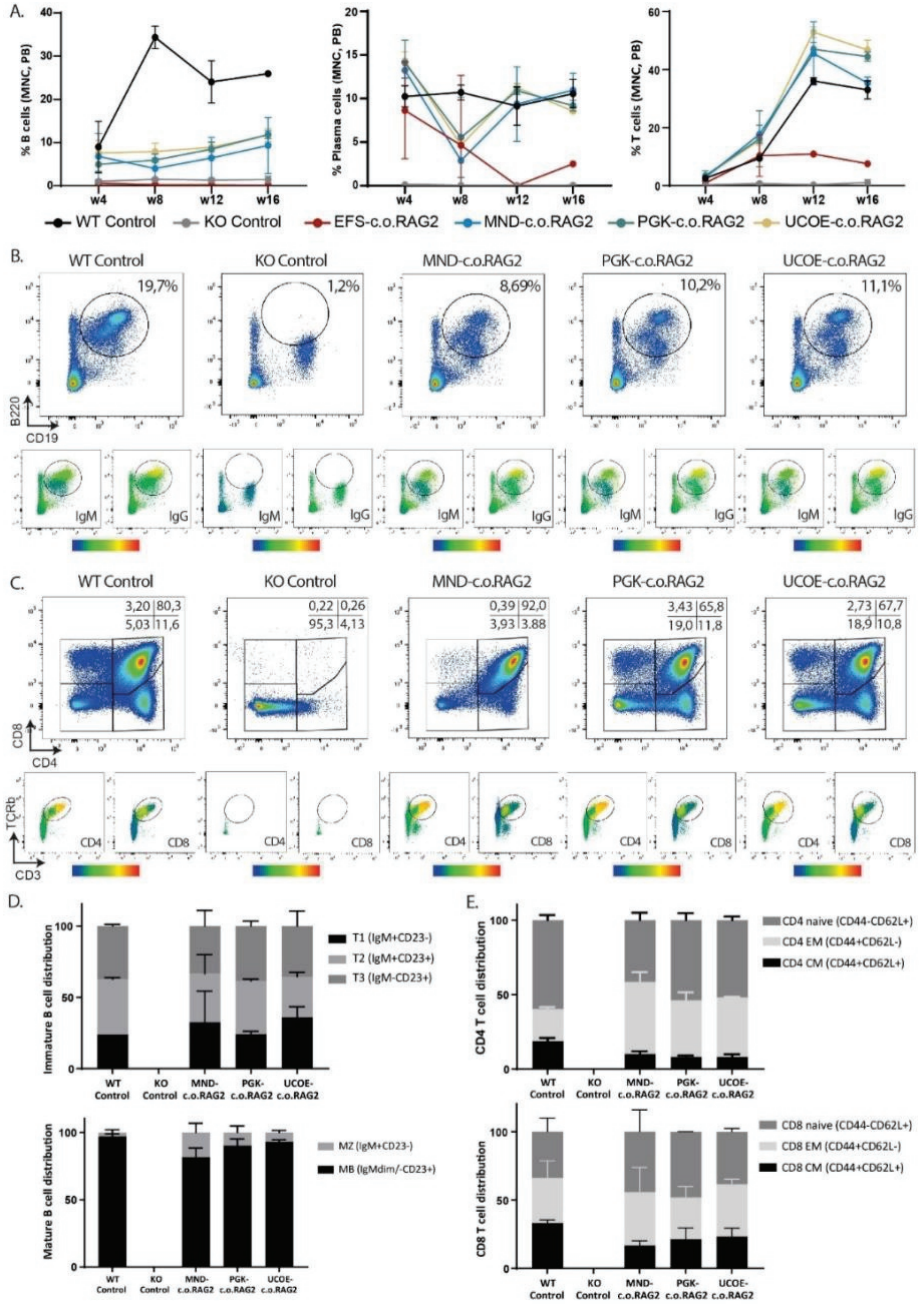

Figure S1: *A) Percentage of B cells (CD11b/CD43-B220+ cells; left panel), plasma cells (B220+CD138+; middle panel) and T cells (CD3+TCRαβ+ cells; right panel) over time in PB after stem cell transplantation with the different constructs. B) Representative FACS plots showing the*

restoration of expressing IgD and IgM B220hi+ B cells in the BM after gene therapy with 3 different constructs. C) Representative plots of T-cell development in the thymus (CD4 vs CD8 cells) and T-cell reconstitution in the blood (CD3+TCRab+ cells) 24 weeks after transplantation with different gene therapy constructs. D) Immature (B220+CD93+ cells; upper panel) and mature (B220+CD93- cells; lower panel) B-cell subsets distribution in spleen. E) CD4 (upper panel) and CD8 (lower panel) T cell subset distribution in the spleen (naïve, effector memory, central memory cells)

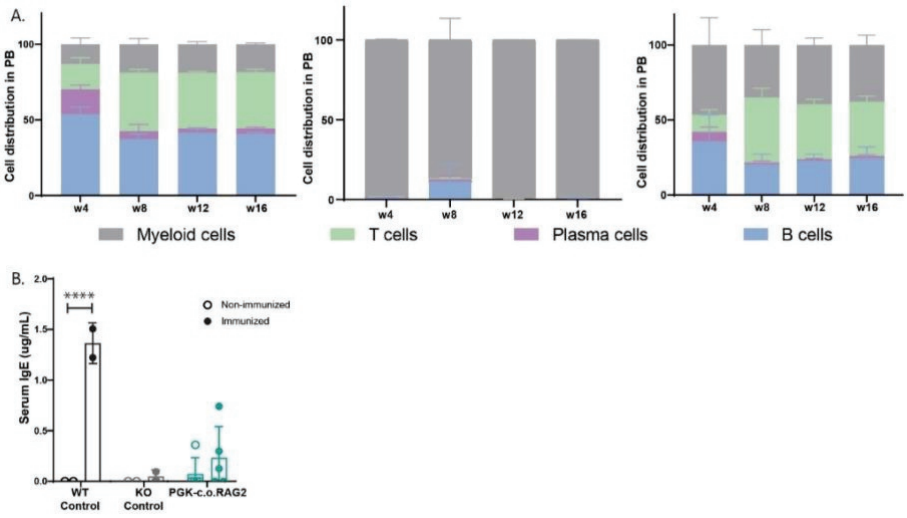

Figure S2: *A) Distribution of B cells (CD11b/CD43-B220+ cells), plasma cells (B220+CD138), myeloid cells (CD11b+CD43+) and T cells (CD3+TCRαβ+ cells) over time in PB after stem cell transplantation in WT control, KO control and PGK=c.o.RAG2 transplanted mice. B) Quantification of total IgE in serum by ELISA. Each dot represents a value obtained in one mouse, immunized and non-immunized mice. Two-way Annova test (two tailed, *p<0,05; ****p<0,0001)*

Table S1: Determination VCN and c.o.RAG2 expression

Description	***Orientation***	***DNA sequence 5'-3'***
ABL1	*FW*	*5'-TGGAGATAACACTCTAAGCATAACTAAAGGT-3'*
	RV	*5'-GATGTAGTTGCTTGGGACCCA-3'*
	Probe	*5'FAM-CCATTTTTGGTTTGGGCTTCACACCATT- TAMRA 3'*
c.o.Rag2	*FW*	*5'-TCTGAAACCGGGTATTGGAT- 3'*
	RV	*5'-GGCACCCATGTATTAATGTCC-3'*
	Probe	*Probe: 56 probe library Roche, FAM NFQ*
PTBP2	*FW*	*5'-TCTCCATTCCCTATGTTCATGC-3'*

	RV	*5'-GTTCCCGCAGAATGGTGAGGTG-3'*
	Probe	*[JOE]-ATGTTCCTCGGACCAACTTG-[BHQ1]*
WPRE	*FW*	*5'- GAGGAGTTGTGGCCCGTTGT-3'*
	RV	*5'-TGACAGGTGGTGGCAATGCC-3'*
	Probe	*[6FAM]-CTGTGTTTGCTGACGCAAC-[BHQ1]*

Table S2: Repertoire analysis (murine) primers

Description	**Orientation**	**DNA sequence**
V gene segment-specific oligonucleotide		(5' -> 3', coding strand)
mVβ1	FW	CTGAATGCCCAGACAGCTCCAAGC
mVβ2	FW	TCACTGATACGGAGCTGAGGC
mVβ3.1	FW	CCTTGCAGCCTAGAAATTCAGT
mVβ4	FW	GCCTCAAGTCGCTTCCAACCTC
mVβ5.1	FW	CATTATGATAAAATGGAGAGAGAT
mVβ5.2	FW	AAGGTGGAGAGAGACAAAGGATTC
mVβ5.3[#]	FW	AGAAAGGAAACCTGCCTGGTT
mVβ6	FW	CTCTCACTGTGACATCTGCCC
mVβ7	FW	TACAGGGTCTCACGGAAGAAGC
mVβ8.1	FW	CATTACTCATATGTCGCTGAC
mVβ8.2	FW	CATTATTCATATGGTGCTGGC
mVβ8.3	FW	TGCTGGCAACCTTCGAATAGGA
mVβ9	FW	TCTCTCTACATTGGCTCTGCAGGC
mVβ10	FW	ATCAAGTCTGTAGAGCCGGAGGA
mVβ11	FW	GCACTCAACTCTGAAGATCCAGAGC
mVβ12	FW	GATGGTGGGGCTTTCAAGGATC
mVβ13	FW	AGGCCTAAAGGAACTAACTCCCAC
mVβ14	FW	ACGACCAATTCATCCTAAGCAC
mVβ15	FW	CCCATCAGTCATCCCAACTTATCC
mVβ16	FW	CACTCTGAAAATCCAACCCAC
mVβ17[#]	FW	AGTGTTCCTCGAACTCACAG
mVβ18	FW	CAGCCGGCCAAACCTAACATTCTC
mVβ19[#]	FW	CTGCTAAGAAACCATGTACCA
mVβ20	FW	TCTGCAGCCTGGGAATCAGAA
C gene segment specific oligonucleotide		(5' -> 3', non-coding strand)
muTCB1-FAM	RV	FAM-CTTGGGTGGAGTCACATTTCTC

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Chapter 6

A Novel branched DNA-based flowcytometric method for single-cell characterization of gene therapy products and expression of therapeutic genes

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ABSTRACT

Many preclinical and clinical studies of hematopoietic stem cell-based gene therapy (GT) are based on the use of lentiviruses as the vector of choice. Assessment of the vector titer and transduction efficiency of the cell product are critical for these studies. Efficacy and safety of the modified cell product are commonly determined by assessing the vector copy number (VCN) using qPCR. However, this optimized and well-established method in the GT field is based on bulk population averages, which can lead to misinterpretation of the actual VCN per transduced cell. Therefore, we introduce here a single cell-based method that allows to unmask cellular heterogeneity in the GT product, even when antibodies are not available. We use Invitrogen's flow cytometry-based PrimeFlow™ RNA Assay with customized probes to determine transduction efficiency of transgenes of interest, promoter strength, and the cellular heterogeneity of murine and human stem cells. The assay has good specificity and sensitivity to detect the transgenes, as shown by the high correlations between PrimeFlow™-positive cells and the VCN. Differences in promoter strengths can readily be detected by differences in percentages and fluorescence intensity. Hence, we show a customizable method that allows to determine the number of transduced cells and the actual VCN per transduced cell in a GT product. The assay is suitable for all therapeutic genes for which antibodies are not available or too cumbersome for routine flow cytometry. The method also allows co-staining of surface markers to analyze differential transduction efficiencies in subpopulations of target cells.

INTRODUCTION

Stem cell-based gene therapy is a promising area of medicine that is rapidly expanding with regards to clinical trials and marketing authorization. Although allogeneic hematopoietic stem cell (HSC) transplantation remains the prevailing therapeutic treatment for the correction of several types of inherited diseases, including primary immunodeficiencies, autologous genetically corrected HSCT is an encouraging alternative. The first *ex vivo* gene therapy product using autologous HSC for the treatment of Adenosine Deaminase (ADA) Severe Combined Immunodeficiency (SCID) was Strimvelis (GlaxoSmithKline), and was approved in 2016 by the European Medicines Agency (EMA)[1-4]. This paved the way for the clinical development of HSC-based gene therapy to treat other immunodeficiencies, including the SCIDs X-linked [5-9], Artemis [10-13], RAG1 [14], as well as X-linked chronic granulomatous disease (CGD) [15, 16] and Wiskott–Aldrich Syndrome (WAS) [17-21].

For the treatment of primary immunodeficiencies, hematopoietic stem and progenitor cells (HSPCs) are corrected *ex vivo* (reviewed by Staal et al. (2019) [22]). The patient's HSPCs are isolated, modified with the therapeutic transgene by viral transduction and the corrected cells, *i.e.* the gene therapy product, are transplanted back into the patient. Genome-integrating vectors like self-inactivating (SIN) gamma-retroviral (γ-RV) and lentiviral vectors (LV) have been used safely over the past two decades to achieve a long-lasting therapeutic effect of the transgene [23]. One of the main release criteria for the treatment with a gene therapy product is to reach sufficient transgene expression, measured by the number of integrated transgene copies per target cell known as the vector copy number (VCN). The therapeutic potency of the transgene correlates positively with the proportion of transduced cells, therefore, a threshold is set for the minimal transduction efficiency required to guarantee the correction of enough cells with sufficient transgene expression for a successful and safe therapeutic outcome. However, VCN is an important parameter to control because multiple vector copies per cell can result in genotoxicity.

The golden standard technique to reliably measure VCN has been quantitative polymerase chain reaction (qPCR) of a LV sequence relative to a housekeeping gene to calculate the number of inserted vectors [24]. This strategy determines the average VCN per cell in the bulk population, while only a proportion of cells carry the therapeutic vector. Therefore, the presence of non-transduced cells in the bulk population invariably underestimates the VCN of the therapeutic cells. Measuring the distribution of vector copies in corrected cells at a single-cell level is important to assess that the actual VCN is in the therapeutic range of sufficient integration without the risk of genotoxicity. Attempts to refrain from the bulk population average have been accomplished by measuring VCN in individual colony-forming cell units (CFC). Transduction efficiency determination in CFC has evolved from a green fluorescent protein (GFP) detection method [25] to more reliable and simplified qPCR assays [26, 27] that have been further validated with clinically relevant experimental data [28]. Although this strategy is a step forward towards a better understanding of the cellular heterogeneity of the therapeutic product, proper single cell information is still missing. Thus, determining transgene expression with a multiparametric

technology such as flow cytometry represents a quick single-cell alternative to CFC assays and an attractive alternative to bulk methods.

Here, we introduce a method based on the PrimeFlow RNA Assay® (Thermo Fisher Scientific) [29, 30] generally referred to as “Branched DNA” method, as a potential new tool to characterize the gene therapy product at the single-cell level. This flow cytometry-based detection platform is inspired by fluorescent *in situ* RNA hybridization coupled with branched DNA signal amplification techniques. The technique has been adapted to single-cell suspensions and modified to detect as many as four different mRNA transcripts simultaneously at the single-cell level. Developed gene-specific oligonucleotide target probe set containing 20–40 probe pairs bind across the length of the mRNA. The method is a quantitative technique that preserves the cellular architecture and can provide a theoretical 8000 to 16000-fold signal amplification of the targeted mRNA to be detected by a standard flow cytometer [29-31]. The branched DNA technique can be beneficial in the gene therapy field by three of its main applications. First, the technique is suitable to quantify viral RNA in infected cells, and therefore can be used to detect transduction efficiency in gene therapy products. Second, customized probes can detect target-specific RNA, *e.g.* from expanding codon-optimized therapeutic transgenes used in GT, for which available antibodies for flow cytometry are non-existent or lack sensitivity. Finally, the branched DNA technique enables heterogeneity analysis of gene expression at single-cell level, unmasking bulk cellular heterogeneity of the gene therapy product.

Taken together, we here report on adaptions of the PrimeFlow™ RNA Assay to be used in the gene therapy field as a reproducible and reliable tool to unmask the heterogeneity of the modified cell product. The percentage of transduced cells and the different promoter strengths can be analyzed, providing a unique tool to accurately assess the actual VCN per transduced cell. Furthermore, this technique shows high specificity, sensitivity and versatility, allowing it to be customized for the different therapeutic transgenes, especially when antibodies against the gene products are not available. Such valuable insights enable a more extensive characterization of gene therapy products, which helps improve the safety and therapeutic outcomes.

MATERIALS AND METHODS

Lentiviral vectors

Optimized *RAG1* and RAG2 sequences were synthesized by GeneArt (Regensburg, Germany) and GenScript, USA) respectively. Codon optimized RAG1 (c.o.RAG1) and codon optimized RAG2 (c.o.RAG2) were cloned into self-inactivating lentiviral pCCL plasmid resulting in pCCL-MND-c.o.RAG1 (hereafter: MND-c.o.RAG1; myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer binding site substituted promoter) [32] or pCCL-EFS-c.o.RAG2 (hereafter: EFS-c.o.RAG2; elongation factor 1α short promoter) [33], pCCL-MND-c.o.RAG2 (hereafter: MND-c.o.RAG2), pCCL-PGK-c.o.RAG2 (hereafter: PGK-c.o.RAG2; human phosphoglycerate kinase-1 promoter) [34] and pCCL-UCOE-c.o.RAG2 (hereafter: UCOE-c.o.RAG2; the modified chromatin-remodeling element, devoid of unwanted splicing activity and minimized read-through

activity) [35]. The native RAG1 and RAG2 constructs were derived from the pRRL.PPT.PGK.GFPpre plasmid. The pRRL.PPT.SFFV.RAG1.pre (hereafter: SFFV-NativeRAG1) and the pRRL.PPT.SFFV.RAG2.pre (hereafter: SFFV-NativeRAG2) transfer vectors were constructed by replacing the PGK promoter by a MLV-derived enhancer–promoter from the spleen-focus-forming virus and the GFP sequence was replaced by human *RAG1* or *RAG2* cDNA [36]. DNA sequencing of the transgene was performed to validate the gene transfer constructs. Helper plasmids pMDLg/pRRE, pRSV-Rev and pMD2.VSVG for lentiviral production were kindly provided by L.Naldini (San Raffaele Telethon Institute for Gene Therapy, Milan, Italy) [34]. Large-scale helper-plasmids were obtained from Plasmid Factory (Bielefeld, Germany).

Vector production

293T cells were transiently transfected with the transfer and helper plasmids using X-tremeGene HP DNA transfection reagent (Sigma-Aldrich). Lentiviruses were harvested 24h, 30h and 48h after transfection, filtered through 0.3µm pore filters (Whatmann) and stored at -80°C. Pooled lentiviral supernatant was concentrated by ultracentrifugation (Beckman Optima^{TM} LE-80K, rotor SW32Ti) for 16 hours at 10.000 rpm and 4°C under vacuum conditions. Pellets were resuspended in StemSpan Serum-Free expansion medium (SFEM; Stemcell Technologies Inc) and aliquoted to avoid multiple freeze/thaw cycles. Since no suitable anti-RAG1 antibodies were available, we determined the viral titer using qPCR as described later on. A clinical GMP-grade MND-c.o.RAG1 vector was generated by Batavia Biosciences (Leiden, The Netherlands).The GMP-grade vector was tested and validated on murine Rag1 deficient bone marrow cells and human CD34+ cells [14].

Human CD34+ cell isolation from Cord Blood (CB), Bone Marrow (BM) and mobilized Peripheral Blood (mPB)

Human cord blood, bone marrow and peripheral blood was obtained according to the Medical Ethical Committee and IRB guidelines at Leiden University Medical Center. Cord blood mononuclear cells were separated by Ficoll (Pharmacy Leiden Academic Hospital) gradient centrifugation, frozen in fetal bovine serum (Hyclone)/10% DMSO (Sigma-Aldrich) and stored in liquid nitrogen. After thawing, human CD34^{+} cells were isolated using αCD34 MicroBead UltraPure Kit (Miltenyi Biotec). In short, cells were incubated with FcR blocking reagent and αCD34 Microbeads Ultrapure following manufacturer protocol for 30 min at 4ºC. Subsequently CD34+ cells were positively selected using the appropriate ferromagnetic columns and the MACS separator (Miltenyi Biotec). CD34+ cells from BM and mPB were freshly isolated using CliniMACS (Miltenyi Biotec) by the Stem Cell lab of the Immunology department (LUMC). Hematopoietic progenitor Stem Cells (HSPC) count and purity after isolation was evaluated using a customized Flexicyte Program on NucleoCounter3000 (Chemometec). Directly isolated CD34+ cells were stimulated overnight in X-VIVO15 without Gentamycin and phenol red (Lonza) with 200g/L Human Albumin Serum (HAS; Sanquin) or SCGM medium (CellGenix), both supplemented with 50x Pen/Strep (Gibco), 300 ng/ml human Stem Cell Factor (huSCF; Miltenyi Biotec), 100 ng/ml human Thrombopoietin (huTPO; Miltenyi Biotec), 300 ng/ml

human Flt3-Ligand (huFlt3L; Miltenyi Biotec) and 10 ng/ml human Interleukin-3 (huIL3; Miltenyi Biotec).

Murine HSPC isolation

Lineage negative depletion was performed using the Direct Lineage Depletion kit from Miltenyi Biotec, to isolate hematopoietic stem cell from frozen murine bone marrow. In short, cells were magnetically labeled with the Direct Lineage cell depletion cocktail and incubated for 10min at 4ºC. Lineage negative cells were subsequently depleted using the appropriate magnetic columns and the MACS separator (Miltenyi Biotec). Directly enriched HSPC were cultured in StemSpan (SFEM) medium supplemented with Pen/Strep (Gibco), 50ng/mL recombinant mouse (rm) Flt3L, 100ng/mL rmSCF and 10ng/mL rmTPO (all from R&D Systems) at 37˚C with 5%CO_2. Depletion efficiency and purity of lineage negative population was analyzed by flow cytometry with FACSCanto (BD).

Cell Transduction

After overnight stimulation, human CD34+ or murine lineage negative bone marrow cells were transduced in the appropriate complete medium with the different lentiviruses. Various transduction methods were used. 1] Cells were transduced using 4 ug/ml protamine sulphate (PS; Sigma-Aldrich) and with or without spin-occulation at 800xg and 32°C for 1 hour. 2] Human cells were transduced adding 100 mg/mL LentiBOOST™ (Sirion Biotech) together with the lentiviral supernatant. 3] Cells were transduced using the combination of 4 ug/ml protamine sulphate and 100 mg/mL LentiBOOST™. Cells were cultured at 37°C, 5% CO_2 for 24h in medium supplemented with cytokines as described above. Cells were cultured for 9 days in the appropriate culture medium for further DNA, RNA and branched DNA assay analysis.

PrimeFlow™ RNA assay

PrimeFlow™ RNA assay (ThermoFisher) was performed on HSPCs after 9 days in culture following the manufacturer protocol divided over 2 days. All buffers are included in the PrimeFlow™ RNA assay kit and specific target probe sets for huRPL13A (type 4), muACTB (type 4), c.o.RAG1 (type 1 and type 6), c.o.RAG2 (type 1 and type 6), native RAG1 (type 10) and Native RAG2 (type 1) were designed by and purchased from ThermoFisher. When applicable, cell surface staining was performed for 30 min at 2-8ºC with fluorochrome conjugated antibodies such as CD34-PE (8G12; BD Biosciences), CD34-BV510 (581; Biolegend; AB_2563856) and CD90-BV605 (5E10; Biolegend; AB_2562281). Cells were then fixed for 30 min at 2-8ºC. After permeabilization, cells were fixed a second time for 1h at RT with Fixation buffer 2. A hybridization step was performed by incubating the cells with the appropriated target probe sets for 2h at 40ºC. Samples were stored over night at 2-8C in the dark. The day after, pre-amplification and amplification of the hybridization was performed by 2 consecutive incubations of 1,5h at 40ºC with the pre-Amplification mix and subsequently the Amplification mix. Finally, cells were incubated with the label probe sets for an hour at 40ºC. Cells were measured by flow cytometry on FACS-CantoII and LSR Fortessa X-20 (BD Biosciences) or sorted on FACSAria II (BD Biosciences) and the data was analyzed using FlowJO software (Tree Star).

Determination vector copy number (VCN) and *gene* expression by RT-qPCR

After 9 days in culture (to prevent detection of pseudotransduction), qPCR was used for the quantitative analysis of genomic lentiviral RNA, proviral DNA copies and transgene mRNA expression of the transduced HSPCs using WPRE (Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element), c.o.RAG1, c.o.RAG2, ABL-I and PTBP2 (Polypyrimidine Tract Binding Protein 2) as targets (Table S1). Total RNA from single cell suspensions was purified using RNeasy Mini kit (Qiagen) and reverse transcribed into cDNA using Superscript III kit (Invitrogen). Genomic DNA was extracted from single cell suspensions using the GeneElute Mammalian Genomic DNA kit (Sigma-Aldrich). DNA and RNA concentration were measured by NanoDrop (ThermoFisher). VCN was determined on DNA samples by the detection of viral WPRE normalized to genomic household gene PTBP2. The levels of transgene expression were determined on cDNA samples, by normalizing the transgene to the expression of the *ABL-I* gene. qPCR was performed using TaqMan Universal Master Mix II (ThermoFisher) in combination with specific probes for indicated genes from Universal Probe Library (Roche). Primers and probes used are listed in Table S1. PCR reactions were performed on the StepOnePlus Real-Time PCR system (ThermoFisher). All samples were run in triplicate.

Statistical analysis

Statistics were calculated and graphs were generated using GraphPad Prims 8. Statistical significance was determined with two-tailed Pearson r correlation coefficients. Analysis such as linear regression, non-linear regression and Area Under the Curve have been used across the experiments

RESULTS

Branched DNA assay: suitable to detect transgene transcription in clinically relevant HSPC

Gene therapy for immunodeficiencies is commonly performed by inserting a normal copy (native or codon optimized) of the defective gene into the patient's CD34+ enriched HSPCs isolated from bone marrow (BM) or mobilized peripheral blood (mPB). The branched DNA technique was therefore tested on isolated CD34+ cells from BM, mPB and cord blood (CB). Cells were transduced with the therapeutic codon optimized (c.o.) RAG1 LV [14], cultured for 9 days (to limit the detection of early, but temporary expression from non-integrated transgenes also referred to as pseudo-transduction) and analyzed by the branched DNA assay with customized probes developed for *c.o.RAG1* mRNA and the housekeeping *RPL13a* mRNA as an internal control (See method overview in **Figure 1.A**). As shown in **Figure 1.**B, *RPL13a* but not *c.o.RAG1* was detected in non-transduced HSPCs. However, transduced cells from all sources revealed an evidently positive *c.o.RAG1* population. Although cells were transduced with the same number of viral particles per cell (VP/cell) and infectious genomes (MOI) (1000 VP/cell; MOI 3.6), a different transduction efficiency can be observed both by the percentage of transduced

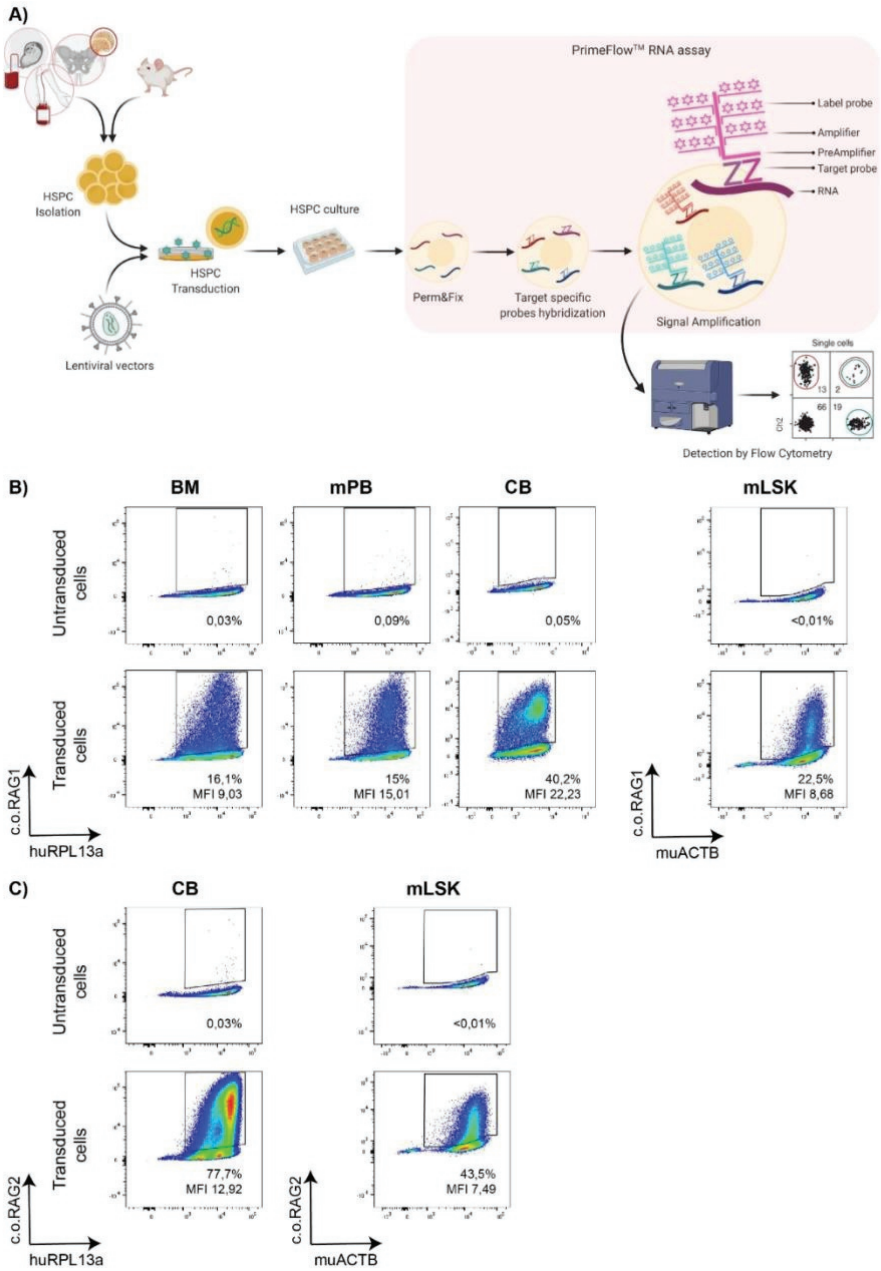


Figure 1: Branched DNA method is suitable to detect diverse therapeutic transgenes in cells of interest. *A) Schematic overview of the analytical process and the branched DNA methodology. Human and murine hematopoietic progenitor stem cells (HSPC) isolated from various sources like cord blood (CB), bone marrow (BM) or mobilized peripheral blood (mPB) were transduced with lentiviral vectors and cultured for 9 days. Cells were harvested and split for DNA isolation, RNA isolation and branched DNA assay, in which cells are first permeabilized and fixed, then target-*

specific probes are hybridized with the mRNA and finally signal is amplified. Cells can then be analyzed by standard flow cytometry [Created with BioRender.com]. B) FACS plot showing the detection of the therapeutic c.o.RAG1 transgene of transduced human CD34+ cells (1000 viral particles/cell; MND-c.o.RAG1 LV) isolated from BM, mPB or CB as well as in murine isolated HSPC. C) FACS plot confirming the detection of the therapeutic c.o.RAG2 transgene in transduced cord blood CD34+ cells and in murine HSPCs (1800 Viral particles/cell; MND-c.o.RAG2 LV). Human RPL13a and murine ACTB housekeeping mRNAs have been used as internal controls. Percentage of positive cells and MFI (ratio MFI positive/negative population) are depicted in each plot.

cells and the mean fluorescent intensity (MFI) with different values for CB (14.8%; 16.4 MFI),BM cells (16,1%; 9,03 MFI) and mPB cells (15%; 15,01 MFI), indicating differences in cell permissiveness of human HSPCs depending on the source. Preclinical development of gene therapy is primarily performed in animal models like mice. The branched DNA assay was therefore tested on isolated transduced LSK cells (Lineage negative Sca1+ c-Kit+ cells). A positive *c.o.RAG1* population was clearly detected (22,5%; 8,68 MFI), while no signal was detected in non-transduced LSK cells (percentage below 0,01). For murine cells, *ACTB* was used as household internal mRNA control. Interestingly, the probe sets can be customized to any gene of interest, and consequently this assay can be adapted to a broad variety of therapeutic transgenes. For example, *c.o.RAG2* probes were also developed and validated (**Figure 1.C**) on transduced CD34+ CB cells and murine LSK cells. In both cases, a well-defined positive *c.o.RAG2* transduced population was detected (77,7%; 12,92 MFI and 43,5%; 7,49 MFI respectively). Hence, the novel branched DNA technique allows the reliable detection of therapeutic transgenes in progenitor cells of human and mouse origin.

High specificity to detect transduced cells within the bulk HSPC population

A variety of LVs and probe sets were used to assess the specificity of this novel branched DNA technique. CD34+ cells enriched from CB were transduced with LVs expressing the native or codon optimized *RAG1* (SFFV-RAG1 or MND-c.oRAG1). Transduction efficiency was assessed by the branched DNA assay with specific probe sets developed against native *RAG1* and *c.o.RAG1* mRNAs (**Figure 2.A_left panel**). A positive native *RAG1* population (14,9%) was only detected in transduced cells with the specific native RAG1 probe sets, but not with the c.o.RAG1 probe sets. Similarly, *c.o.RAG1* transduced cells were only detected with the c.o.RAG1 probe sets (29,5%). The same approach was used to assess specificity of the RAG2 probe sets (**Figure 2.A_right panel**) where native *RAG2* expression was only detected by the native probe sets (33,2%) and *c.o.RAG2* positive cells were only identified with the c.o.RAG2 probe sets (59%). Quantitative PCR of *c.o.RAG2* or *RAG2* relative to *ABL-I* expression confirmed these results (inset below **Figure 2A_right panel**). Thus, our developed probe sets were highly specific and accurately discriminated between native sequences and codon optimized mRNAs, without cross-reactivity between similar sequences (e.g. 82% similarity between c.o.RAG1 and native RAG1 sequences and 94,5% similarity between c.o.RAG2 and native RAG2 sequences).

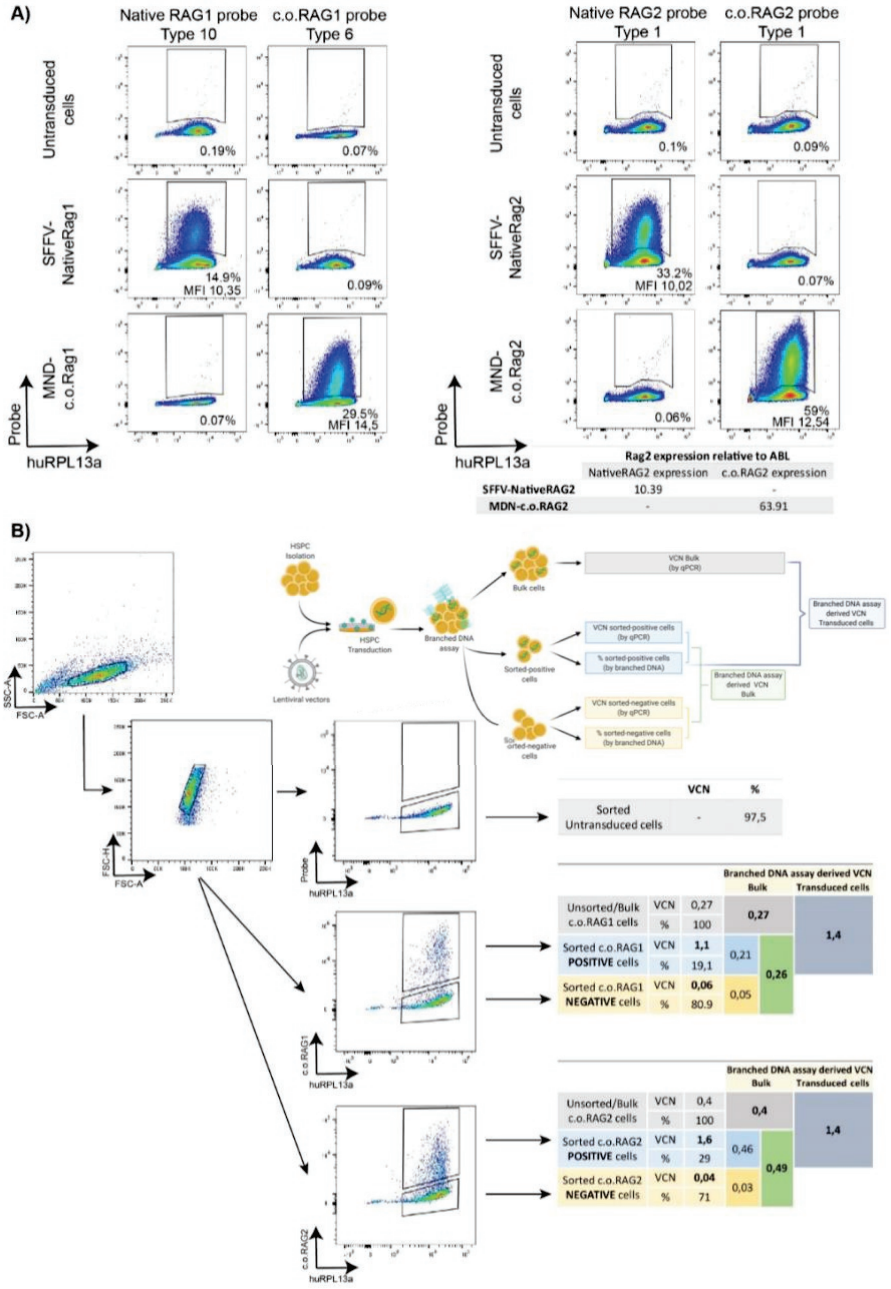


Figure 2: Specificity of the branched DNA technique to detect transduced cells within the bulk HSPC population. *A) FACS plots representing the specificity of the DNA branched method using different lentiviral vectors (SFFV-NativeRAG1, SFFV-Native RAG2, MND-c.o.RAG1 and MND-c.o.RAG2) and specific probe sets for the detection of Native RAG1, RAG2 or codon optimized RAG1 or RAG2. Percentage of positive cells and MFI (ratio MFI positive/negative population) are depicted per plot. Human RPL13a housekeeping mRNAs have been used as internal control. Table showing*

the detection by qPCR of RAG2 expression (Native or codon optimized) in the different transduced cells. B) FACS plots depicting the sorting strategy of non-transduced, MND-c.o.RAG1 and MND-c.o.RAG2 transduced cells. C.o.RAG1 or -2 positive and negative populations were sorted after branched DNA assay, and VCN was determined by qPCR in the unsorted and sorted populations. Diagram and tables show the VCN determined by qPCR and percentages (%) measured by flow cytometry after branched DNA assay, as well as the branched DNA derived VCN of the sorted cells in the bulk population (VCN sorted population x % sorted population) and the branched DNA derived VCN of the transduced cells from the bulk VCN and the known percentage of positive cells (VCN bulk population / % positive population). Human RPL13a housekeeping mRNAs have been used as internal control.

Another relevant parameter is to elucidate whether the positive detected cells are accurately reflecting the proportion of cells with the transgene insertion. Therefore, CD34+ cells transduced with either c.o.RAG1 or c.o.RAG2 were sorted after 9 days in culture based on positivity using the branched DNA assay. Positive and negative c.o.RAG1 or c.o.RAG2 populations were sorted (all RPL13a positive cells) and VCN was determined by qPCR in the sorted populations, as well as in the bulk population before sorting (**Figure 2.B** and **Figure S1**). Both transduced positive populations revealed high VCNs (1,1 for c.o.RAG1 and 1,6 for c.o.RAG2) whereas the negative sorted populations showed insignificant VCNs (0,06 and 0,04 respectively). Importantly, positively sorted cells presenta considerably higher VCN than the VCN assessed in the bulk population by qPCR (1,1 sorted *vs.* 0,27 bulk for c.o.RAG1 and 1,6 sorted *vs.* 0,4 bulk for c.o.RAG2). With 19,1% of the bulk properly c.o.RAG1 transduced with a VCN of 1,1 and a negative population representing 80,1% (VCN of 0,06), the calculated bulk VCN obtained was 0, 26; very close to the 0,27 assessed directly by qPCR in bulk cells. Similarly, calculated c.o.RAG2 VCN in the bulk (derived from sorted cell data) is 0,49 while VCN determined by qPCR in the bulk was 0,4. These data show that both the branched DNA and qPCR methods are extremely accurate. Conversely, knowing the VCN measured by qPCR in bulk cells (0,27 for c.o.RAG1 and 0,4 for c.o.RAG2) together with the fraction of actual transduced cells (19,1% c.o.RAG1 cells and 29% c.o.RAG2 cells) is sufficient to determine the VCN of the actual transduced cells. Here this was 1,4 in both cases, relatively close to the VCN determined in the positively sorted cells (1,1 and 1,6 respectively).

Altogether, the branched DNA method shows high specificity and accuracy in discriminating between close sequences and detecting positively transduced cells that express the therapeutic transgene. Moreover, this assay provides key information on transduction efficiency of the gene therapy product, allowing a reliable calculation of the real actual VCN of the portion per transduced cells.

Proper sensitivity to detect therapeutic transgenes

To test the sensitivity of the branched DNA technique, serial dilution of transduced cells was performed and analyzed both by the branched DNA assay and qPCR (**Figure 3.A**). C.o.RAG2 transduced cells were cultured for 9 days and serially diluted by mixing with non-transduced cells. While MFI values stayed constant with the serial dilution, the percentage of positive cells decreased with each dilution. The VCN and expression level measured by qPCR also decreased accordingly. A significant correlation between the standard parameters VCN and gene expression measured by qPCR (R^2=0.979, p<0,001)

is found (**Figure 3.B**). The percentage of positive cells as measured by the branched DNA method also reveal a high correlation with the VCN (R^2=0,9945, p<0,001) and gene expression level (R^2=0.9761, p<0,001). Finally, data from up to 23 experiments of c.o.RAG1 transduced cells, either from BM or mPB, shows high reproducibility of the novel assay (**Figure 3.C**) with significant correlation between VCN and the percentage of transduced cells (R^2=0.8939, p<0,001). As seen in **Figure 3.C**, an average of 40% of cells are properly transduced in mPB or BM with our MND-c.o.RAG1 LV when a VCN of 1 was detected by qPCR in the bulk population, resulting in an actual VCN of 2,5 in the transgene-positive cells (40% of the gene therapy product). This can vary depending on the cell source and the vector used. When c.o.RAG2 LV was used to transduce CD34+ cells isolated from CB, on average 31% of the population was transduced when a VCN of 1 was detected, increasing to a VCN of 3,2 in the transduced cells (**Figure S2**). As modified cells only represent a portion of the bulk HSPCs, the VCN of the modified, potentially therapeutic cells is higher than the determined by qPCR (See **Table 1**) and the transgene insertion is generally underestimated by bulk measurements.
Collectively, this data indicate that the results of the branched DNA technique correlates to those of qPCR methods and constitutes a highly sensitive and reproducible method to measure the percentage of transduced cells.

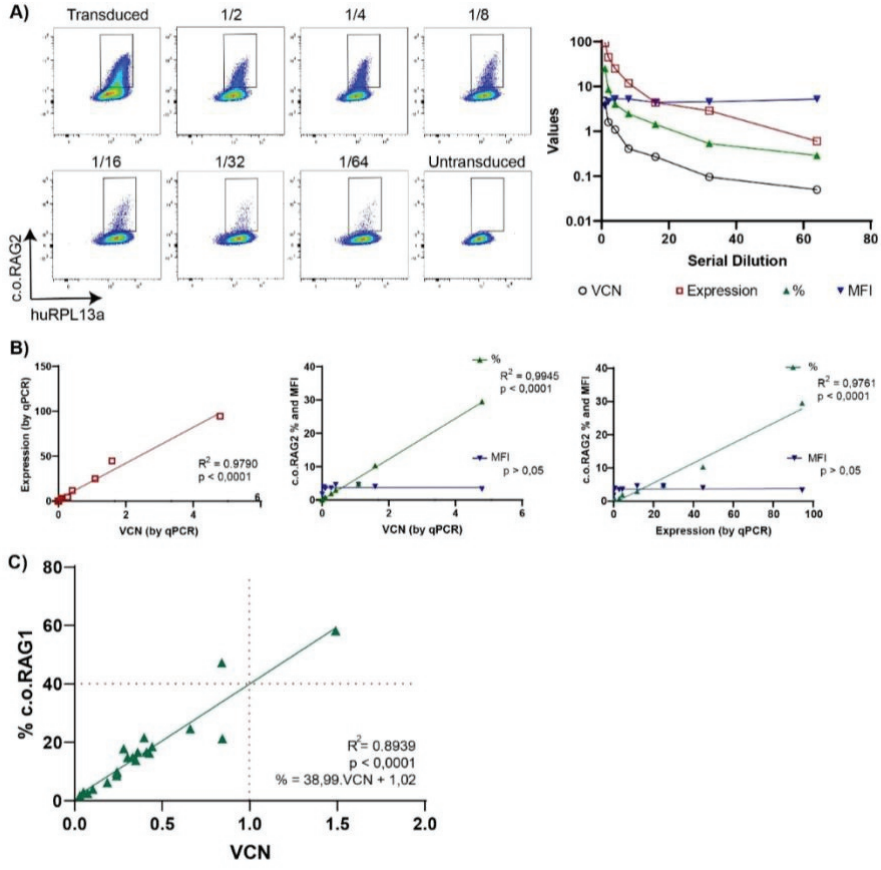


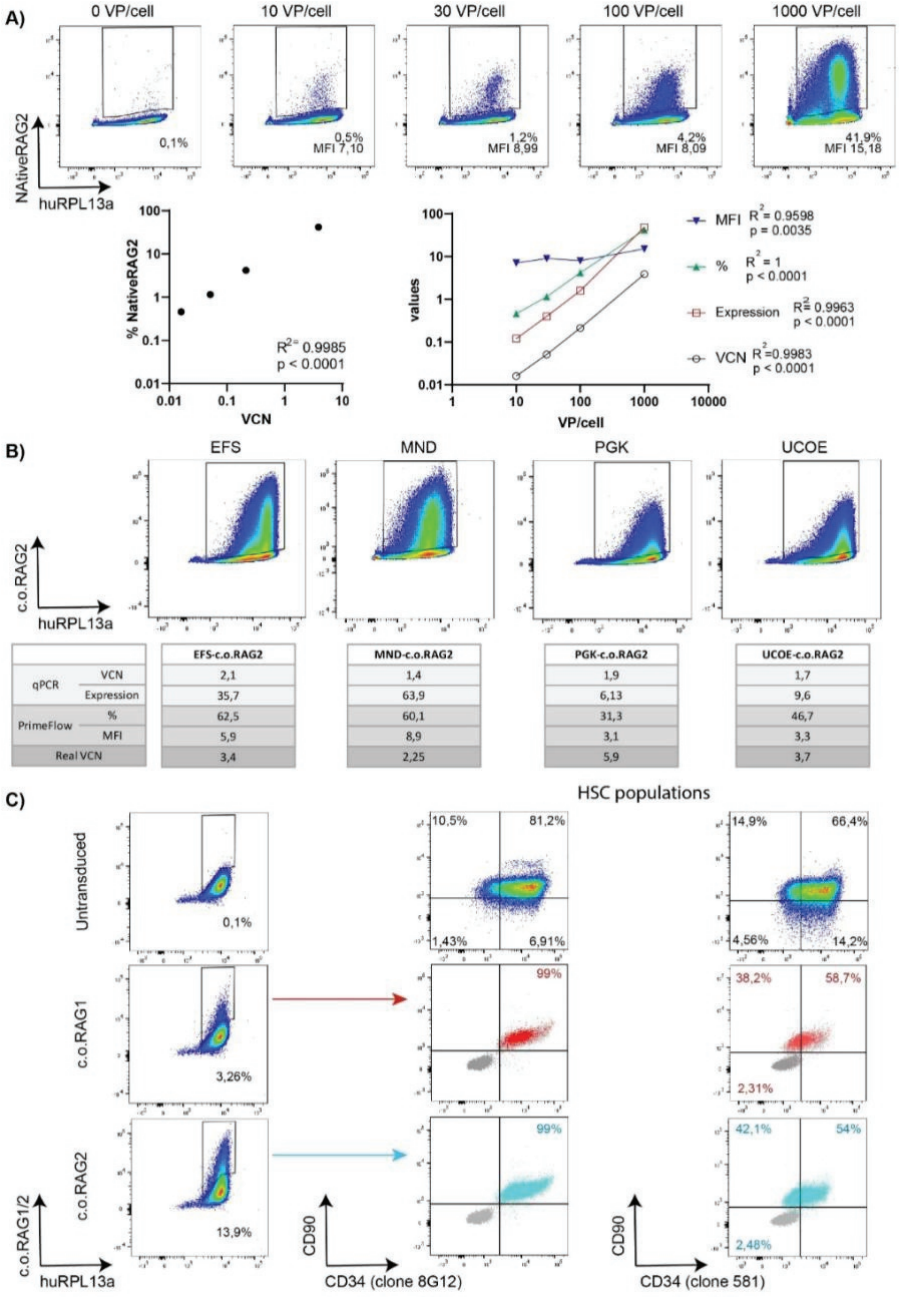
***Figure 3: Branched DNA assay sensitivity to detect therapeutic transgenes**. A) FACS plots showing the positive c.o.RAG2 population of transduced CD34+ cells in a serial dilution with non-transduced cells, from 1/2 to 1/64. VCN, c.o.RAG2 expression, % and MFI measured in the serial dilution are represented in a graph. Human RPL13a housekeeping mRNAs have been used as internal control. B) Correlation between different parameters have been studied: VCN (by qPCR) vs c.o.RAG2 expression (by qPCR), VCN vs % and MFI (by flow cytometry) and c.o.RAG2 expression (by qPCR) vs % and MFI. (Two-tailed, Pearson r correlation coefficients). C) Correlation between VCN detected by qPCR and % or MFI determined by flow cytometry including a total of 24 independent experiments on CD34+ cells (mPB and BM) transduced with MND-c.o.RAG1 at different viral particles per cell and different transduction protocols. Red dashed line= % c.o.RAG1 cells at VCN=1. (Two-tailed, Pearson r correlation coefficients; linear regression).*

Branched DNA assay as a novel gene therapy tool.

Determining the virus titer is important to calculate the quantity of virus needed to achieve efficient and reproducible transduction of primary cells. Enriched CD34+ cells from CB were transduced with increasing amounts of native RAG2 LV (SFFV-Native RAG2) and analyzed by qPCR and branched DNA technique after 9 days in culture (**Figure 4.A**). An increasing percentage of positive native RAG2 cells was detected by branched DNA-flow cytometry (0,5%, 1,2%, 4,2% and 41,9%) with increasing numbers of VP/cell and MOI (10 VP/cell [0,02 MOI]; 30 VP/cell [0,07 MOI]; 100 VP/cell [0,21 MOI] and 1000 VP/cell [2,13 MOI] respectively). Just as in **Figure 3B**, there was a high and significant correlation (R^2=0,9985, p<0,0001) between the VCN determined by qPCR and the percentage of positive cells detected by the branched DNA technique. In addition, all parameters measured by qPCR (VCN and expression) and the branched DNA assay (% and MFI) significantly correlated with the VP/cell and MOI with R^2 values higher than 0.9804 and p<0.0001 (**Figure 4A, graphs**).

Therefore, both the standard qPCR and the branched DNA assay correlate with the viral titration. Interestingly, the percentage of transduced cells can reach a plateau at a lower VP/cell and MOI) than the VCN determined in bulk (**Figure S.3A&B**). Overall VCN kept increasing with rising viral particles but not the proportion of modified cells, implying a potential increase of insertions within the same cell population.

Identifying differences in promoter strengths could reveal different gene expression intensity patterns. Isolated HSPCs were transduced with various c.o.RAG2 LVs carrying different promoters (EFS [33], MND [32], PGK [34] and UCOE [35], see Material & Methods section). After 9 days in culture, transduced cells were split and analyzed by qPCR and branched DNA technique (**Figure 4.B**). Bulk VCN detected in the different transductions was measured as 1,76 +/- 0,27. Although similar VCN were determined along the conditions, a wide range of c.o.RAG2 gene expression was detected by qPCR ranging from 6,13 with the PGK promoter to 63,9 with the strong MND promoter. Similarly, different percentages and MFIs were detected by the branched DNA assay, depending on the promoter used (from 31,3% and 3,1 MFI with PGK to 60,1% and 8,9 MFI with MND promoter). Even though bulk VCN were similar across the conditions, the calculated VCN per transduced cells differs across promoters because the actual percentage of transduced cells highly differ from 60% to 31%. Taken together, the branched DNA method



		EFS-c.o.RAG2	MND-c.o.RAG2	PGK-c.o.RAG2	UCOE-c.o.RAG2
qPCR	VCN	2,1	1,4	1,9	1,7
	Expression	35,7	63,9	6,13	9,6
PrimeFlow	%	62,5	60,1	31,3	46,7
	MFI	5,9	8,9	3,1	3,3
Real VCN		3,4	2,25	5,9	3,7

Figure 4: Branched DNA method as a novel gene therapy tool. *A) Branched DNA technique as a virus titration tool: Representative FACS plots of the detected positive NativeRAG2 population in CD34+ cells with increasing viral concentration. % and MFI depicted in each case. Human RPL13a housekeeping mRNAs have been used as internal control. Graphs represent correlation between VCN vs % and Viral Particles/cell or MOI vs VCN (by qPCR), expression (by qPCR), % (by branched*

DNA assay) and MFI (by branched DNA assay). (Two-tailed, Pearson r correlation coefficients). B) Branched DNA assay for promoter strength visualization: FACS plots representing the c.o.RAG2 population detected by flow cytometry after transduction of CD34+ cells at similar VCN with different constructs including different promoters (EFS-c.o.RAG2, MND-c.o.RAG2, PGK-c.o.RAG2 and UCOE-c.o.RAG2). Human RPL13a housekeeping mRNAs have been used as internal control. VCN, c.o.RAG2 expression, % and MFI results are depicted in the table. "Real" VCN is calculated in each case as VCN (by qPCR) / % (by flow cytometry). C) FACS plots showing c.o.RAG1 or c.o.RAG2 transduced cells determined after 4 days in culture. Transduced cells co-expressed CD34 and CD90 markers (dark grey = unstained control, light grey = non-transduced control, blue=c.o.RAG2 positive cells, red=c.o.RAG1 positive cells). HSC population FACS plots represent the bulk population. Human RPL13a housekeeping mRNAs have been used as internal control.

accurately reflects the MFI of transduced cells that together with the percentage gives an indication of the strength of the promoter used.

The last benefit of the branched DNA technique is that it can be combined with the staining of other cell markers, allowing to more thoroughly study transduction of HSPCs sub-populations. Transduced cells stained for *c.o.RAG1* or *c.o.RAG2* using the branched DNA method were co-stained with CD34 and CD90 antibodies to define $CD34^{+}CD90^{+}$ HSCs in the bulk CD34-enriched HSPC population (**Figure 4.C**). Positive c.o.RAG1 and c.o.RAG2 cells were detected 4 days after transduction (3,26% c.o.RAG1 and 13,9% c.o.RAG2 positive populations). Importantly, cells adequately expressing c.o.RAG1 or c.o.RAG2, co-expressed CD34 and CD90 (CD34 staining with two different clones) visualizing the transduction of HSCs within a CD34-enriched HSPC population with our therapeutic transgenes by flow cytometry. The single cell-based data make the branched DNA technique a valuable tool to be used in determining the optimal virus titration, to compare different constructs and to depict transduction of sub-population defined by extra markers. Notably, it is the first time to depict single cell information directly in clinically applicable therapeutic plasmids. Most importantly, determination of the VCN per effectively transduced cell represents a determinant factor to define the composition of a heterogeneous gene therapy product.

DISCUSSION

We herein describe and validate a branched DNA method coupled with flow cytometry to directly measure mRNA and thus assess transduction efficiency and transgene expression at the single-cell level. As an additional parameter, the MFI indicates the expression level of the therapeutic gene on a per cell basis. Until now, most of our assumptions of transgene expression are based upon the VCN as a bulk average that changes with a different transduction efficiency. Using the branched DNA assay, we accurately evaluate the proportion and VCN of the therapeutic cells present in the heterogeneous bulk CD34+ product, particularly with a relevant c.o.RAG1 vector [14]. In fact, the lack of information regarding expression levels in individual cells became a problem in our initial experiments aimed at developing RAG1 gene therapy [37]. It was unclear based on bulk Q-PCR data whether there was a modest level of transduction in all target cells, or if a small subset was preferentially targeted with much higher efficiencies. Our later studies indicated a

significant heterogeneity in transduction levels, even when using an envelope protein (VSV-G) that should transduce all cells [14]. Thus, using this assay, large numbers of events can be reliably collected by standard flow cytometry, generating sufficient statistical power which is especially important when analyzing rare events.

This branched DNA assay proves to be suitable to detect transgene expression like *c.o.RAG1* in potentially therapeutic CD34+ enriched cells from numerous sources like BM, mPB or CB, as well as in the murine HSPCs used in most of the pre-clinical studies. Enriched CD34+ cells from different sources have different cell composition, proliferation index and lifespan [38-42], resulting in diverse transduction permeability as reflected by the different percentages of gene marking efficiencies when transduced with the same amount of virus and infectious genomes. With its customized probe sets, the branched DNA technique is versatile because it can be adjusted to detect a broad variety of clinically applicable therapeutic genes. The developed probe sets are highly specific, discriminating between similar sequences of the native and the codon-optimized mRNA of a gene without cross-reactivity. Moreover, this method allows an accurate detection of the modified cells invariably containing and expressing the therapeutic transgene versus the unmodified cells. The correlation between the detection of transduction with the standard qPCR (VCN) and novel method (%) is significant and reproducible over independent experiments and constructs. However, the reliable detection of transduced cells using the branched DNA method shows better sensitivity than the standard qPCR method in detecting small portions of positive cells. While the branched DNA technique is a robust assay to study transduction efficiency by means of percentage of transduced cells, the mean fluorescent intensity (MFI) is more variable as it depends on the quality of the lasers in each instrument. To standardize MFI values, the ratio between the positive population MFI and the negative population was calculated. In addition, intensity of the expressed transgene reflected by the MFI also depends on the expression level at the harvesting time (days in culture, cell proliferation), adding an extra variable within the MFI parameter. While MFI is stable for the same promoter, it can of course differ for different promoters and can be used to determine relative promoter strength. Finally, the branched DNA method is an attractive alternative when proper antibodies against the transgene product are not available. Although the protein level is not measured directly, a single-cell flow cytometry readout of mRNA transcribed from the introduced transgene was previously not available. Co-staining with other cellular markers can provide more specific information on target sub-populations. Ideally, a complete antibody panel including all CD34+ sub-populations can reveal single-cell information on the specific targeted subpopulation and their differences with the bulk CD34+ cells. Notably, this novel method to detect single cell information directly in clinically applicable therapeutic cells eliminates the need for GFP carrying constructs. However, the successful use of cell surface co-staining requires individual optimization as antibody epitopes and fluorochromes are not always compatible with the permeabilization/fixation protocol. Altogether, the branched DNA assay shows high specificity, sensitivity and reproducibility to reliably determine transduction efficiency of HSPCs by measuring the precise frequency of transduced cells at a single cell level. Previously unavailable single-cell data is unmasking the heterogeneity of the gene therapy cell product.

Knowing the suitable specificity, sensitivity and reproducibility; the branched DNA assay can be used as a tool to analyze virus titration in primary cells, compare promoter strength of different constructs, to identify the transduced HSPCs sub-populations by co-staining with extra markers. The novel single-cell data supports a reliable re-interpretation of the actual VCN within the modified therapeutic cells as opposed to the average in the bulk population becoming a determinant factor to measure the composition of the gene therapy product and to evaluate the risk of genotoxicity of the clinical vectors. The higher actual VCN on modified cells may indicate an increased risk of genotoxicity on the transduced cells which could be underestimated when relying on bulk measurements. Correspondingly, the branched DNA technique revealed an underestimation of the actual VCN in the potentially therapeutic cells by the presence of a rather sizable fraction of non-transduced cells in the bulk CD34+ product population. Indeed, around 40% of the bulk HSPCs are modified with the clinical c.o.RAG1 vector when aiming at an average of one insertion per cell (VCN=1); a lower portion than the 63% expected from a mathematical approach (Poisson distribution) generated by Fehse et al (2004) [43]. Therefore, the VCN of transduced cells has been underestimated, indicating an actual 2,5 times higher VCN in the therapeutic cells than what has been assessed by qPCR in the bulk. Assessment of this correction factor of VCN in therapeutic cells can vary between vectors (research vs GMP grade or transgene) like for our research grade c.o.RAG2 LV with a lower percentage of targeted cells (30%) than c.o.RAG1 LV. Independent calculation of this factor per clinically applicable vector might therefore be advisable.

In the perspective of clinical gene therapy, the branched DNA method is intended to be implemented as an extra assay to collect additional safety data and better characterization of the gene therapy product and the potentially therapeutic cells. The murine model for X-linked SCID (IL2rg) suggests that the threshold of functional corrected HSPCs required to reconstitute immune function is around 10% of the total transplanted cells [44]; although additional studies with the branched DNA assay are needed to assess this with a clinically relevant vector. A more accurate analysis of transduction efficiency and the number of potentially therapeutic cells to be transplanted can be correlated to the immune reconstitution and transplantation outcome. This additional criterion for transduction efficiency would reveal the minimum number of transduced, therapeutic cells with a safe “real” VCN with a successful transplantation outcome. The increased usage of codon optimized transgenes in the new versions of SIN LV that are being developed allows a successful customization of the probe sets for the different therapies. This novel strategy could be implemented for ongoing clinical trials for immunodeficiencies like X-linked SCID [5-9], ADA SCID [45], Artemis SCID [10-13], X-linked chronic granulomatous disease (CGD) [15, 16, 46] or WAS [17-21, 47], as well as for other diseases with a similar gene therapy approaches like Pyruvate Kinase deficiency [48], Fanconi Anemia [49] or hemoglobinopathies [50-52].

To conclude, we introduce a novel branched DNA technique as an additional tool to accurately assess transduction efficiency at a single cell level and to measure VCN of transduced cells in gene therapy for immunodeficiencies and other modalities, revealing underestimated VCN and heterogeneity of the gene therapy product.

SUPPLEMENTARY MATERIAL

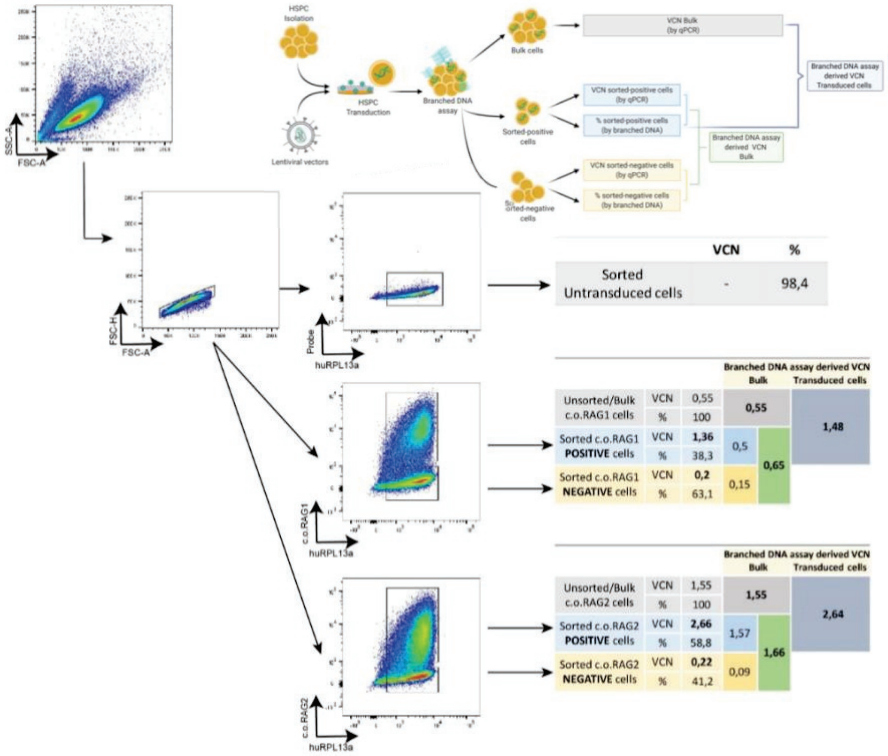


Figure S1: Specificity of the branched DNA assay to detect transduced cells *(duplicate sorting). FACS plots depicting the sorting strategy of non-transduced, MND-c.o.RAG1 and MND-c.o.RAG2 transduced cells. C.o.RAG1 or 2 positive and negative populations were sorted after branched DNA assay, and VCN was determined by qPCR in the unsorted and sorted populations. Diagram and tables show the VCN determined by qPCR and percentages (%) measured by flow cytometry after branched DNA assay, as well as the branched DNA derived VCN of the sorted cells in the bulk population (VCN sorted population x % sorted population) and the branched DNA derived VCN of the transduced cells from the bulk VCN and the known percentage of positive cells (VCN bulk population / % positive population). Human RPL13a housekeeping mRNAs have been used as internal control.*

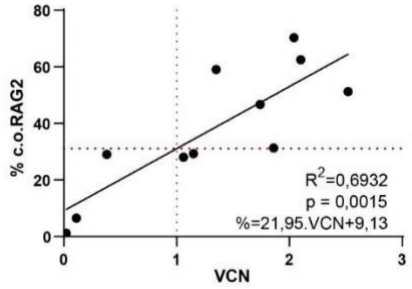


***Figure S2: Expected portion of transduced cells when VCN of 1 is obtained by qPCR**. Correlation VCN determined by qPCR and percentage of transduced cells detected by branched DNA technique. A total of 11 independent experiments of transduced CD34+ cells isolated from cord blood and transduced with various c.o.RAG2 LVs at different multiplicity of infection. Red dashed line= % c.o.RAG2 cells at VCN=1. (Two-tailed, Pearson r correlation coefficients; linear regression).*

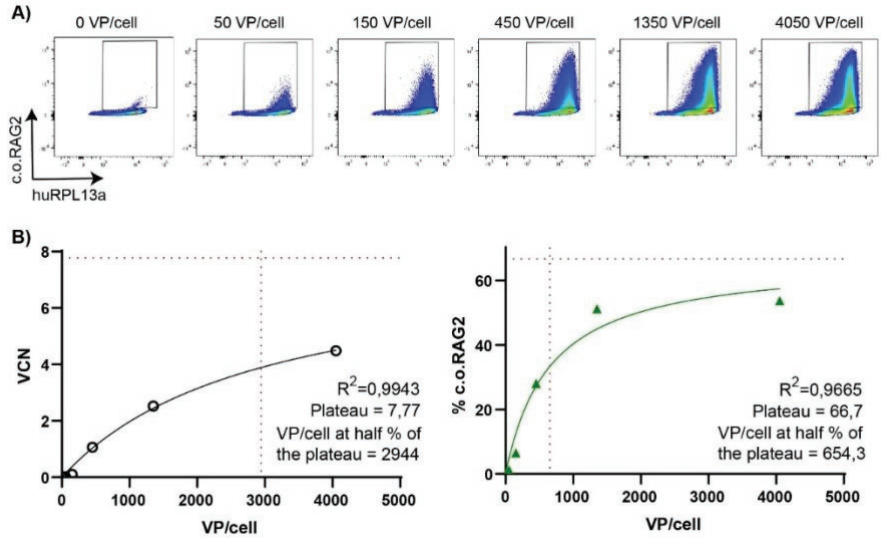


***Figure S3: Proportion of transduced cells reached plateau earlier than bulk VCN.** A) Branched DNA technique as a titration tool: Representative FACS plots of the detected positive c.o.RAG2 population in CD34+ cells with increasing viral concentration. Human RPL13a housekeeping mRNAs have been used as internal control. B) Graphs represent correlation between VCN and % vs Viral Particles/cell (VP/cell) or MOI. (Non-linear fit regression).*

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Chapter 7

Combining mobilizing agents with busulfan to reduce chemotherapy-based conditioning for hematopoietic stem cell transplantation

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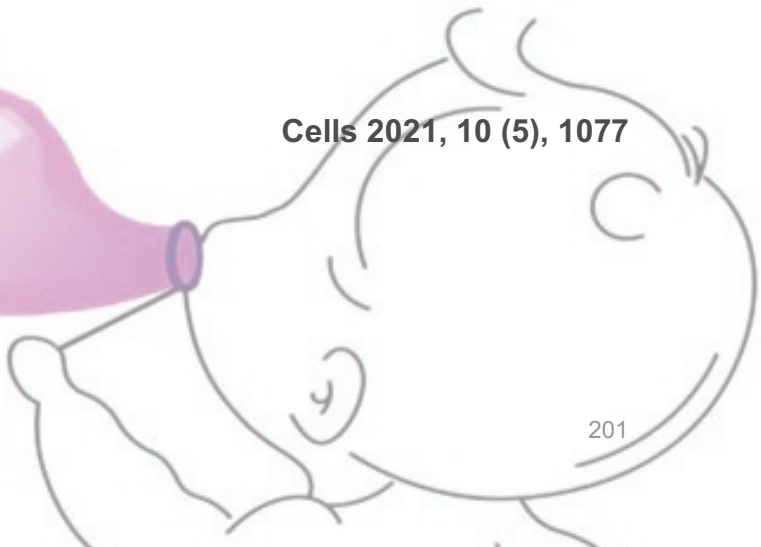

ABSTRACT

In the context of hematopoietic stem cell (HSC) transplantation, conditioning with myelo- and immune-ablative agents is used to eradicate the patient's diseased cells, generate space in the marrow and suppress immune reactions prior to the infusion of donor HSCs. While conditioning is required for effective and long lasting HSC engraftment, currently used regimens are also associated with short and long-term side effects on extramedullary tissues and even mortality. Particularly in patients with Severe Combined Immunodeficiency (SCID), which are generally less than 1-year-old at the time of transplantation and often suffer from existing co-morbidities. There is a pressing need for developing alternative, less toxic conditioning regimens. Hence, we here aimed to improve efficacy of currently used myeloablative protocols by combining busulfan with stem-cell niche-directed therapeutic agents (G-CSF or Plerixafor) that are approved for clinical use in stem cell mobilisation. T, B and myeloid cell recovery was analysed in humanized NSG mice after different conditioning regimens. Increasing levels of human leukocyte chimerism were observed in a busulfan dose-dependent manner, showing comparable immune recovery as with total body irradiation in CD34-transplanted NSG mice. Notably, a better T cell reconstitution compared to TBI was observed after busulfan conditioning not only in NSG mice but also in SCID mouse models. Direct effects reducing the stem cell compartment in the bone marrow were observed after G-CSF and Plerixafor administration, as well as in combination with low doses of busulfan. Unfortunately, these direct effects on the stem population in the bone marrow were not reflected by increased human chimerism nor immune recovery after CD34 transplantation in NSG mice. These results indicate moderate potential of reduced conditioning regimens for clinical use relevant for all allogeneic transplants.

INTRODUCTION

Allogeneic and gene-corrected autologous hematopoietic stem cell (HSC) transplantation may result in limited engraftment of progenitors without preceding conditioning regimen due to the occupation of bone marrow (BM) and thymic niches by host cells, which results in incomplete graft function, immune reconstitution and cure [1]. Conditioning agents can be employed to create space in the BM niches thus allowing transplanted HSCs to engraft efficiently. Although conditioning contributes to an improved HSCT outcome by increasing HSC engraftment, immune chimerism, immune function and by reducing the risk of graft rejection, it may also have negative impact on patient well-being due to short-term and long-term treatment-related morbidity and mortality [2, 3]. The use of irradiation-based regimens and alkylating chemotherapy in infants has an unfavorable impact on growth and fertility, and is associated with an increased risk for secondary malignancies [4-6]. Therefore, particularly in pediatric patients, total body irradiation regimens have been gradually replaced by chemotherapy-based conditioning [7]. Busulfan is a myeloablative alkylating agent that prevents DNA replication through DNA crosslinking and therefore triggering cell apoptosis [8]. Busulfan is used as conditioning agent prior HSCT as it is known to be cytotoxic to host hematopoietic stem and progenitor cells (HSPCs) [9].

In the first stem cell gene therapy protocols for Severe Combined immunodeficiency (SCID) conditioning was omitted. The absence of conditioning prior to both allogeneic and gene-corrected autologous HSCs transplantation led to limited engraftment of transplanted HSC and thus only partial correction of the immune deficiency, especially B cell function, resulting in suboptimal clinical benefit. [3, 10]. Subsequent clinical trials on gene therapy for SCID included the use of a non-myeloablative reduced intensity conditioning (RIC) regimen consisting of a low dose busulfan-based conditioning (4mg/kg), approximately 25% of the total dose usually used in myeloablative protocols. The use of RIC regimens enables the engraftment of early progenitor cells and therefore allow both T and B cell long-term correction, while limiting potential short- and long-term toxicities [11-13]. However, insufficient conditioning is associated with the risk of mixed chimerism in the HSC compartment [14] and therefore reduce the chance for a favorable outcome. Current gene therapy protocols for SCID, especially ADA-SCID and X-linked SCID, rely on the use of HSC corrected cells and a reduced-intensity busulfan-based conditioning regimen which have been shown to be successful in achieving a lasting effective engraftment with limited toxicity [11, 15, 16].

However, this reduced-intensity busulfan-based conditioning may be insufficient in other forms of SCID like the Recombinase-Activating gene 1 and 2 (RAG1/2) SCID where there is a more prominent occupancy of BM niches by precursors B cells blocked in development. For this patient group, insufficient HSC engraftment resulting in poorer T- and B-cell reconstitution have been reported in the absence of conditioning. [17-19]. In RAG1/2 SCID, precursor B cells completely occupy bone marrow niches and strongly compete with transplanted cells leading to poor immune reconstitution [20, 21]. Therefore, to achieve proper engraftment of transplanted cells, a myeloablative regimen is required to empty precursor niches. Conditioning benefits should also be weighed against its short and long-term toxicity, especially in for instance Artemis deficiency with inherent radio-

sensitivity due to impaired DNA repair and in new-born patients [3, 6]. Accordingly, a critical balance for successful engraftment together with the risk of dose-limiting toxicities must be carefully considered and highlight the need to develop alternative non-/less genotoxic conditioning regimens.

Thus, although current conditioning agents are often successfully employed, there is a pressing need for alternative, less toxic conditioning regimens to create space in the BM niches for a durable engraftment of stem cells/HSC without adverse effects on extramedullary tissues. Development of effective, non-toxic, non-alkylating-based conditioning regimens are essential to ensure a successful transplantation and good quality of life in patients with SCID or related inborn errors. In SCID, where patients are predominantly less than 1 year-old at the time of treatment and where co-morbidities including viral infections, are frequently present, reducing conditioning-related toxicity and improving the rate of immune recovery are of great importance.

Hence, we here explored alternative approaches including the added value of clinically approved mobilizing agents like G-CSF (Granulocyte-Colony Stimulating Factor) or Plerixafor. G-CSF together with Plerixafor are used to mobilize HSCs from the BM niche to the bloodstream for HSC collection in autologous transplants. G-CSF mobilizes by impairing HSC niche function in the BM by suppressing niche-supportive cells and cytokines whereas Plerixafor (also known as AMD3100) directly targets HSC without altering HSC niche function by directly antagonizing the CXCR4-mediated sensing that retains HSCs within the BM [22]. Therefore, we studied whether combining chemotherapy regimens similar to those used in clinical setting with stem cell niche directed therapeutic agents (HSC mobilizing agents) would result in engraftment of transplanted progenitor cells with equivalent efficacy at lower chemotherapy exposure in comparison with current standard chemotherapy-based conditioning. With this aim we first assessed the efficacy and tolerability of busulfan conditioning in mice. Secondly, we examined the direct effect of the chemotherapy and the HSC mobilizing agents in the BM and the HSC niches. Finally, we analysed whether alternative low toxicity conditioning regimens allowed successful and equivalent immune reconstitution in NSG mice compared to high standard chemotherapy dose.

MATERIALS AND METHODS

Human CD34+ cell enrichment

Human cord blood was obtained according to the Medical Ethical Committee and IRB guidelines at Leiden University Medical Center. Cord blood mononuclear cells were separated by Ficoll (Pharmacy Leiden Academic Hospital) gradient centrifugation, frozen in fetal bovine serum (Hyclone)/10% DMSO (Sigma-Aldrich) and stored in liquid nitrogen. After thawing, human $CD34^+$ cells were isolated using CD34 MicroBead UltraPure Kit (Miltenyi Biotec). In short, cells were incubated with FcR blocking reagent and CD34 Microbeads Ultrapure following manufacturer protocol for 30 min at 4ºC. Subsequently CD34+ cells were positively selected using the appropriate ferromagnetic columns and the MACS separator (Miltenyi Biotec). Hematopoietic progenitor Stem Cells (HSPC) count

and purity after isolation was evaluated using a customized Flexicyte Program on NucleoCounter3000 (Chemometec). Directly isolated CD34+ cells were stimulated for 2 days in StemSpan serum-free expansion medium (StemSpan-SFEM; STEMCELL Technologies) supplemented with 10 ng/ml human Stem Cell Factor (huSCF; Miltenyi Biotec), 20 ng/ml human Thrombopoietin (huTPO; R&D Systems), 20 ng/ml recombinant mouse insulin-like growth factor 2 (IGF2; R&D Systems) and 10 ng/ml recombinant human fibroblast growth factor-acidic (hIFG1; PeproTech).

Murine HSPC isolation

Lineage negative depletion was performed using the Lineage Cell Depletion kit from Miltenyi Biotec, to isolate hematopoietic stem cell from frozen murine bone marrow. In short, cells were magnetically labelled with the Biotin-Antibody Cocktail and incubated for 10min at 4ºC and subsequently incubated for 15 minutes at 4ºC with the Anti-Biotin Microbeads. Lineage negative cells were subsequently depleted using the appropriate magnetic columns and the MACS separator (Miltenyi Biotec). Directly enriched HSPC were cultured in StemSpan (SFEM) medium supplemented with Pen/Strep (Gibco), 50ng/mL recombinant mouse (rm) Flt3L, 100ng/mL rmSCF and 10ng/mL rmTPO (all from R&D Systems) at 37˚C with 5%CO_2. Depletion efficiency and purity of lineage negative population was analyzed by flow cytometry with FACSCanto (BD).

Mice

BALB/c Rag2/Il2rg double-knockout mice were a kind gift from Dr. E.J. Rombouts from the Department of Hematology at Erasmus MC (University Medical Center Rotterdam, The Netherlands). C57BL/6 wild-type, BALB/c wild-type and NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were purchased from Charles River (Netherlands & France). Mice were bred and maintained in the animal facility of Leiden University Medical Center (LUMC). All animal experiments were approved by the Dutch Central Commission for Animal experimentation (Centrale Commissie Dierproeven, CCD).

Pre-conditioning of mice

Rag2$^{-/-}$ mice were conditioned with a total body single dose irradiation 24h prior the transplantation using orthovoltage X-rays (8.08Gy) or with two consecutive doses of 25 mg/kg Busulfan (1mg/ml; Sigma-Aldrich) (48h and 24h prior transplantation). NSG mice were conditioned with injected busulfan intraperitoneally, single dose (5mg/kg, 12,5mg/kg and 25mg/kg) 24h prior to cell transplantation or with 2 consecutive doses of 25 mg/kg Busulfan (48h and 24h prior transplantation) for the highest dose (50mh/kg).

HSPC mobilization was performed with G-CSF (Neulasta®, Amgen) up to a total dose of 125µg/kg. Mice were injected subcutaneously 2 consecutive days, 24h apart with the last injection 24h before the transplantation or analysis. Plerixafor (AMD3100, Sigma) was also used as HSC mobilization agent. A single dose of 10mg/kg was injected subcutaneously 1h prior transplantation or analysis. Pre-conditioning of NSG mice with the different regimens described in the paper (Busufan, G-CSF, Plerixafor and combinations) were weight-adjusted per mice.

HSPC transplantation

Cells were harvested and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) without phenol red (Gibco) for intravenous injection into the tail veins of pre-conditioned mice. Human CD34+ enriched cells (100.000 cells per mice) were transplanted into 5-6 week old female NSG mice, while murine HSPCs (mixed with supportive $Rag2^{-/-}$ spleen cells ($3x10^6$ cells/mouse) and transplanted into pre-conditioned $Rag2^{-/-}$ recipient mice (8-12 week old mice).

Mice used for transplantation were kept in a specified pathogen-free section. The first four weeks after transplantation mice were fed with additional DietGel recovery food (Clear H2O) and antibiotic water containing 0.07 mg/mL Polymixin B (Bupha Uitgeest), 0.0875 mg/mL Ciprofloxacin (Bayer b.v.) and 0.1 mg/mL Amfotericine B (Bristol-Myers Squibb) and their welfare was monitored daily. Peripheral blood (PB) from transplanted mice was drawn by tail vein incision and analysed every 4 weeks until the end of the experiment (20 to 24 weeks after transplantation). PB, thymus, spleen and bone marrow were obtained from CO_2 euthanized mice.

Flow cytometry analysis

Single cell suspensions from thymus and spleen were prepared by squeezing the organs through a 70 µM cell strainer (BD Falcon). Bone marrow single cell suspension was obtained from flushed or crushed bones (femur and tibias) and cells were also passed through a 0,7 µm cell strainer (BD Falcon). Erythrocytes from PB and spleen were lysed using NH_4Cl (8,4 g/L)/$KHCO_3$ (1 g/L) solution (LUMC Apotheek). Mononuclear cells were counted and stained with the antibodies listed in Table S1. Briefly, cells were incubated for 30 min at 4°C in the dark with the antibody-mix solution including directly conjugated antibodies at the optimal working solution in FACS buffer (PBS pH 7.4, 0.1% azide, 0.2% BSA). After washing with FACS buffer, a second 30 min incubation step at 4°C was performed with the streptavidin-conjugated antibody solution. When necessary, 7AAD (BD Biosciences) was used as viability dye. Data was acquired on a FACS-CantoII and a LSR Fortessa X-20 (BD Biosciences) and analysed using FlowJO software (Tree Star).

Statistics

Statistics were calculated and graphs were generated using GraphPad Prism6 (GraphPad Software). Statistical significance was determined by one-way or two-way ANOVA test (*p<0.05, **p < 0.01, ***p < 0.001 and ****p<0.0001).

RESULTS

Busulfan conditioning as an alternative to TBI in mice

The standard pre-conditioning method in mice for hematopoietic stem cell (HSC) transplantation is total body irradiation (TBI), varying the irradiation dose depending on the mouse strain. $Rag2^{-/-}$ mice were transplanted with wild-type BALB/c hematopoietic and progenitor stem cells (HSPC) after conditioning with TBI (8,09Gy) or busulfan (50mg/kg) as previously published for immunodeficient mice [23, 24]. An improved welfare and well-being of the animals was observed for mice pre-conditioned with busulfan compared to TBI, with a lower loss of weight and a faster recovery after transplantation (**Figure 1A**).

The survival rate of busulfan-conditioned mice was higher than TBI treated mice (**Figure 1B**), where mice died from irradiation side effects which requires strict and careful animal support and can lead to high mortality rates [24]. In addition, busulfan-conditioned mice showed increased T-cell reconstitution from week 12 after transplantation, represented by a more significant population in the peripheral blood (PB) (**Figure 1C**). Although T-cell development in the thymus including all development stages was comparable between busulfan and TBI conditioned mice (**Figure 1D**), the T-cell output in PB at 20 weeks after transplantation was higher for busulfan-conditioned mice (**Figure 1E**). The immune outcome of busulfan-conditioned (50mg/kg dose) NSG transplanted mice was also comparable to TBI-treated NSG mice previously published [25].

Overall human engraftment, HSC engraftment in the bone marrow (BM) and immune cell distribution of mice pre-conditioned with 50mg/kg dose busulfan (**Figure 2A, 2B** and **2C**) matched TBI-conditioned reference values (horizontal black dot line). Busulfan conditioning may lead to better conservation of tissue integrity than TBI, allowing for a higher immune output after transplantation, mainly seen in the T-cell compartments. Importantly, the welfare and well-being of the animals were improved, without compromising the overall immune recovery. Therefore, busulfan conditioning represents a favourable regimen to use in pre-clinical studies in mice, bringing the model a step closer towards mirroring clinical protocols.

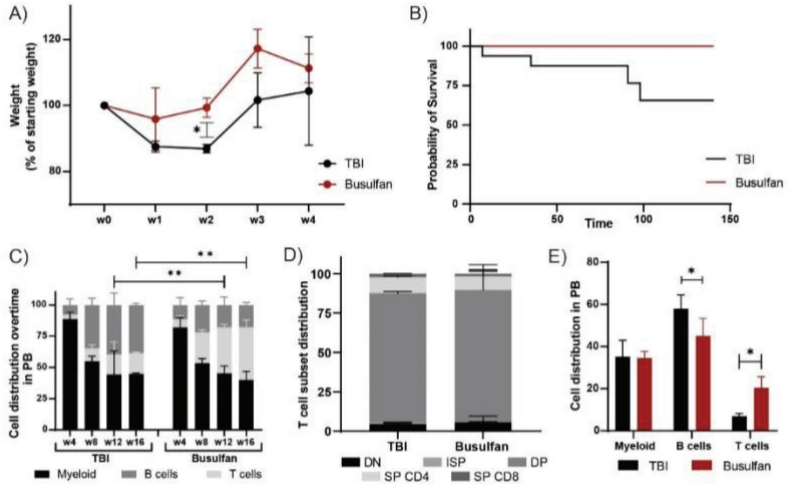

Figure 1: Busulfan conditioning as an alternative of TBI in immunodeficient mice. *Rag2-/- mice transplanted with wild-type BALB/c HSPCs were pre-conditioned by total body irradiation (TBI, 8,09 Gy) or busulfan (50mg/kg). Immune reconstitution was analysed up to 20 weeks after transplantation. A) Mice were weighted weekly during the first month after transplantation. Change of weight normalized to the starting weight before conditioning is depicted in the graph for TBI (2 mice) and busulfan (3 mice) treated mice. (Unpaired t-test; *p<0,05). B) Survival analysis of the TBI-conditioned (16 mice from historical data) and busulfan-conditioned mice after transplantation (4 mice). C) Cell distribution (myeloid, B and T cell) in peripheral blood (PB) over time of mice pre-conditioned with TBI (2 mice) or busulfan (4 mice). (two-way ANOVA; **p<0,01). D) Proportion of the different T-cell*

*developmental subsets in the thymus after TBI or busulfan conditioning. E) Cell distribution (myeloid, B and T cells) in PB 20 weeks after transplantation. (two-way ANOVA; *p<0,05).*

Modelling busulfan conditioning in NSG mice; determining a suitable dose

We first focused on setting the optimal busulfan dose in NSG mice to investigate the possibility of reducing busulfan conditioning before HSCT to reduce associated side effects. A dose of 50mg/kg busulfan was used as starting dose [23, 26-28] reducing it gradually until 5mg/kg. Mice were pre-conditioned with different doses of busulfan (control without busulfan, 5mg/kg busulfan, 12,5mg/kg busulfan, 25mg/kg busulfan and 50mg/kg busulfan as described in Material & Methods) and transplanted intravenously with $1x10^5$CD34+ cells/kg isolated from cord blood (5mice/group). Human chimerism and human immune cell reconstitution were followed up to 20 weeks after transplantation (**Suppl. Figure 1**). Mice were sacrificed and immune organs were thoroughly analysed for human HSC engraftment and human B- and T-cell development. Increasing levels of human chimerism were observed in PB, spleen and BM with increasing busulfan doses, with a significant increase in the group receiving the maximum dose (50mg/kg) compared to the control group and the lower 5mg/kg and 12,5mg/kg doses (**Figure 2A**). As NSG thymi are devoid of murine cells, human engrafted cells completely repopulated the thymus in all dosing groups, showing close to 100% human chimerism in this organ. Although comparable number of human HSC engrafted in BM across the groups (**Figure 2B**), the distribution of immune cell lineages in PB, mainly B and T cells, significantly differed for the highest dose compared to other groups, leading to a higher T-cell contribution (**Figure 2C**). All busulfan doses contributed to an overall normal B cell development in BM (**Figure 2D**) and T cell development in the thymus (**Figure 1F**) with a normal population distribution over the developmental stages. However, significantly higher B-cell (**Figure 1E**) and T-cell (**Figure 1G**) numbers were detected in the periphery (spleen and PB) with the highest dose, while following more moderate doses, immune output was comparable to that of control transplanted mice.

High dose busulfan (50mg/kg) gave reliably higher immune reconstitution and was set as high dose group for the following experiments. Consistent immune development and chimerism were detected for lower busulfan doses, and therefore we set the 12,5mg/kg dose as our low dose busulfan for following experiments where we aimed to improve our low dose busulfan immune outcome by combining with stem cell niche directed non-chemotherapeutic agents.

Short-term effect of busulfan and mobilizing agents on BM HSCs

The principal purpose of conditioning is to make space in BM before transplantation to improve HSC engraftment and immune recovery. Our aim was to reduce the dose of busulfan used, without compromising immune recovery, by combining a low dose busulfan with mobilizing agents. G-CSF (Granulocyte Colony-Stimulating Factor) and Plerixafor are clinically used mobilising agents to collect HSC cells directly from PB instead of BM. We therefore investigated the effect of busulfan, G-CSF and plerixafor as single agents, and G-CSF or plerixafor in combination with low dose busulfan on the HSC compartment of NSG mice (3 mice/group) 24h after the last injection of G-CSF and Busulfan and 1h after Plerixafor. High dose busulfan resulted in a significant reduction of total BM cells (**Figure**

3A). Spleen cell numbers and viability were also significantly compromised with the highest dose of busulfan (**Figure 3B**). In addition, only the high dose busulfan showed a reduction of the HSPC population (named LSK in mouse; lineage-Sca1+ckit-) in NSG mice (**Figure 3C**), mostly explained by the decrease of hematopoietic progenitor cells (HPC; **Lin-**Sca1+cKit+ CD48+**)** and to a lesser extent multipotent progenitor cells (MPP; Lin-Sca1+cKit+CD150-CD48-**)** in BM but no long-term HSCs (Lin-Sca1+cKit+CD150+CD48-) (**Figure 3D**).

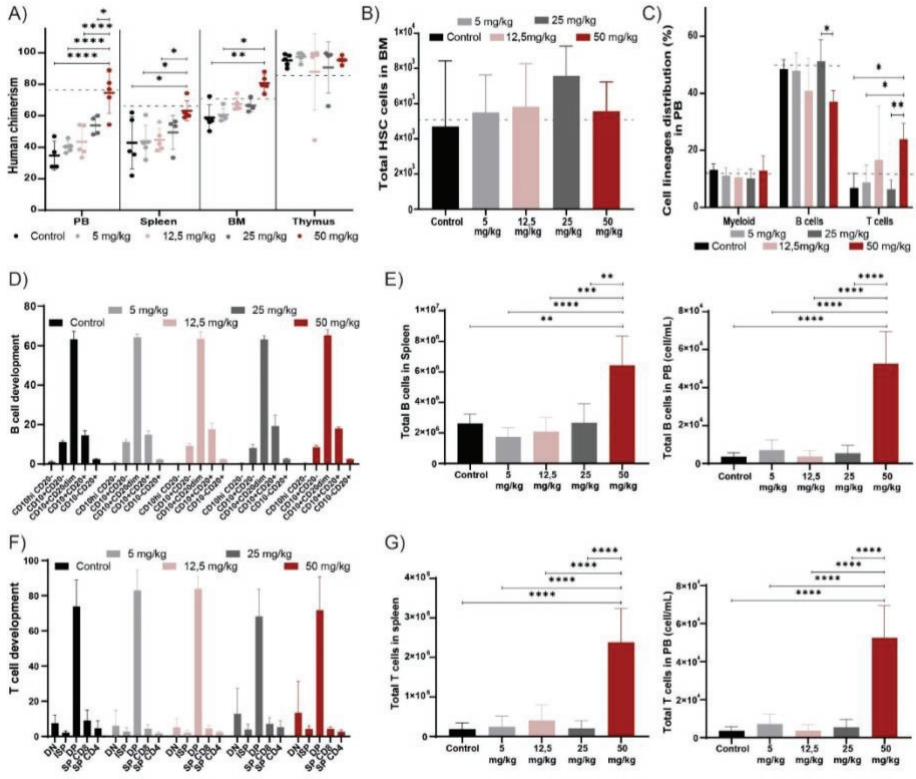

Figure 2: Modelling busulfan conditioning in NSG mice, determining a suitable dose*. NSG mice were pre-conditioned with increasing doses of busulfan (control, 5mg/kg, 12,5mg/kg, 25mg/kg and 50mg/kg) and transplanted with 100.000 human CD34 cells (5 mice/group). A) Human chimerism (% hCD45 cells) achieved in PB, spleen, bone marrow (BM) and thymus 20 weeks after transplantation. Human chimerism achieved by TBI represented by dashed line. (2-way-ANOVA; *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001). B) Total number of human hematopoietic stem cells (HCS) in NSG BM 20 weeks after transplantation. C) Cell lineage distribution (myeloid, B and T cells) in PB 20 weeks after transplantation of the different conditioned groups. (2-way-ANOVA; *p<0,05, **p<0,01). D) Proportion of B cell developmental stages in BM in the different busulfan treated mice. E) Total B cell counts in spleen and in PB 20 weeks after transplantation. (One-way ANOVA; **p<0,01, ***p<0,001, ****p<0,0001). F) Proportion T cell developmental stages in the thymus in the different busulfan treated mice. G) Total T cell counts in spleen and in PB 20 weeks after transplantation. (One-way ANOVA; ****p<0,0001).*

The mobilizing efficiency to peripheral blood of G-CSF and Plerixafor was tested on NSG mice (3 mice/group) as previously published for different mouse strains and with doses adjusted to the NSG mouse strain (**Suppl. Figure 2**) [22, 29-31]. An increased HSPC (LSK) population was detected in PB of NSG mice treated with G-CSF (total 250µg/kg) or Plerixafor (10mg/kg) 24h or 1h after the last injection respectively (**Figure 3E**). In addition, in accordance with Winkler et al (2012) [22] the counts in PB highly increases after G-CSF administration due to the increased release of myeloid cells to the periphery (**Suppl. Figure 2A**). Knowing that G-CSF and Plerixafor are able to mobilise HSPCs in NSG mice, we analysed their effect directly in the BM. G-CSF alone or in combination with the low dose busulfan had no impact on BM cellularity (**Figure 3F, upper graph**). However, significant decrease of the HSPC (LSK) compartment was observed after G-CSF treatment, even more prominent than the decreased induced by the high dose busulfan (**Figure 3F, middle graph**). As for high dose busulfan, this decreased was mainly explained by a reduction of the progenitor compartment, but not of long-term HSCs (**Figure 3F, lower graph**). In contrast, total BM cells were reduced by Plerixafor comparable to high dose busulfan dose (**Figure 3G, upper graph**). Although no significant decrease of the total HSPC (LSK) population was detected, an interesting but nor significant reduction of the long-term HSCs as well as MPPs was observed in mice treated with the combination of plerixafor and low dose busulfan (**Figure 3G, lower graph**).

In summary, high dose busulfan and G-CSF administration alone showed consistent reduction in the number of progenitor cells in BM. However, while the low dose busulfan did not impact the HSPC population in BM, interesting effects were observed when combined with plerixafor, the only condition agent leading to a potential reduction of long-term HSCs**.**

Long-term immune recovery after reduced busulfan conditioning

Finally, we aimed to study if the direct effects of the different conditioning regimens on the cellular composition of the BM would also lead to better engraftment *in vivo* after CD34 transplantation. NSG mice (5 mice/group) were pre-conditioned with different conditioning regimens (low dose busulfan, high dose busulfan, G-CSF, G-CSF+low dose busulfan, Plerixafor and Plerixafor+low dose busulfan) and transplanted with $1x10^5$ CD34/kg enriched cells from cord blood. As previously described, human chimerism increased with increasing busulfan dose. Combining low dose busulfan with either of the mobilizing agents did not increase human chimerism, achieving similar engraftment as with low dose busulfan only. In addition, G-CSF or Plerixafor alone yielded lower human chimerism in BM (**Figure 4A** & **Suppl. Figure 3**). However, no significant differences in the number of human HSC engrafted cells in BM was detected across the conditions (**Figure 4B**). B-cell development in BM was consistent across all conditions (**Figure 4C**), however a lower number B cells was observed in spleen of mice conditioned with the single mobilizing agents. In addition, no difference was observed between the combinations and the low dose busulfan group (**Figure 4D**). In parallel, T-cell development in the thymus was uniform across all conditions, both in early (**Figure 4E**) and late developmental stages (**Figure 3F**). The T-cell output, both CD4+ and CD8+ T cells, was significantly lower after

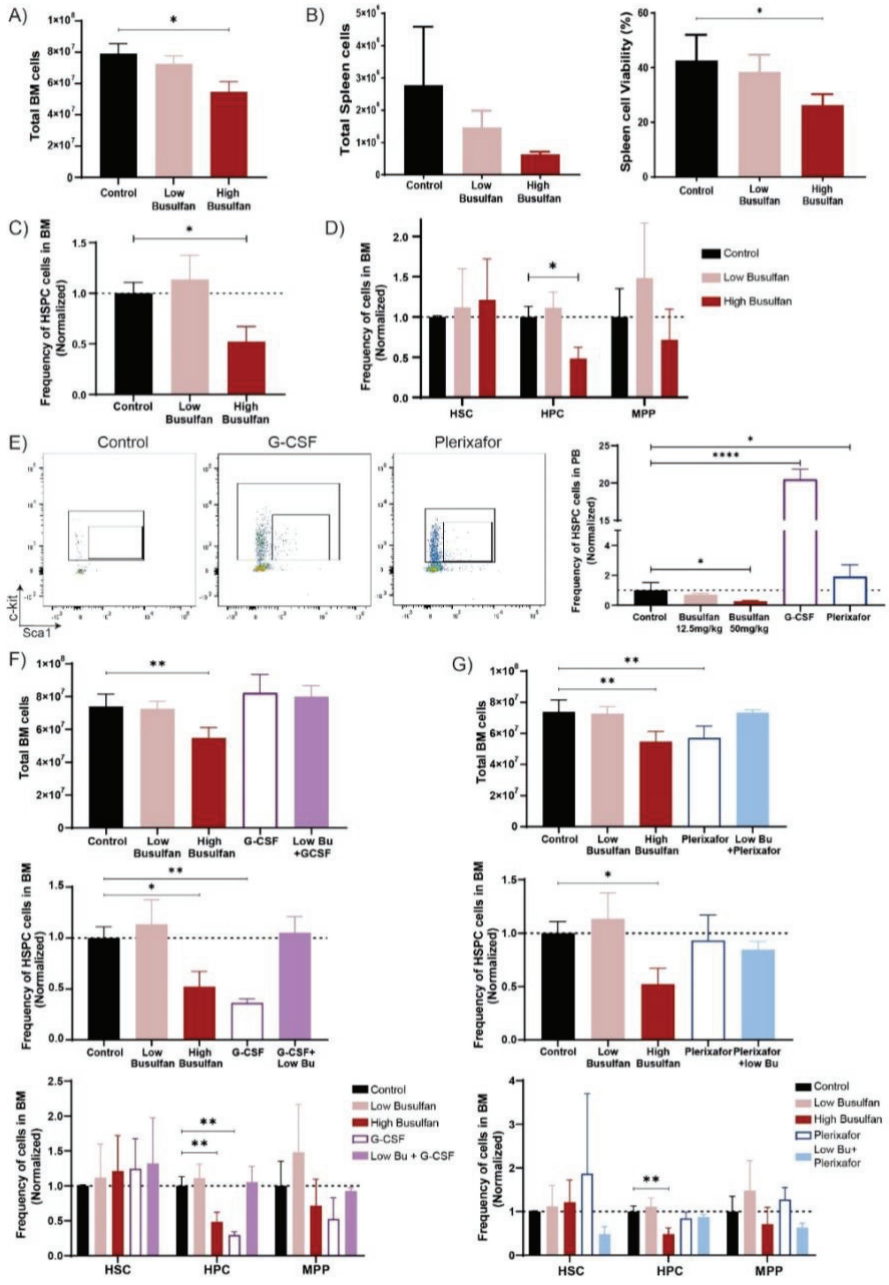

Figure 3: Effect of busulfan and mobilizing agents on BM HSCs. *A) Total BM cell numbers, 24h after busulfan conditioning (low and high dose) compared to the control group (without busulfan). (One-way ANOVA, *p<0,05). B) Total spleen cell numbers and cell viability after busulfan conditioning (24h after). (One-way ANOVA; *p<0,05). C) Frequency of HSPCs (LSK; Lin-Sca1+cKit+) cells in BM 24h after busulfan conditioning, normalized to control mice. (One-way ANOVA; *p<0,05). D) Frequency of long-term HSCs (Lin-Sca1+cKit+CD150+CD48-), hematopoietic progenitor cells (HPC;*

*Lin-Sca1+cKit+CD48+) and multipotent progenitor cells (MPP; Lin-Sca1+cKit+CD150-CD48-). (2-way-ANOVA; *p<0,05). E) Representative FACS plots of PB HSPCs after mobilizing agents injection. G-CSF was measured 1 day after the last injection and Plerixafor 1h after injection. Quantification of HSPCs in PB is depicted in the graph. (One-way ANOVA; **p<0,01, ***p<0,001). F) Mice were conditioned with busulfan (low and high dose), G-CSF or the combination G-CSF+low dose busulfan and analysed 24h after the last injection (3 mice/group). Upper graph: Total BM cells count after conditioning. (One-way ANOVA; **p<0,01). Middle graph: Frequency of HSPCs (LSK; Lin-Sca1+cKit+) cells in BM 24h after conditioning, normalized to control mice. (One-way ANOVA; *p<0,05, **p<0,01). Lower graph: Frequency of long-term HSCs, HPC and MPP cells. (2-way-ANOVA; **p<0,01). F) Mice were conditioned with busulfan (low and high dose), Plerixafor or the combination Plerixafor+low dose busulfan and analysed after the last Busulfan injection or 1h after Plerixafor administration (3 mice/group). Upper graph: Total BM cells count after conditioning. (One-way ANOVA; **p<0,01). Middle graph: Frequency of HSPCs (LSK; Lin-Sca1+cKit+) cells in BM 24h after conditioning, normalized to control mice. (One-way ANOVA; *p<0,05). Lower graph: Frequency of long-term HSCs, HPC and MPP cells. (2-way-ANOVA; **p<0,01).*

single G-CSF or Plerixafor conditioning and no improvement was observed with the combinations compared to using only low dose busulfan (**Figure 4G**). Only an enhanced naive T-cell compartment, most prominent for CD8+ naive cells, was detected by combining plerixafor with a low dose busulfan (**Figure 4H**).

Hence, single mobilizing agents did not yield sufficient immune reconstitution in NSG mice by themselves. In addition, the combination of a low dose busulfan with mobilizing agents did not reveal additive effects, and reconstitution efficiency was primarily driven by busulfan. Only the naïve T-cell compartment seemed to be boosted by Plerixafor. None of the novel combinations reached high dose busulfan reconstitution levels. However, Plerixafor apparently could have more impact on the lymphoid progenitors than on the myeloid which could be interesting to further investigate from a clinical perspective.

DISCUSSION

The NSG mouse model is suitable to study *in vivo* detection and quantification of human HSCs and human immune cells, and can therefore be used to evaluate the effects of stem cell based therapies. Pre-conditioning of mice prior to human HSCT is important to ensure successful homing and HSC development. In murine pre-clinical experiments, the most commonly used conditioning regimen is based on total body irradiation (TBI; x-rays or γ-rays). However, the irradiation procedure induces high stress levels and intestinal damage in the mice, and leads to weight loss and potentially death of the animal in some occasions. Therefore, it is critical to maintain irradiated mice under strict aseptic conditions and continuous health control. In addition, mice can absorb different doses of irradiation depending on their weight and position during the procedure resulting in a heterogenous group of conditioned mice. Alternative conditioning with chemotherapy like busulfan which is commonly used in human HSCT, represents a suitable alternative offering simple, convenient, individual, weight-adjusted and less-toxic conditioning regimen. Busulfan is indeed an attractive and effective alternative conditioning model that allows an improved human immune reconstitution and better well-being and survival of the mice, which is highly important when working with precious patient material.

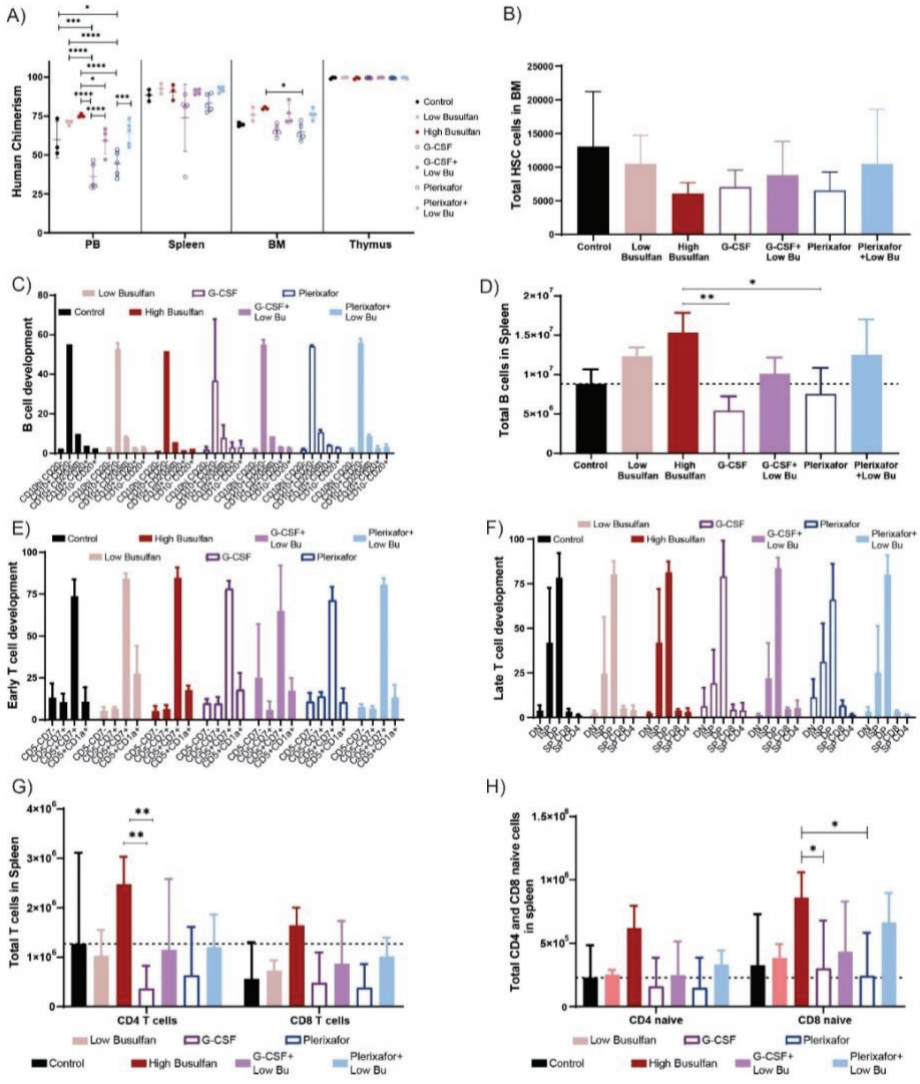

Figure 4: Long-term immune recovery after reduced busulfan conditioning*. NSG mice (5 mice/group) were pre-conditioned with different conditioning regimens (low dose busulfan, high busulfan, G-CSF, G-CSF+low dose busulfan, Plerixafor and Plerixafor+low dose busulfan) and transplanted with 100.000 CD34 enriched cells from cord blood. A) Achieved human chimerism (% hCD45 cells) in PB, spleen, bone marrow (BM) and thymus 20 weeks after transplantation. (2-way-ANOVA; *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001). B) Total number of human hematopoietic stem cells (HCS) in NSG BM 20 weeks after transplantation. C) Proportion of B cell developmental stages in BM in the different conditioned regimen groups. D) Total B cell counts in spleen 20 weeks after transplantation. (One-way ANOVA; *p<0,05; **p<0,01). E and F) Proportion T-cell developmental stages (early and late) in the thymus in the different conditioning regimen groups. G) Total T-cell numbers (CD4+ and CD8+ cells) in spleen 20 weeks after transplantation. (2-way*

*ANOVA; **p<0,01). H) Total naïve T-cell numbers (CD4+ and CD8+ cells) in spleen 20 weeks after transplantation. (2-way ANOVA; *p<0,05).*

Although previous groups already set the most suitable dose of busulfan to condition NSG mice [23, 24, 27, 28], we present here a more extensive analysis of the thymus and T-cell development, leading to higher T cells in the periphery after busulfan compared to TBI as identified also by Choi et al [24]. A more preserved and less damaged thymic tissue after busulfan conditioning compared to TBI may explain the higher T-cell outcome observed. While busulfan may have a more targeted effect on BM, TBI is a general therapy causing damage in thymic and lymphoid tissue that will impact T-cell output. A dose of 50mg/kg busulfan (split in 2 doses 24h apart) provides optimal human cell engraftment not only in NSG mice, but also for other immunodeficient mice like Rag2-/- or Rag1-/- [32]. Normal human B-and T-cell development was obtained also with lower doses of busulfan, but the output of B- and T-cells in the periphery was dose dependent. Chevaleyre et al (2013) [23] described that although increasing human CD45 chimerism was observed with increasing doses of busulfan (as we also described), no impact on the number of colony-forming cells was detected, which would explain that B- and T- cell developmental pattern we observed across the conditions. The direct effect of busulfan and mobilizing agents used in this study (G-CSF and Plerixafor) on BM and HSPC population was analysed in NSG mice. To the best of our knowledge, G-CSF and Plerixafor have not been used previously in the NSG mouse model. Therefore, G-CSF and Plerixafor doses were derived from published literature on other mouse strains [22, 29, 31] and the HSC mobilizing capacity was analysed on PB of the NSG mice. NSG mice show a significant capacity to mobilize HSC to the periphery after G-CSF or Plerixafor administration. While busulfan and G-CSF affect more mature progenitor populations such as HPC and MPP in BM, Plerixafor boosts the reduction in BM and increases mobilization of long-term HSCs.

Although interesting effects on different HSPC populations were observed in BM shortly after administration, the longer term human cell engraftment and immune development after CD34 transplantation did not reflect that direct effect. G-CSF and Plerixafor alone allowed appropriate immune development as described previously by Huston et al [29]. However, when combined with low dose busulfan, no additive effect was observed between the mobilizing agents and the chemotherapy. The main parameters of chimerism and immune development observed in the combination groups were comparable to the low dose busulfan group, meaning that immune reconstitution was triggered by the chemotherapy conditioning rather than the non-chemotherapy agents. Only the naive T-cell compartment tended to be improved by the addition of plerixafor to low dose busulfan, which can be caused by the effect of plerixafor on the long-term HSC cells in BM. More extensive pharmacokinetics and pharmacodynamics studies of busulfan, G-CSF and Plerixafor in NSG mice will help to select the most suitable doses and timings to ensure a proper model for humanized mice. As G-CSF and Plerixafor are clinically approved as mobilizing agents, a small trial with patients has been already performed where patients were pre-conditioned with myeloablative regimen together with G-CSF and Plerixafor prior transplantation. No suitable engraftment was achieved with minimal myeloablative regimen [33], however the addition of G-CSF and Plerixafor to TCRαβ+/CD19+-depletion

regimen appears to solve the problem of graft failure after HSCT, with no additional risks of toxic complications and associated morbidity [34].

To note, the conditioning field is moving towards antibody-based conditioning that will target and potentially deplete stem cells without causing off-target toxicity. Antibody-based conditioning regimens are being developed, which may ultimately achieve long-term myeloid engraftment without the associated toxicities of current chemotherapy-based regimens. Different variations of antibody-based conditioning are being tested both pre-clinically and clinically, such as antibody-drug conjugates specifically targeting HSPCs. Antibody-drug conjugates (ADC) like CD177-ADC [35-37] or CD45-ADC [38-42] have proven to be a safer conditioning regimen than conventional chemotherapy in pre-clinical models. In addition, monoclonal antibodies targeting CD117 [43-46] have been successfully developed and paved the way for the use of anti-CD117 antibody in a currently ongoing clinical trial (NCT02963064). Less toxic and more directed conditioning regimens are needed to improve outcome of all allogeneic and autologous gene therapy stem cell transplantations. The possible implications of these improvements are substantial and could potentially impact allogeneic and autologous transplants worldwide.

SUPPLEMENTARY MATERIAL

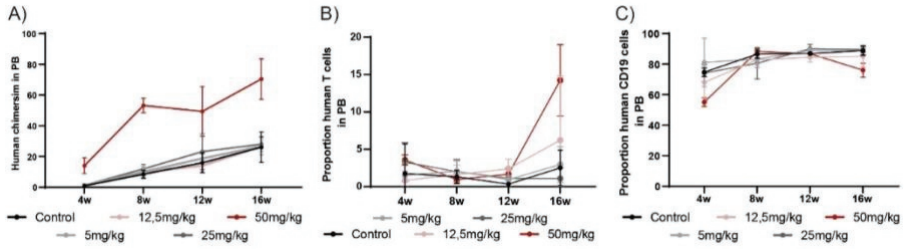

***Figure S1: Human immune reconstitution kinetics after different busulfan dose conditioning in NSG mice**. A) Human chimerism kinetics, B) human T-cell development kinetics and C) Human B-cell development kinetics over time in PB of CD34 transplanted NSG mice after pre-conditioning with different doses of busulfan (Control, 5mg/kg, 12,5mg/kg, 25mg/kg and 50mg/kg).*

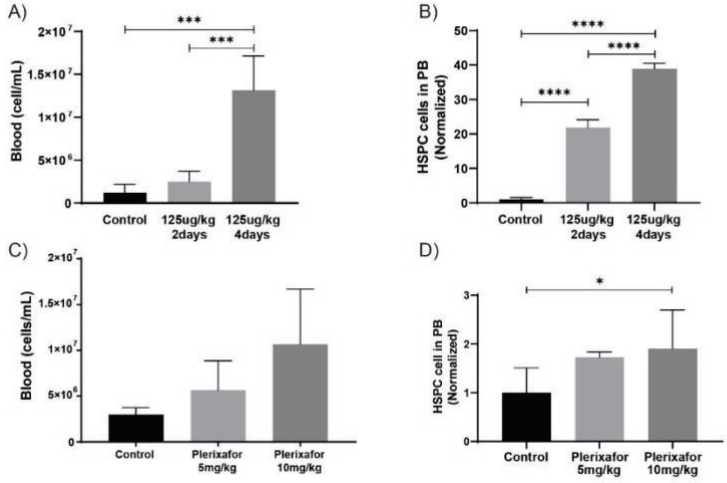

***Figure S2: Mobilizing agents (G-CSF and Plerixafor) dosage in NSG mice.** Effect of different doses of G-CSF in mice A) PB cell numbers and B) HSPC mobilization capacity. Effect of different doses of G-CSF in mice C) PB cell numbers and D) HSPC mobilization capacity. (One-way ANOVA; *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001).*

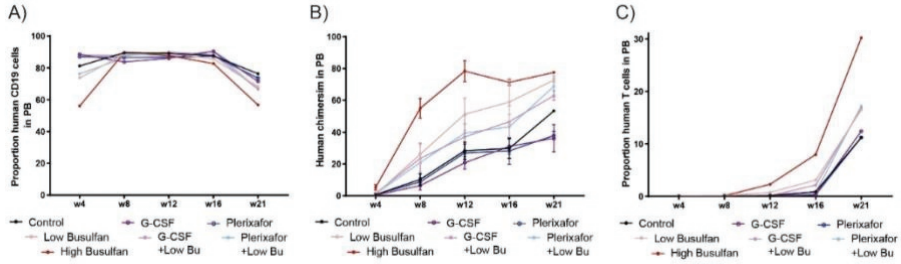

***Figure S3: Human immune reconstitution kinetics after conditioning regimens in NSG mice.** A) Human chimerism kinetics, B) human T-cell development kinetics and C) human B-cell development kinetics over time in PB of CD34 transplanted NSG mice after pre-conditioning with*

different regimens (Control, low dose busulfan, high busulfan, G-CSF, G-CSF+low dose busulfan, Plerixafor and Plerixafor+low dose busulfan).

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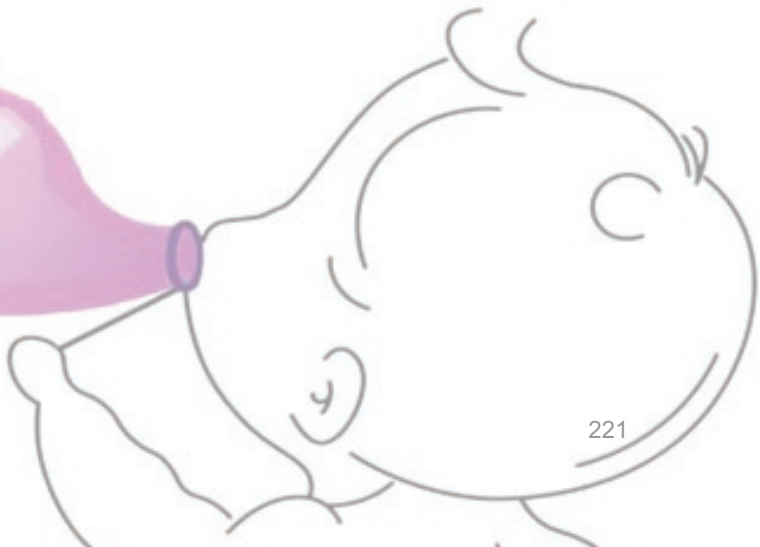
Chapter 8

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.

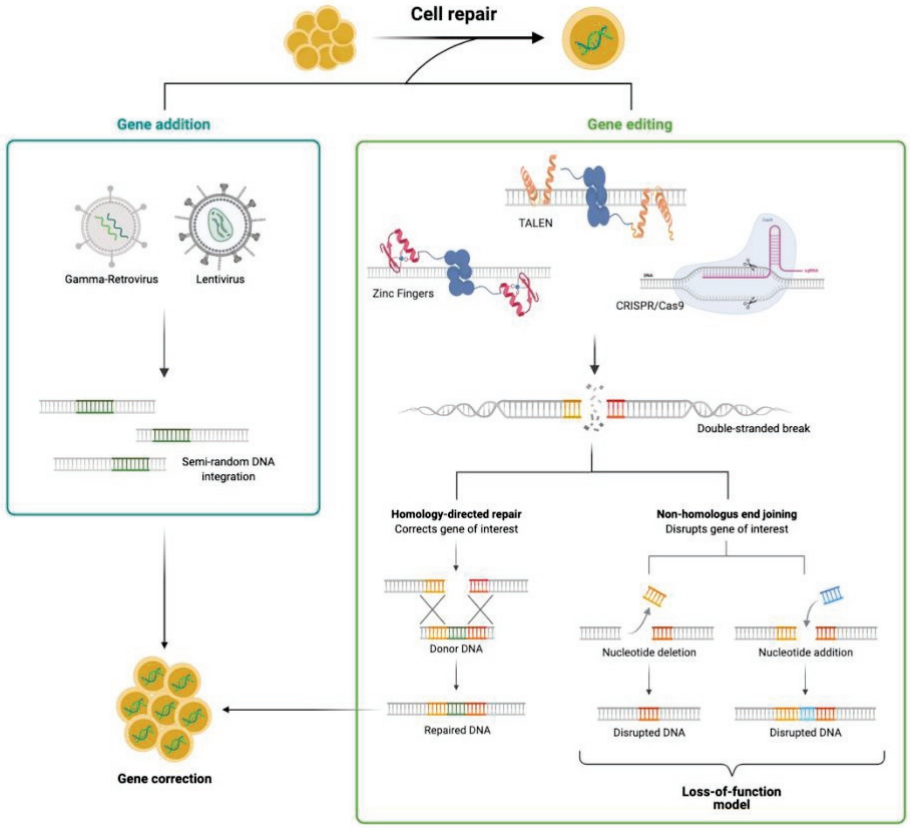


Figure 1: Gene addition vs gene editing approach overview for gene correction on HSCs. *Gene correction of HSCs by gene addition is by semi-random integration into the DNA using retroviral or lentiviral vectors. Gene editing can be achieved by programmable artificial nucleases (Zinc Fingers, Transcription 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.

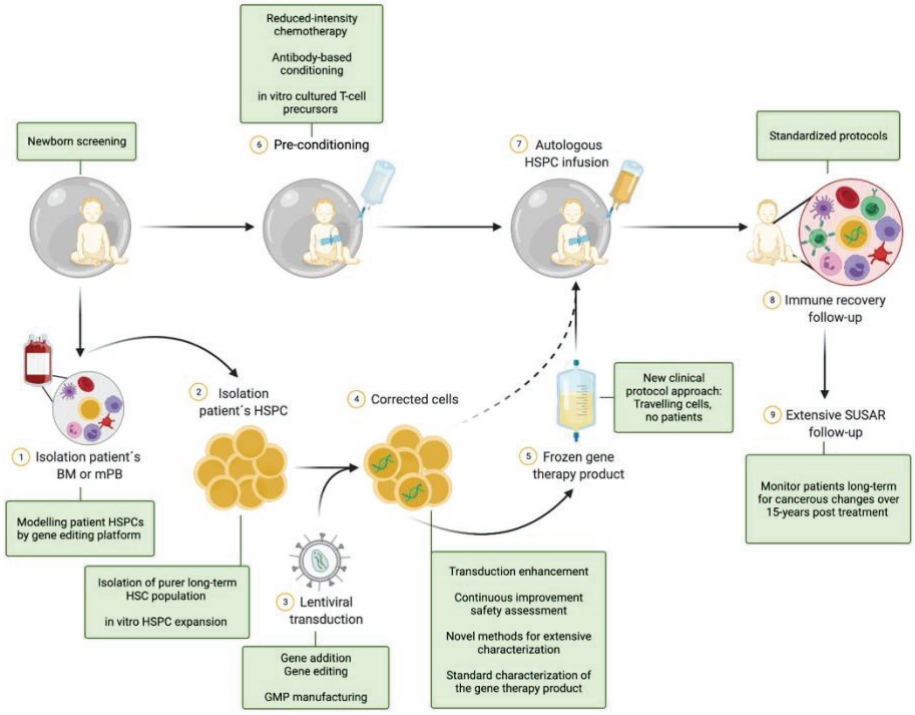


Figure 2: Overview of the autologous HSC gene therapy treatment and potential improved/to improve milestones during the overall procedure.

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Chapter 9

SUMMARY (ENGLISH)

The immune system is a complex, layered mechanism of defence to prevent or limit infections and maintain homeostasis. It consists of an interactive network of lymphoid organs, humoral factors and several types of specialized cells, including B and T lymphocytes that constitute the hallmark of the adaptive immune system. These immune cells develop from hematopoietic stem cells (HSC) that undergo differentiation through a highly regulated succession of developmental steps. Each cell type of the immune system performs a unique specialized role, and their development is strictly regulated by the action of many transcription factors and their target genes.

In **Chapter 2** of this thesis, we gained additional knowledge on normal immune development, with detailed focus on the transcriptional network orchestrating T-cell development. Tcf1, the first T cell-specific protein induced in the thymus regulates the expression of two major target genes, Gata3 and Bcl11b. Tcf1 deficiency results in partial arrests in T cell development, high apoptosis, and altered development of alternative (i.e., non-T) lineages. Phenotypically, seemingly fully T cell-committed thymocytes with Tcf1 deficiency have promiscuous gene expression, an altered epigenetic profile and can dedifferentiate into more immature thymocytes and non-T cells. Restoring Bcl11b expression in Tcf1-deficient cells rescues T cell development but does not strongly suppress the development of non-T cells. In contrast, expressing Gata3 suppresses the development of non-T cells, but does not rescue T cell development. Thus, a minimal transcription factor network ensuring a properly regulated T-cell gene expression program was described: Notch signaling induces Tcf1 expression which subsequently has two target genes, *Gata3* and *Bcl11b*, that accomplish a division of labor with Gata3 suppressing non-T cell lineages and Bcl11b inducing the expression of T-cell specific genes.

The disruption of normal lymphoid development can lead to severe illnesses known as immunodeficiencies. 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 administered. Therapeutic treatments are limited and confined to allogeneic HSC transplantation and emerging autologous stem-cell gene therapy.

In this thesis we focused on developing an efficient and safe lentiviral-based gene therapy approach to correct both Recombinase Activating Gene 1 and 2 (RAG1 and RAG2) immune defects. In **Chapter 3**, I discuss the pre-clinical development, safety and regulatory hurdles across the process and steps to successfully develop gene therapy for immunodeficiencies from the bench to the clinic. Autologous lentiviral-based HSC gene therapy was described in **Chapter 4** and **Chapter 5** as an efficient and safe therapy to correct RAG-SCID deficiency. Full functional immune reconstitution with our MND-c.o.RAG1 lentiviral vector and our PGK-c.o.RAG2 lentiviral vector in murine HSC was observed in murine models (Rag1-/- mice and Rag2-/- mice). B- and T- cell development in BM and Thymus were observed after gene therapy together with a successful functional restoration including immunoglobulin production, diverse T-cell receptor rearrangements

and an effective immune response. Additionally, patient HSCs transduced with our clinical RAG1 vector and transplanted into NSG mice led to improved human B- and T-cell development**.** Successful pre-clinical development together with a favorable safety described in **Chapter 4** substantiates the first phase I/II clinical trial worldwide for RAG1-SCID gene therapy. In parallel, functional immune reconstitution was achieved in the murine Rag2-/- mouse model with a safe clinical PGK-c.o.RAG2 lentiviral vector in **Chapter 5**, although RAG2 gene therapy seems to be more challenging due to the crucial role of optimal RAG2 expression levels.

Additionally, we optimized related protocols by developing novel tools and conditioning regimens for a successful allogeneic and autologous gene therapy transplantation outcome. A novel method for single-cell characterization of the transduction efficiency and transgene expression in the gene therapy product is introduced in **Chapter 6**. The branched DNA technique used showed high specificity, sensitivity, reproducibility and versatility. This method allows to study the heterogeneity within gene therapy products and to reconsider the actual proportion of potential therapeutic cells and the underestimated transgene vector copy number.

Lastly, alternative reduced-chemotherapy conditioning regimens were tested to achieve suitable cell engraftment and immune recovery while reducing short and long-term side effects. In **Chapter 7**, I describe our efforts to develop a reduced chemotherapy (busulfan) regimen by combining it with clinically approved mobilizing agents (G-CSF and Plerixafor) 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 differences were achieved in the transplantation outcome using NSG mouse model. 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.

Altogether, the work described in this thesis moves towards a regular enforcement of gene therapy treatment for immunodeficiencies for which potential milestones during the overall procedure, e.g., newborn screening, cell isolation and transduction, pre-conditioning, standardized protocols and patient monitoring, have been optimized.

NEDERLANDSE SAMENVATTING

Het immuunsysteem is een complex, meerlagig verdedigingssysteem om infecties tegen te gaan en om homeostase te behouden. Het immuunsysteem is opgebouwd uit een interactief netwerk van lymfoide organen, oplosbare componenten en verschillende gespecialiseerde celtypen zoals B en T-lymfocyten. Deze gespecialiseerde witte bloedcellen vormen de belangrijkste cellen van het specifieke immuunsysteem. Alle typen witte bloedcellen ontstaan uit bloedstamcellen in het beenmerg waar ze een aantal sterk gereguleerde, opeenvolgende ontwikkelingsstappen ondergaan. Binnen het immuunsysteem heeft elk type witte bloedcel zijn eigen specifieke rol en hun ontwikkelingstraject wordt gestuurd door verschillende transcriptiefactoren die de genexpressie van hun target genen reguleren en zorgen dat de cellen richting hun definitieve celtype differentiëren.
In **Hoofdstuk 2** van dit proefschrift hebben we meer inzicht verkregen over de normale ontwikkeling van het immuunsysteem, in het specifiek over het transcriptie factor netwerk dat de ontwikkeling van T-cellen reguleert. Tcf1, het eerste T-cel specifieke eiwit dat in de thymus aangezet wordt, reguleert de expressie van twee belangrijke target genen, Gata3 en Bcl11b. Het ontbreken van Tcf1 zorgt voor een gedeeltelijke blokkade in T-cel ontwikkeling, verhoogde celdood en de ontwikkeling van andere afweercellen dan T-cellen in de thymus. Het fenotype van volledig T-cel gecommitteerde thymocieten met een Tcf1 deficiëntie wordt gekenmerkt door heterogene genexpressie, een afwijkend epigenetisch profiel en het feit dat ze terugvallen in hun ontwikkeling naar meer onrijpe stadia waarna ze zich tot andere afweercellen kunnen ontwikkelen. Expressie van Bcl11b in Tcf1-deficiente cellen herstelt de T-cel ontwikkeling maar zorgt niet voor sterke onderdrukking van de ontwikkeling tot een ander type afweercel. De expressie van Gata3 blokkeert, in tegenstelling tot Bcl11b, de ontwikkeling tot andere afweercellen maar herstelt T-cel ontwikkeling niet. Concluderend hebben deze bevindingen laten zien dat er voor een goed gereguleerd T-cel genexpressie programma een minimaal netwerk van transcriptie factoren nodig is: Notch signalering induceert Tcf1 expressie dat vervolgens Gata3 en Bcl11b activeert, waarbij Gata3 zorgt voor de onderdrukking van de ontwikkeling van andere afweercellen en Bcl11b de expressie van T-cel specifieke genen induceert.

Een verstoorde ontwikkeling van lymfocyten kan leiden tot ernstige ziektes zoals immuundeficiënties. Ernstige gecombineerde immuundeficiëntie of “severe combined immunodeficiency” (SCID) is een zeer ernstige afweerstoornis die voorkomt bij kinderen zonder een functioneel immuunsysteem waarbij de afwezigheid van T cellen kenmerkend is. Zonder een gepaste behandeling zullen kinderen met SCID overlijden voor hun eerste levensjaar. Behandelingen beperken zich tot beenmergtransplantatie en recentelijk ook tot stamcel-gentherapie.

In dit proefschrift hebben we ons gericht op het ontwikkelen van een efficiënte en veilige lentivirale gentherapie om genetische defecten te kunnen corrigeren die worden veroorzaakt door zowel Recombinase Activating Gene 1 als 2 (RAG1 en RAG2) deficiëntie. In **Hoofdstuk 3** worden de preklinische ontwikkeling, veiligheid en regulatoire stappen beschreven die genomen moeten worden om een succesvolle gentherapie voor immuundeficiënties naar de kliniek te brengen. Autologe stamcelgentherapie gebaseerd

op lentivirale vectoren wordt beschreven in **Hoofdstukken 4** en **5** als een efficiënte en veilige methode om RAG-SCID deficiëntie te corrigeren. In Rag1-/- en Rag2 -/- muizenmodellen zagen we een volledig functioneel herstel van het immuunsysteem na het toedienen van onze MND-c.o.RAG1 en PGK-c.o.RAG2 lentivirale vector in stamcellen van de muizen. In het beenmerg en de thymus konden we B- en T cel ontwikkeling zien samen met een herstel van antistof productie, hoge mate van diversiteit in herschikkingen van T-cel receptor genen en een effectieve immuunrespons. Daarnaast zagen we ook een verbeterde ontwikkeling van B- en T-cellen vanuit stamcellen afkomstig van patiëntenmateriaal na transductie met onze klinische RAG1 vector en daaropvolgende transplantatie in NSG muizen.

Succesvolle preklinische ontwikkeling samen met bewezen veiligheid vormen een stevige basis voor de start van de eerste wereldwijde klinische fase I/II studie voor RAG1-SCID gentherapie. Parallel hieraan werd bij Rag2-/- muizenmodellen functionele immuun reconstitutie bewerkstelligd met behulp van een veilige klinische PGK-c.o.RAG2 lentivirale vector, wat in **Hoofdstuk 5** besproken wordt. Hierin worden ook de uitdagingen van RAG2 ten opzichte van RAG1 gentherapie besproken, zoals de noodzaak voor het bereiken van optimale RAG2 expressie niveaus.

Ook hebben we bestaande protocollen geoptimaliseerd door het ontwikkelen van nieuwe toepassingen en conditioneringsmethoden met als doel de uitkomsten van allogene en autologe transplantatie voor gentherapie te verbeteren. Een nieuwe methode om op single-cel niveau transductie efficiëntie en transgene expressie in het gentherapie product te kunnen meten wordt geïntroduceerd in **Hoofdstuk 6**. De zogenoemde “branched DNA” techniek laat een hoge mate van specificiteit, gevoeligheid, reproduceerbaarheid en veelzijdigheid zien. Met deze methode kan de heterogeniteit binnen gentherapie producten in kaart gebracht worden, waardoor de daadwerkelijke hoeveelheid van potentiele therapeutische cellen wellicht opnieuw overwogen zou moeten worden; mede door de onderschatting van het aantal kopieën van het transgen per cel.

Als laatste hebben we een alternatieve conditionerings behandeling getest waarin het gebruik van chemotherapie kan worden teruggebracht, met als doel het beperken van korte- en lange termijn bijwerkingen en het behouden van voldoende nesteling van stamcellen en herstel van het afweersysteem. In **Hoofdstuk 7** beschrijven we hoe we een behandeling ontwikkelen waarin het gebruik van chemotherapie (busulfan) verminderd kan worden door het te combineren met klinisch toegepaste mobilisatie middelen (G-CSF en Plerixafor). Deze middelen worden gebruikt om stamcellen te laten verhuizen van het beenmerg naar het perifere bloed. Bij het gebruik van deze nieuwe combinaties inclusief G-CSF of Plerixafor werd in het beenmerg een interessante afname van het stamcel compartiment waargenomen, terwijl in het NSG-muizenmodel geen verschil werd bereikt in de transplantatie resultaten. Recente veelbelovende ontwikkelingen op het gebied van conditionering op basis van antilichamen zijn zeer interessant voor het veld, omdat deze methodes in het ideale geval donor chimerisme oplevert zonder het gebruik van toxische chemotherapeutische middelen.

Samengevat beschrijft het werk in dit proefschrift de route naar de regulering van een behandeling voor immuundeficiënties op basis van gentherapie, waarbij potentiële

mijlpalen voor de algehele procedure zoals screening bij pasgeborenen, cel isolatie en transductie, pre-conditionering, het standaardiseren van protocollen en het monitoren van patiënten allemaal zijn geoptimaliseerd.

RESUMEN (ESPAÑOL)

El sistema inmunitario es un mecanismo de defensa complejo para prevenir o limitar infecciones y mantener la homeostasis. Consiste en una red interactiva de órganos linfoides, factores humorales y varios tipos de células especializada, incluyendo linfocitos B y T que constituyen el sello distintivo de la respuesta inmunitaria adquirida. Estas células inmunes se originan a partir de células madre hematopoyéticas de la medula ósea que se desarrollan y maduran mediante una serie de procesos altamente regulados. Cada tipo celular del sistema inmune desempeña una función especializada única y su desarrollo está estrictamente regulado mediante distintos factores de transcripción y sus genes diana.

En el **Capítulo 2** de esta tesis nos concentramos en obtener conocimientos adicionales del desarrollo inmune, con un enfoque detallado en el conjunto transcripcional que orquesta el desarrollo de células T. Tcf1, la primera proteína especifica de las células T inducida en el timo, regula la expresión de dos de sus principales genes diana, Gata3 y Bcl11b. La deficiencia de Tcf1 provoca un arresto parcial en el desarrollo de células T, una apoptosis prominente y un desarrollo alterado de células alternativas (es decir no-células T). Fenotípicamente, las células T aparentemente especializadas con deficiencia de Tcf1 tienen una expresión génica heterogénea, un perfil epigenético alterado y pueden des-diferenciarse en células T inmaduras y otros tipos celulares. Al restaurar la expresión de Bcl11b en células madre con deficiente Tcf1 conseguimos rescatar el desarrollo de las células T, pero no suprime el desarrollo de células alternativas. Al contrario, la expresión de Gata3 suprime el desarrollo de células no-T, pero no consigue rescatar el desarrollo de linfocitos T. Por lo tanto, describimos una red mínima de factores de transcripción que garantizan una regulación optima del programa génico de las células T: Notch induce la expresión de Tcf1 que posteriormente actúa sobre dos genes diana, Gata3 y Bcl11b, que logran dividirse esfuerzos con Gata3 suprimiendo el desarrollo de células no-T y Bcl11b induciendo la expresión de genes específicos de células T para el desarrollo de estas células.

La alteración del desarrollo normal de los linfocitos puede causar enfermedades graves conocidas como inmunodeficiencias. La inmunodeficiencia combinada severa (llamada en ingles SCID, Severe Combined Immunodeficiency) es un desorden inmune devastador que afecta a bebés carentes de un sistema inmune funcional, en particular células T. Desafortunadamente, los bebes con SCID mueren durante su primer año de vida a menos que reciban un tratamiento eficaz. Los tratamientos terapéuticos para SCID se limitan al trasplante de células madre de donante sano y a la emergente terapia génica basada en células madre del paciente corregidas.

En esta tesis nos enfocamos en desarrollar una terapia génica lentiviral eficiente y segura para corregir los defectos inmunes derivados del mal funcionamiento del gen activador de la recombinación (RAG) 1 y RAG2. En el **Capítulo 3**, analizo el desarrollo preclínico, la seguridad y los obstáculos regulatorios a lo largo del proceso y pasos para realizar con éxito terapia génica para inmunodeficiencias, desde el laboratorio hasta la clínica. En el **Capítulo 4** y **Capitulo 5** se describe una terapia génica lentiviral eficaz y segura para

corregir RAG-SCID. Observamos una completa reconstitución funcional del sistema inmune en modelos de ratón (Rag1-/- mutante y Rag2-/- mutante) con nuestro vector lentiviral MND-c.o.RAG1 y PGK-c.o.RAG2 en células madre de ratón. Tras la terapia génica se percibe el desarrollo de células B y T en la medula ósea y el timo, junto con una reparación funcional incluyendo la producción de inmunoglobulinas, receptores de células T con gran diversidad y una respuesta inmune eficiente. Además, al probar la terapia génica con el vector clínico de RAG1 en células madre de paciente se observó gratamente una mejora en el desarrollo de células B y T humanas. Un desarrollo preclínico exitoso junto con una seguridad favorable descrita en el **Capítulo 4** reafirma el primer ensayo clínico de fase I/II en todo el mundo para terapia génica para pacientes RAG1-SCID. En paralelo, la reconstitución inmune se logró en el modelo de ratón Rag2-/- con un vector lentiviral PGK-c.o.RAG2 clínico seguro en el **Capítulo 5**, aunque la terapia génica para RAG2 parece ser más desafiante debido al papel crucial de los niveles óptimos de expresión de esta proteína.

Además, nos centramos en optimizar protocolos relacionados mediante el avance de nuevas herramientas y regímenes de condicionamiento para obtener un resultado exitoso tras el trasplante de células madre. En el **Capítulo 6** presentamos un método novedoso para la caracterización (uni)celular de la eficiencia de corrección y expresión transgénica en el producto de terapia génica. La técnica de ADN ramificada utilizada muestra una alta especificidad, sensibilidad, reproducibilidad y versatilidad. Este método permite estudiar la heterogeneidad dentro de los productos de terapia génica y reconsiderar la proporción real de células potencialmente terapéuticas y el número de copias del transgén subestimado hasta ahora.

Por último, experimentamos regímenes de condicionamiento alternativos de quimioterapia reducida para lograr un injerto celular adecuado y una recuperación inmune, al tiempo que se reducen los efectos secundarios a corto y largo plazo. En el **Capítulo 7**, describo nuestros esfuerzos para desarrollar un régimen de quimioterapia reducida (busulfán) combinándolo con agentes movilizadores clínicamente aprobados (G-CSF y Plerixafor) utilizados para movilizar las células madre hematopoyéticas de la médula ósea hacia la sangre. Si bien se observó una reducción interesante de un grupo de células madre en la médula ósea con las nuevas combinaciones que incluyen G-CSF o Plerixafor, no se lograron diferencias significativas tras el trasplante utilizando el modelo de ratón humanizado. Los desarrollos más recientes y prometedores de condicionamiento basados en anticuerpos son extremadamente atractivos para este campo, ya que idealmente proporcionarán la capacidad de lograr un trasplante exitoso sin el uso de agentes quimioterapéuticos tóxicos.

En conjunto, el trabajo descrito en esta tesis progresa hacia una aplicación regular del tratamiento de terapia génica para tratar inmunodeficiencias. Varios procesos importantes durante el procedimiento general se han optimizado, como por ejemplo la detección precoz en recién nacidos, el aislamiento y corrección de las células madre, los regímenes de condicionamiento, unos protocolos estandarizados y el monitoreo de pacientes.

RESUM (CATALÀ)

El sistema immunitari és un mecanisme complex de defensa per prevenir o limitar les infeccions i mantenir l'homeòstasi. Consisteix en una xarxa interactiva d´òrgans limfoides, factors humorals i diversos tipus cel·lulars especialitzats, inclosos el limfòcits B i T que constitueixen el segell distintiu de la resposta immunitària adquirida. Aquestes cèl·lules immunitàries provenen de cèl·lules hematopoètiques de la medul·la.la òssia que es desenvolupen i maduren a través d'una sèrie de processos altament regulats. Cada tipus cel·lular del sistema immunitari té un paper especialitzat únic i el seu desenvolupament està estrictament regulat per diferents factors de transcripció i els seus gens diana.

En el **Capítol 2** d'aquesta tesi ens concentrem en obtenir coneixements addicionals de el desenvolupament immunitari, enfocant-nos detalladament en el conjunt transcripcional que orquestra el desenvolupament de les cèl·lules T. Tcf1, la primera proteïna específica de cèl·lules T induïda en el timó, regula la expressió de dos dels seus principals gens diana, Gata3 i Bcl11b. La deficiència de Tcf1 provoca una arrest parcial en el desenvolupament de cèl·lules T, una apoptosis prominent i un desenvolupament alterat de cèl·lules alternatives (és a dir, cèl·lules no T). Fenotípicament, les cèl·lules T aparentment especialitzades amb deficiència de Tcf1 tenen una expressió gènica heterogènia, un perfil epigenètic alterat, i poden desdiferenciar-se en cèl·lules T immadures i altres tipus cel·lular. En restaurar l'expressió de Bcl11b en cèl·lules mare amb deficiència de Tcf1, vam aconseguir rescatar el desenvolupament de cèl·lules T, però no vam suprimir el desenvolupament de cèl·lules alternatives. Per contra, l'expressió de Gata3 suprimeix el desenvolupament de cèl·lules no T, però no aconsegueix rescatar el desenvolupament de limfòcits T. Per tant, descrivim una xarxa mínima de factors de transcripció que garanteixen una regulació òptima de el programa gènic de cèl·lules T: Notch indueix l'expressió de Tcf1 que posteriorment actua sobre dos gens diana, Gata3 i Bcl11b, que aconsegueixen dividir esforços amb Gata3 suprimint el desenvolupament de cèl·lules no-T i Bcl11b induint l'expressió de gens específics de cèl·lules T per al desenvolupament d'aquestes cèl·lules.

L'alteració del desenvolupament normal dels limfòcits pot causar malalties greus conegudes com immunodeficiències. La immunodeficiència combinada severa (anomenada en anglès SCID, Severe Combined Immunodeficiency) és un trastorn immunitari devastador que afecta els nadons que no tenen un sistema immunitari funcional, en particular les cèl·lules T.

En aquesta tesi ens enfoquem a desenvolupar una teràpia gènica lentiviral eficient i segura per corregir els defectes immunitaris derivats del mal funcionament del gen activador de recombinació (RAG) 1 i RAG2. En el **Capítol 3** analitzo el desenvolupament preclínic, la seguretat i els obstacles regulatoris al llarg del procés i passos per realitzar amb teràpia gènica per immunodeficiències amb èxit, des del laboratori fins a la clínica. En el **Capítol 4** i **Capítol 5** es descriu una teràpia gènica lentiviral eficaç i segura per corregir RAG-SCID. Observem una completa reconstitució funcional del sistema immune en model de ratolí (RAG1-/- i RAG2-/-) amb el nostre vector lentiviral MND-c.o.RAG1 i PGK-c.o.RAG2 en cèl·lules mare de ratolí. Després de la teràpia gènica es percep el

desenvolupament de cèl·lules B i T a la medul·la.la òssia i el tim, juntament amb una reparació funcional incloent la producció d'immunoglobulines, receptors de cèl·lules T amb gran diversitat i una resposta immunitària eficient. A més, en provar la teràpia gènica amb el vector clínic de RAG1 en cèl·lules mare de pacient es va observar una grata millora en el desenvolupament de cèl·lules B i T humanes. L'èxit del desenvolupament preclínic juntament amb una seguretat favorable descrita al **Capítol 4** reafirma el primer assaig clínic en fase I/II del món pel tractament de teràpia gènica per a pacients amb RAG1-SCID. Paral·lelament, es va aconseguir la reconstitució immunitària en el model Rag2-/- de ratolí amb un vector PGK-c.o.RAG2 clínicament segur en el **Capítol 5**, tot i que aquesta teràpia gènica sembla ser més desafiant a causa de el paper crucial dels nivells òptims d'expressió d'aquesta proteïna.

A més, ens centrem en optimitzar diversos protocols relacionats mitjançant l'avanç de noves eines i règims de condicionament per obtenir un resultat reeixit després del transplantament de cèl·lules mare. En el **Capítol 6** presentem un mètode innovador per a la caracterització (uni)cel·lular de l'eficiència de correcció i expressió transgènica en el producte de teràpia gènica. La tècnica d'ADN ramificada utilitzada mostra una alta especificitat, sensibilitat, reproductibilitat i versatilitat. Aquest mètode permet estudiar l'heterogeneïtat dins dels productes de teràpia gènica i reconsiderar la proporció real de cèl·lules potencialment terapèutiques i el nombre de còpies del transgènic subestimat fins ara.

Finalment, vam experimentar règims de condicionament alternatius de quimioteràpia reduïda per aconseguir un empelt cel·lular adequat i la recuperació immunitària, alhora que reduïm els efectes secundaris a curt i llarg termini. En el **Capítol 7**, descric els nostres esforços per desenvolupar un règim de quimioteràpia reduït (Busulfan) combinant-lo amb agents mobilitzadors clínicament aprovats (G-CSF i Plerixafor) utilitzats per mobilitzar cèl·lules mare hematopoètiques de la medul·la òssia a la sang. Si bé es va observar una reducció interessant d'un grup de cèl·lules mare en la medul·la.la òssia amb noves combinacions que inclouen G-CSF o Plerixafor, no es van aconseguir diferencies significatives després del transplantament utilitzat un model de ratolí humanitzat. Els desenvolupaments de condicionament més recents i prometedors basats en anticossos són extremadament atractius en aquest camp, ja que idealment proporcionaran la capacitat d'aconseguir un transplantament reeixit sense l'ús d'agents quimioterapeutics tòxics.

En conjunt, el treball descrit en aquesta tesi avança cap a una aplicació regular del tractament de teràpia gènica per tractar immunodeficiències. Diversos processos importants durant el procediment general s'han optimitzat, com ara la detecció precoç en nounats, l'aïllament i correcció de les cèl·lules mare, els règims de condicionament, uns protocols estandarditzats i el seguiment de pacients.

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CURRICULUM VITAE

Laura García Pérez was born on June 7th, 1990, in Andorra La Vella, Andorra. After graduating from Lycée Comte de Foix in 2008 (Andorra), she moved to Spain where she followed her bachelor's degree in biotechnology at the University of Lleida. For the last 6 months, she entered the Erasmus Programme and was able to pursue her studies at Hedmark University, Norway. After obtaining her diploma in 2012, she moved to the Netherlands and started in 2013 her master program in Biomedical Sciences, research specialization, at Leiden University Medical Center (LUMC). During this period, she performed two research internships within the LUMC: one at the Nephrology Department on type-2 diabetes and a second one in the Stem Cells and Lymphocyte Development group at the Department of Immunology. Her master thesis focused on understanding the role and functional definition of T cell-specific transcription factors regulating T lymphocyte lineage commitment. She graduated with her master's degree in 2015.

Soon after, January 2016, she started her PhD at the same department in the Stem Cells and Lymphocyte Development research under the supervision of Prof.F.J.T. Staal and Dr. K.Pike-Overzet. Her main focus is to develop hematopoietic stem cells-based gene therapy for severe combined immunodeficiencies due to Recombinase-Activating Gene deficiency and optimizing related protocols under the European Union's Horizon 2020 research and innovation programme SCIDNET.

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