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

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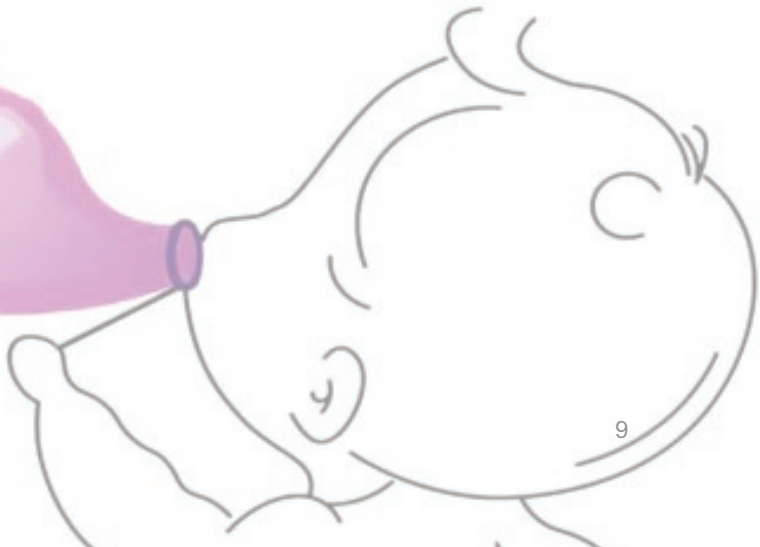
Chapter 1

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%

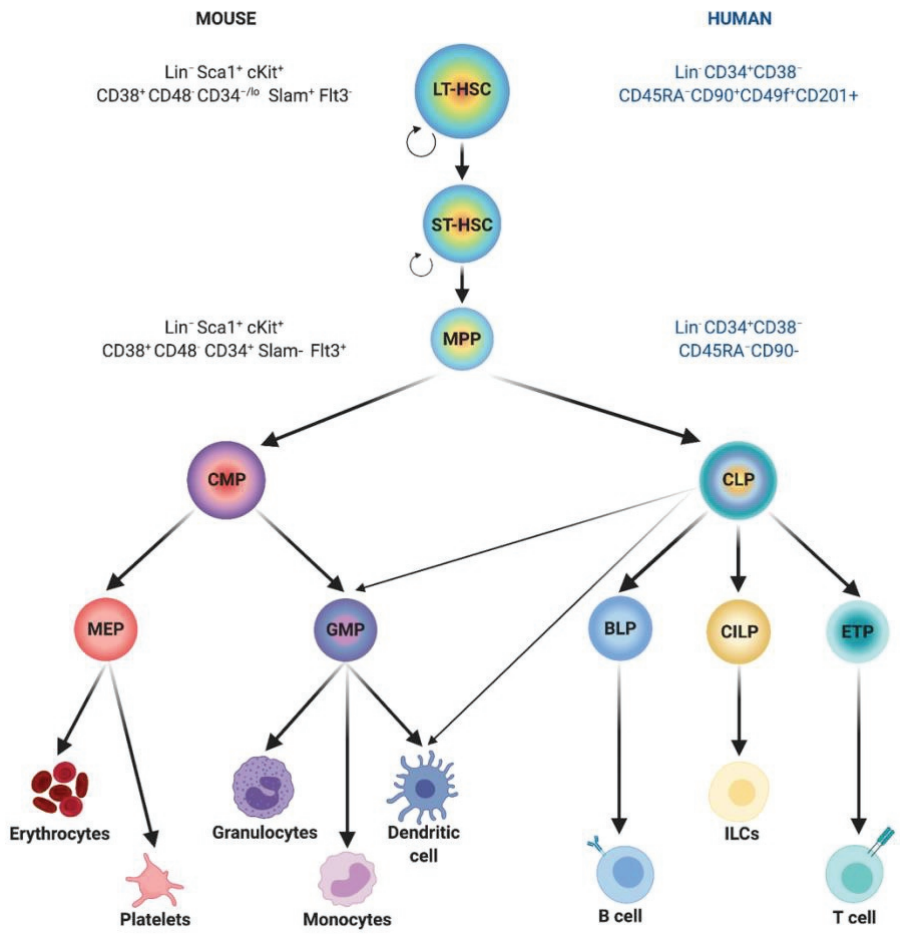


Figure 1: Overview of the classic model of hematopoiesis. *All blood cell lineages arise from hematopoietic stem cells (HSC), a heterogenous rare population that have the unique dual capacity of self-renewal and multipotency to maintain the pool of HSCs, and to differentiate into all blood cell types 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].

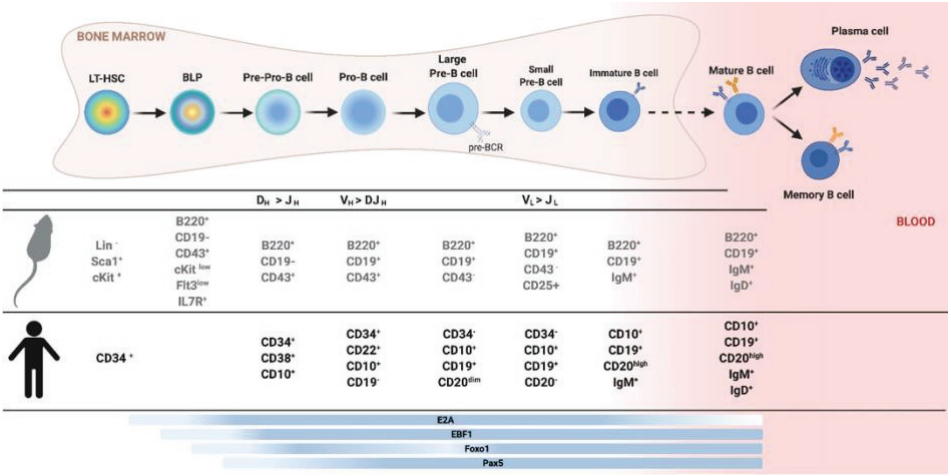


Figure 2: Schematic overview of B cell development in the bone marrow. *Murine and human B cells develop in the bone marrow (BM) from the B-cell-biased lymphoid progenitors (BLP) cells, progenitor cells with the B-cell differentiation program initiated. Cells develop through successive comparable stages 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].

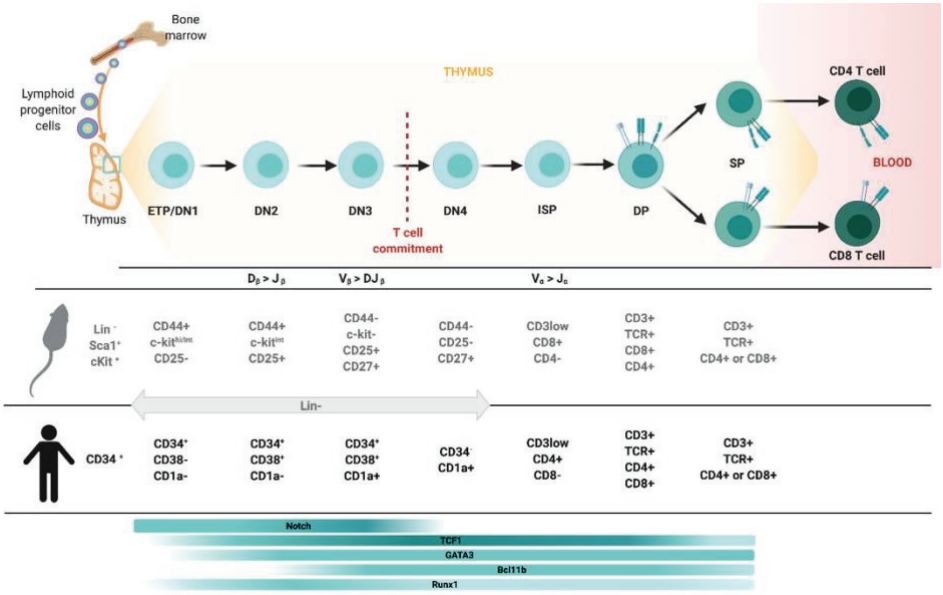


Figure 3: T-cell development in the thymus overview. *T cells develop within the specialized microenvironment of the thymus. Few bone marrow hematopoietic progenitors migrate from the bone marrow and seed the thymus. Subsequent developmental stages are described for mouse and human according to the surface 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].

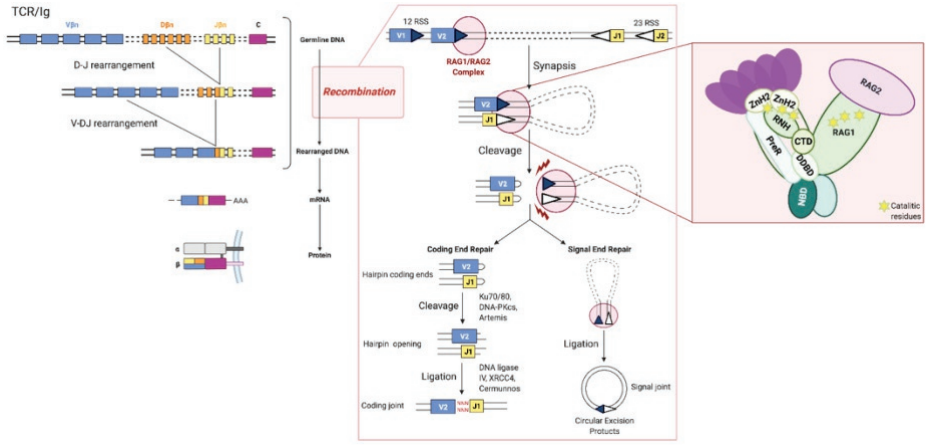


Figure 4: Schematic overview of TCR/Ig gene rearrangement by V(D)J recombination and highlighting the structure of the RAG1/2 recombinase complex. *Highly diverse repertoire of B-cell receptors (BCR) and T-cell receptors (TCR) on mature B and T cells are generated during lymphopoiesis from a substantial 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].

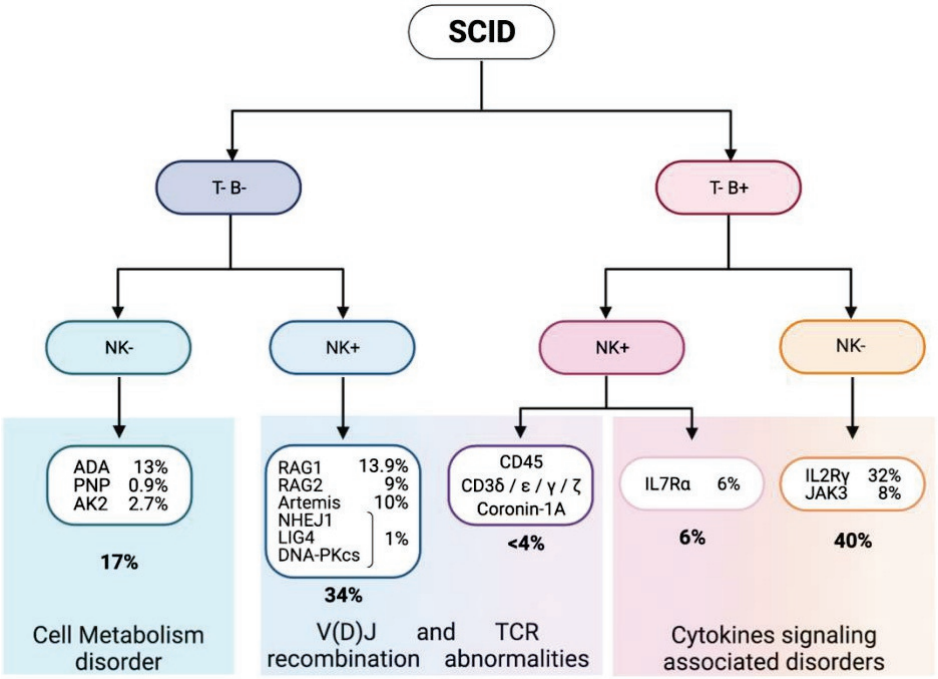


Figure 5: Severe Combined Immunodeficiency (SCID) classification. *Classification of SCID according to 3 major types of deficiency based on the main affected pathways: 1) SCID concerning metabolic enzymes, with a T-B-NK- immune phenotype, affects highly proliferating cells such as Adenosine deaminase (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.

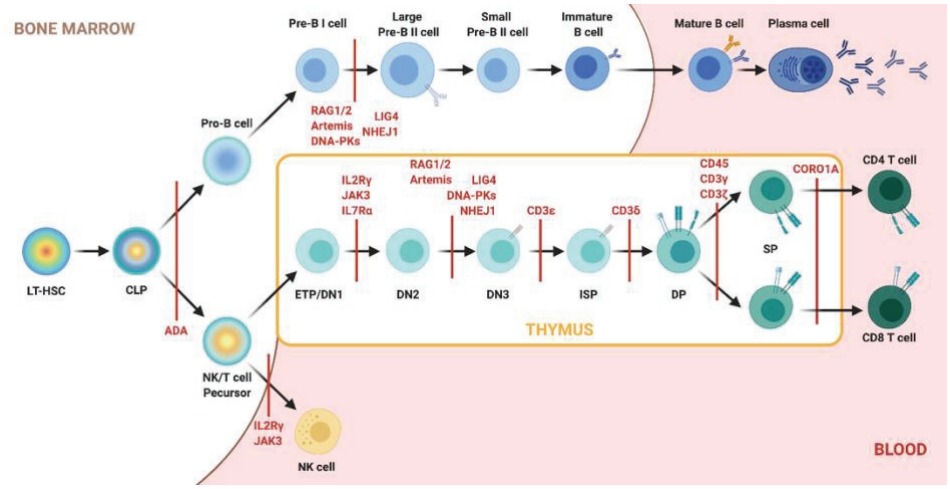


Figure 6: Schematic representation of the developmental blocks in B, T and NK cell development in SCID. *Lymphoid development blocks caused by the different genes are indicated. B–cell developmental arrests are commonly visualized by flow cytometry and Immunoglobulin repertoire analysis on available 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].

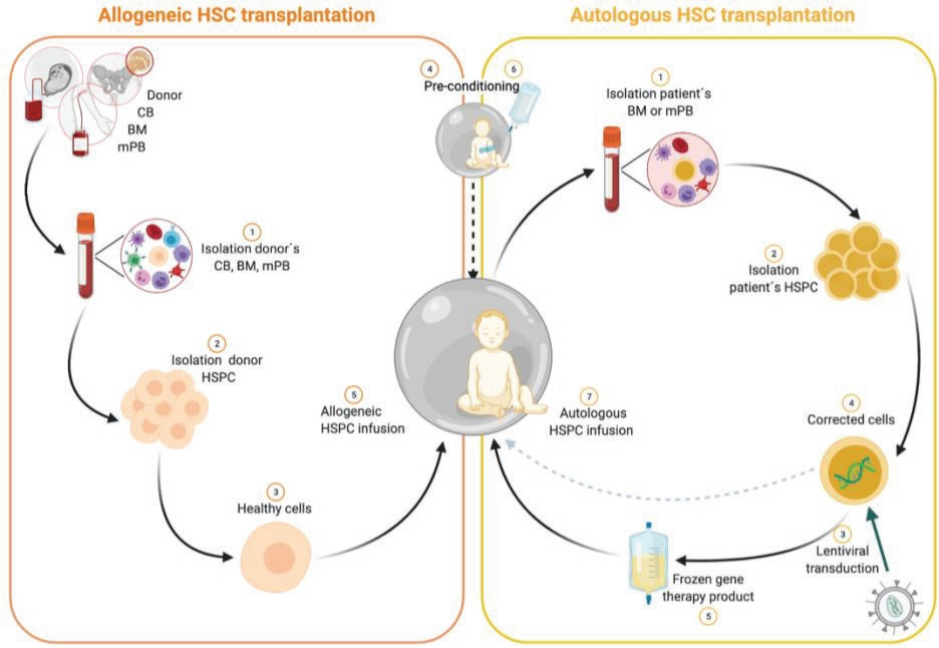


Figure 7: Therapy options for SCID: overview of allogeneic and autologous HSCT. *Conventional allogeneic hematopoietic stem cell (HSC) transplantation relies on the isolation of hematopoietic stem and progenitor cells (HSPCs) from a healthy compatible donor (1). Enriched healthy HPSC (2)(3) can be transplanted 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

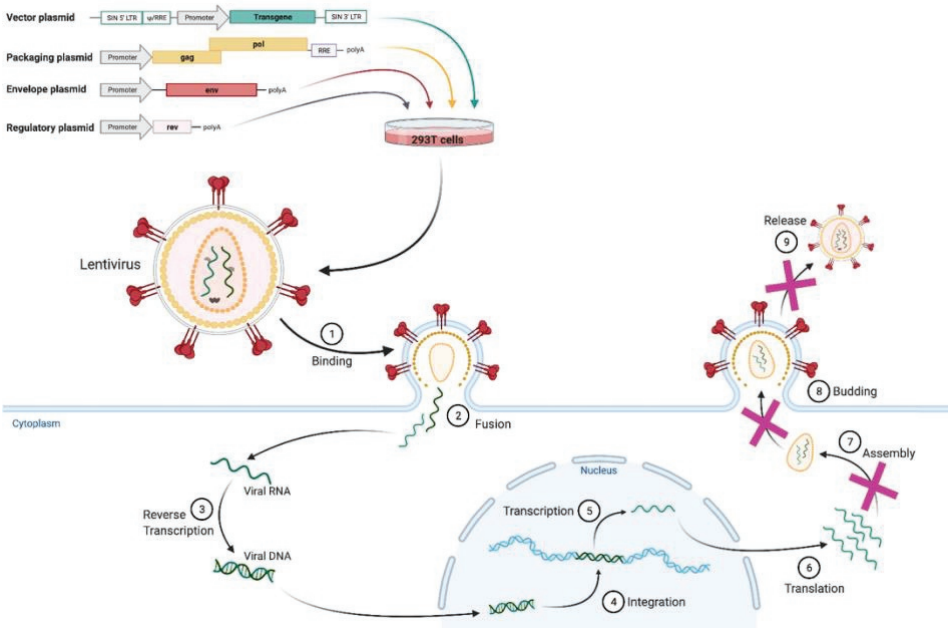


Figure 8: Schematic representation of the 3rd generation lentiviral vector (LV) system. *The 3rd generation LV system consist of a 4-plasmid packaging design in which all essential viral genes have been separated into several plasmids. The 4 plasmids (vector, packaging, envelope and regulatory plasmids) 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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