Gene Therapy for RAG-deficient Severe Combined Immunodeficiency

Gentherapie voor RAG-deficiënte ernstige gecombineerde immuundeficiëntie

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“The gene therapy bubble”
The cover shows a schematic drawing of a retrovirus that contains soap bubbles. Retroviral gene therapy is capable of providing the protective bubble for SCID patients. However, the soap bubbles also symbolize the vulnerable state of a developing technique, which gene therapy still is.

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PROEFSCHRIFT

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Ter nagedachtenis van mijn moeder

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

The human body has several ways of combating bacteria, viruses, and other pathogens, as we usually do not contract an illness from the millions of microorganisms we are exposed to on a daily basis. Physical barriers, such as our epithelial layers, and physiological defense mechanisms such as the low pH in our stomach and a slightly acidic layer on our skin, form a first line of defense. When pathogens succeed in passing the physical and physiological barriers, cells of the immune system play an important role in attacking them. Macrophages and neutrophils are the main cell types involved in the first immunological response to invading pathogens. Both cell types are capable of engulfing and digesting microorganisms and are part of the innate immune system. In addition to being responsible for the early defense against microbes, the innate immune system plays an important role in activating the adaptive immune system.

The adaptive immune system, also called the antigen-specific immune system, consists of lymphocytes and antibodies and is capable of recognizing and fighting a wide range of pathogens. T and B lymphocytes make up the cellular compartment of adaptive immune system. These cells recognize a large variety of antigens, stretches of amino acids, polysaccharides or lipids present on or derived from pathogens. B lymphocytes produce antibodies. T lymphocytes function either as ‘stimulators’ in immune responses, or directly destroy infected cells, mainly virus-infected cells. In addition, the adaptive immune system has the ability to remember and adjust to the pathogens they encounter.

A compromised adaptive immune system can lead to severe illness and death, underscoring its significance. The most striking example of an affected antigen-specific immune system is severe combined immunodeficiency (SCID). Genetic mutations that cause this inherited disease have been found in about a dozen genes. These mutations prevent lymphoid cells from developing. SCID can be cured by transplantation of hematopoietic precursor cells and recently, gene therapy has emerged as a treatment alternative. In this introduction, normal and defective development of lymphoid cells is discussed. In addition, methods for treating SCID through gene therapy are described.

LYMPHOID DEVELOPMENT

T cells, B cells and natural killer (NK) cells are the three major types of cells that can be distinguished within the lymphoid lineage. NK cells are part of the innate immune system and play a key role in the host-defense against virally infected cells as well as tumors. Upon activation of NK cells, granules that contain cytotoxic proteins are released from their cytoplasm, resulting in the destruction of the target cell. T and B lymphocytes make up the antigen-specific adaptive immune system and are both able to recognize a broad spectrum of antigens. T cells bear so called T-cell receptors (TCR) on their cell surface that are capable of recognizing unique antigens when presented in the context of

major histocompatibility complex (MHC) molecules by cells of the innate immune system or by B cells. Similar to T cells, B lymphocytes express specialized antigen receptors, called B-cell receptors or immunoglobulins (Ig), with a single antigenic specificity. These Igs are membrane-bound but can also be secreted when B cells mature into plasma cells. The secreted form of B-cell receptors are called antibodies, and are part of the humoral immune system. Like all blood cells, lymphocytes are highly specialized cells that develop from a common stem cell through a process called lymphopoiesis. Key decisions made during the multi-step process of lymphopoiesis depend on a large number of signals conveyed by cell-cell interactions, soluble factors, and the extracellular matrix within stromal microenvironments at specialized sites of maturation.

Hematopoietic stem cells

The stem cells that give rise to all blood lineages are known as hematopoietic stem cells and mainly reside in the bone marrow. Figure 1 shows a schematic overview of hematopoiesis. It is estimated that about 1 in 100,000 cells in the bone marrow is a true hematopoietic stem cell. Despite their low frequency, hematopoietic stem cells have the ability to produce high numbers of new blood cells each day. The potential to sustain the supply of blood throughout an individuals lifespan can be attributed to two features that characterize hematopoietic stem cells: self-renewal and pluripotency. Self-renewal of hematopoietic stem cells is defined as the ability to divide while retaining undifferentiated features. The pluripotency of hematopoietic stem cells refers to their capacity to differentiate into all types of blood cells. Pluripotency of HSC was initially demonstrated by the lack of hematopoietic failure after total-body irradiation of mice that were injected with bone marrow cells.[1] hematopoietic stem cells are cells with a slow division rate. Up to 20% of all divisions are asymmetrical, resulting in one daughter cell that retains stem cell features and the other daughter cells will differentiate.[2-4] It is difficult to identify hematopoietic stem cells by their size and shape, since their morphology and behavior in culture resembles that of white blood cells. The development of monoclonal antibodies recognizing cell surface markers as well as fluorescence activated cell sorting (FACS) has facilitated the purification of small subsets of cells. In combination with *in vivo* and *in vitro* assays, populations that contain cells with hematopoietic stem cell capacities have been identified. Besides from bone marrow, human hematopoietic stem cells can also be isolated from umbilical cord blood, and when mobilized by granulocyte-colony stimulating factor (G-CSF), from peripheral blood.[5, 6]

Murine HSC are characterized by the expression of Sca-1, C-kit, CD38, low expression levels of the Thy-1, low to absent CD34 and the lack of markers specific for differentiated hematopoietic lineages (B220, Mac-1, Gr-1, CD3, CD4, CD8 and Ter119). The most widely used HSC population in mouse is the so called LSK population: lineage marker negative, Sca-1$^+$ and C-kit$^+$. Within this population two subsets can be distinguished, namely, long-term[7-10] and short-term[8] repopulating HSC. Human HSC express CD34, CD59, CD133 and Thy-1 and lack CD38, C-kit and lineage markers. For both mouse and man, other

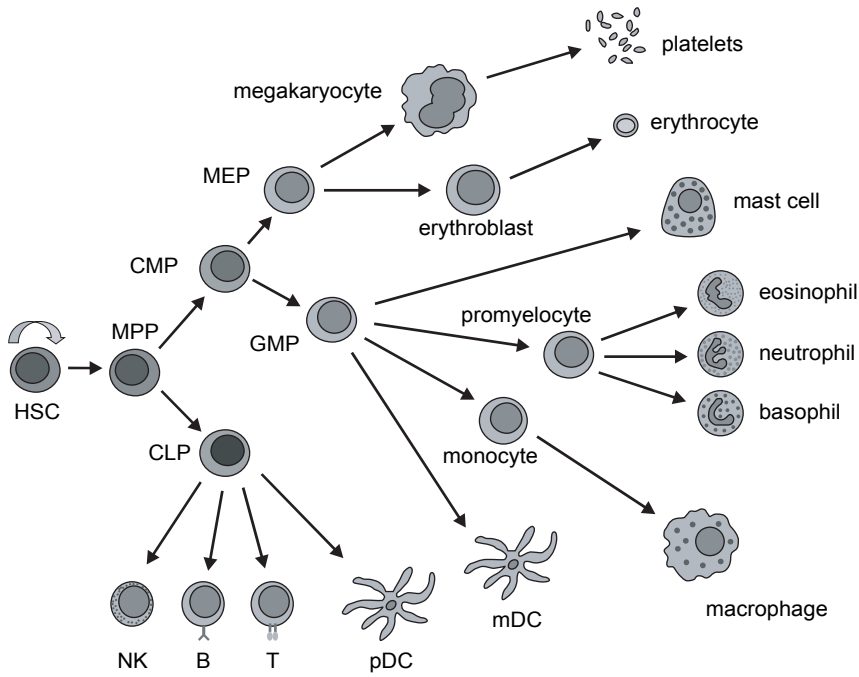

Figure 1. Schematic overview of hematopoiesis.
Hematopoietic stem cells (HSC) are responsible for blood cell production throughout the lifetime of an individual. HSC can give rise to a number of different hematopoietic lineages while retaining the capacity for self-renewal. Lineage-committed progenitor cells produce progeny capable of giving rise to platelets, erythrocytes, mast cells, granulocytes, macrophages, dendritic cells and lymphocytes. MPP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/monocyte progenitor; MEP, megakaryocyte/erythrocyte progenitor; NK, natural killer; pDC, plasmacytoid dendritic cell; mDC, myeloid dendritic cell.

markers are continuously evaluated and added in an attempt to more precisely define true hematopoietic stem cells.

Because they can give sustained reconstitution of all blood lineages, transfer of hematopoietic stem cells has been used in numerous therapeutic protocols. Conditions that are regularly treated by hematopoietic stem cell transfer include leukemia, lymphoma, different types of inherited anemia, inborn metabolic disorders, Wiskott-Aldrich syndrome, and SCID.[11-16]

V(D)J recombination

For hematopoietic stem cells to become specialized B and T lymphocytes, the cells will have to undergo several developmental steps. A common feature of T- and B-cell development is the process of gene rearrangement of TCR and Ig loci, respectively.

This highly specialized process of cutting and pasting gene segments within the Ig and TCR loci, also called V(D)J recombination, assures the generation of antigen receptors with an extensive repertoire. Antigen receptors are made up of constant and variable domains. The constant domains are equipped to provide structure, effector function, and signaling of antigen receptors, while the variable domains are responsible for specific antigen recognition.[17, 18] If each unique antigen receptor would be coded by a separate gene, the size of the human genome would not suffice. Therefore, the exon coding for the variable domain of Ig and TCR proteins is generated by more or less random recombination of variable (V), diversity (D), and joining (J) gene segments. The Ig heavy chain locus consists of V, D and J segments, whereas kappa and lambda light chain loci only have V and J segments. Similarly, the *TCRA* and *TCRG* loci are composed of V and J segments, while the *TCRB* and *TCRD* loci contain V, D and J segments. In loci containing V, D and J segments, the D to J rearrangement takes place first, followed by V to DJ rearrangements. An example of V(D)J recombination is shown in Figure 2. The initiation of recombination is directed by recombination signal sequences (RSSs) that flank the coding gene segments. RSSs are built up of two conserved sequences, a heptamer and a nonamer, separated by a non-conserved spacer sequence of 12 or 23 base pairs. The consensus heptamer sequence is CACAGTG and the nonameric consensus sequence is ACAAAAACC. Generally, recombination occurs between an RSS with a 12-base pair spacer and an RSS with a 23-base pair spacer, the so-called “12/23 rule”. Recombination activating gene (RAG) 1 and RAG2 proteins are indispensable for initiation of V(D)J recombination.

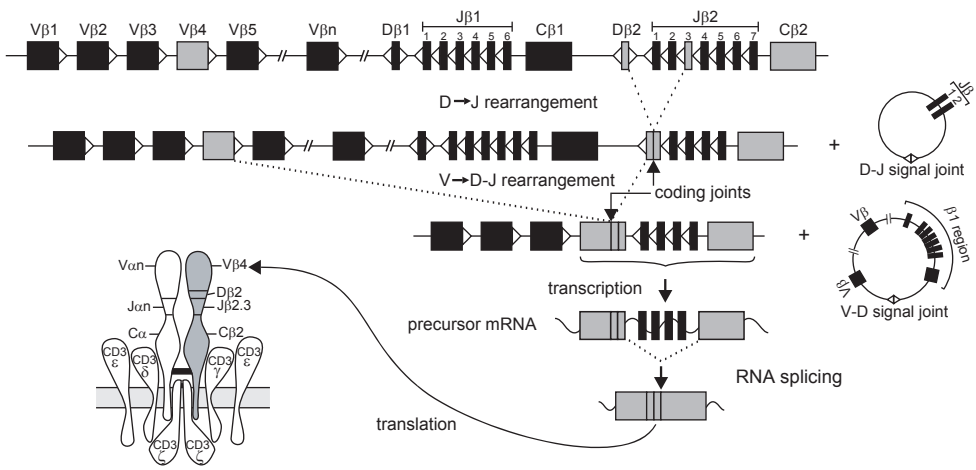

Figure 2. Schematic overview of *TCRB* gene rearrangement.
During *TCRB* gene rearrangement, Dβ to Jβ rearrangement occurs first, followed by a Vβ to Dβ-Jβ rearrangement step. In the process of *TCRB* gene rearrangement, two extra-chromosomal TCR excision circles are formed that bear the D-J signal joint and the V-D signal joint. The rearranged *TCRB* gene is transcribed into precursor mRNA and subsequently translated into protein.

The process of V(D)J recombination can be subdivided into several phases; synapsis, cleavage, and DNA repair (Figure 3). In the first step of the recombination process, RAG1 and RAG2 bind to an RSS as a complex. This RAG-RSS complex then associates with a second RSS, completing the process of synapsis. Subsequently, this synaptic complex facilitates the nucleolytic activity[19, 20] of RAG1 that hydrolyses one strand of the DNA at the junction of the coding segments and the RSSs. Next, the 3'-hydroxyl group that results

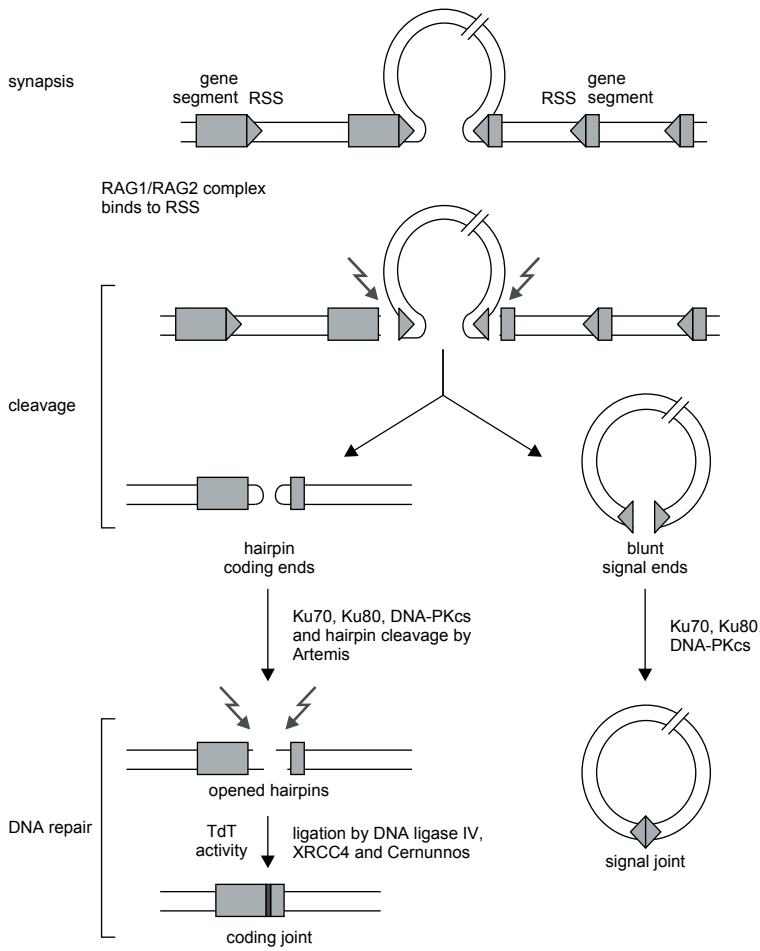

Figure 3. Schematic representation of V(D)J recombination.
The complex of RAG proteins bind the RSSs that flank the antigen receptor gene segments. The RAG protein complex cleaves the DNA at the border of the RSSs, resulting in two coding ends that bear hairpin structures, and two blunt signal ends. During the DNA repair phase, a complex of Ku70, Ku80 and DNA-PKcs directly join the signal ends. This complex, in conjunction with Artemis, is also required for processing of the coding ends. Artemis cleaves the hairpins, after which the coding ends are modified by endonuclease and TdT activity. Finally, a complex of DNA ligase IV, XRCC4 and Cernunnos ligates the ends to form a coding joint.

from the hydrolysis, attacks a phosphodiester bond on the anti-parallel strand forming a hairpin. This cleavage step results in the formation of two blunt ends on the side of the RSS (the signal ends) and two hairpins on the side of the coding sequences (the coding ends). In the next phase of V(D)J recombination, the double strand breaks are repaired, ultimately yielding a signal joint and a coding joint. This ligation requires the use of several proteins of the non-homologous end joining machinery. Formation of a signal joint calls for the presence of the factors Ku70, Ku80, XRCC4 and DNA ligase IV that facilitate ligation of the blunt signal ends. Through this ligation step, a circular excision product is formed. This product is now regularly used as a tool to measure the number of divisions a T cell has undergone.[21, 22] More recently, a similar assay has been developed for calculating the number of divisions in B cells.[23]

The formation of a coding joint is more complex. For coding joint formation, a Ku70/Ku80 heterodimer binds to DNA ends and recruits the DNA-dependent protein kinase catalytic subunit (DNAPKcs). This complex with kinase activity subsequently recruits and activates Artemis by phosphorylation.[24] The activated form of Artemis has nuclease activity with the ability to open the hairpin structures.[24] After opening of the hairpins, several nucleotides can be deleted from or added to the coding ends, creating an extra layer diversity on top of the diversity resulting from the combination of different gene segments. Nucleotides that are added to the junction of two gene segments can either be palindromic (P nucleotides) or non-templated (N nucleotides). P nucleotides are added when the opening of the hairpin is asymmetric. The lymphoid specific enzyme terminal deoxynucleotidyl transferase (TdT) is responsible for insertion of N nucleotides to the coding joint.[25, 26] In the final phase of coding joint formation, ligation of modified coding ends is mediated by XRCC4, DNA ligase IV, and Cernunnos.

In summary, Ig and TCR gene segments are ligated through V(D)J recombination, creating receptors that can recognize a multitude of antigens. During this process junctional diversity is introduced by excision and addition of nucleotides. The stretch of DNA located between the rearranged segments is excised from the genome resulting in a circular excision product bearing the signal joint.

Structure and function of RAG proteins

RAG proteins are fairly large proteins. An amino acid sequence homology of about 90% is found between human and murine RAG proteins. As most research on the function of RAG protein domains was done in murine models, the functional domains of murine RAG proteins are discussed here. Mouse RAG1 contains 1040 amino acids and mouse RAG2 consists of 527 amino acids (Figure 4). The enzymatic activity of the RAG proteins is largely concentrated in the core regions; the minimal regions required for DNA cleavage. Amino acids 384-1008 of RAG1 and amino acids 1-387 of RAG2 retain most of the DNA cleavage activity. The core regions are highly conserved between species.[27, 28] The RAG1 core contains three acidic residues (D600, D708, and E962) making up the DDE motif. This DDE motif forms the active site for DNA cleavage by the RAG-protein complex.[20]

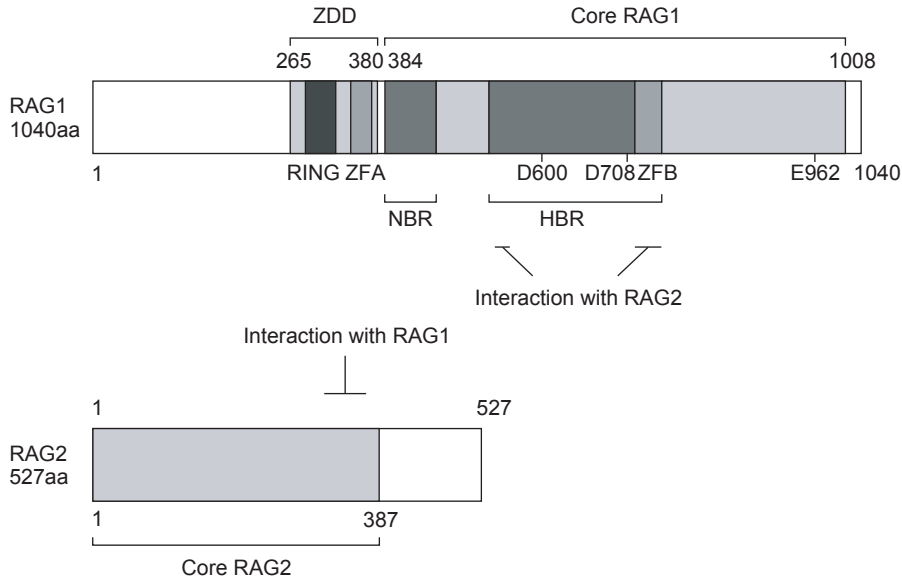

Figure 4. Schematic representation of murine recombination activating gene (RAG) 1 and 2.
Murine RAG proteins are depicted schematically as bars and several functional domains are indicated. The core regions are the minimal regions required for the catalysis of DNA cleavage. The locations of amino acid residues making up the active site for DNA cleavage are indicated (D600, D708, and E962). The nonamer-binding region (NMR), the heptamer-binding region (HBR), and a zinc finger motif (ZFB) are situated within the core region of RAG1. The zinc-binding dimerization domain (ZDD) of RAG1 harbors a RING finger (RING) and a zinc finger motif (ZFA). Areas known to be involved in RAG1-RAG2 interactions are pointed out.

The central domain, amino acids 528-760, of core-RAG1 specifically binds to the RSS heptamer.[29, 30] Amino acids 384-454 within the core region of RAG1 comprise a nonamer-binding region that specifically interacts with the conserved nonamer of the RSS.[31, 32] Regions outside the core domain also contribute to the function of RAG1. Amino acid residues 265-380 contains a zinc-binding dimerization domain that harbors a RING finger motif and a C2H2 zinc finger. The RING finger motif was found to have ubiquitin ligase activity, of which the physiological substrate is currently unknown.[33] Additional functions have been assigned to non-core RAG1 sequences, such as an additional zinc-binding site and three basic regions that bind RNA polymerase 1, a protein that facilitates nuclear transport. The contribution of RAG2 to the recombination reaction is thus far not clear, beyond the fact that its presence is essential for high affinity binding of RAG1 protein to DNA and specificity of DNA cleavage.[29, 34] RAG2 has no reported DNA binding capacity. It was predicted by computational analysis that core-RAG2 forms a six-bladed β-propeller, a structure that mediates protein-protein binding. The non-core sequence of RAG2 has been shown to enhance V to DJ rearrangement, to suppress transposition

events mediated by the core-RAGs and to contain areas that function in cell-cycle dependent RAG2 expression.

V(D)J recombination and transcription factors

Recombination of V(D)J segments is not just dependent on the presence of proteins of the recombination machinery described above. In addition, gene segments have to be made accessible for recombination. This requires remodeling of the chromatin that is tightly associated with the genome. Ig and TCR gene segments are made accessible under the influence of transcription factors in a highly regulated manner. For B-cell development the transcription factors E2A,[35] EBF,[36] and PAX5[37, 38] are important for establishing the accessibility of the Ig loci. It is less clear which factors are involved in opening of the TCR loci. In mice, IL-7R signaling was found to be required for *TCRG* locus accessibility.[39] In addition, E2A and HEB proteins have proven capable of inducing TCR gene rearrangements.[40] Although widely investigated, detailed control of opening of the TCR loci is not clear to date.

T-cell development

T-cell development is a highly regulated, multi-step process aimed at generating mature, functional T cells bearing TCRs that are capable of recognizing a broad range of antigens in the context of self-MHC. In contrast to all other hematopoietic lineages, that develop in the specialized microenvironment of the bone marrow, development of T cells from pluripotent hematopoietic stem cells takes place in the thymus. Throughout life, T-cell precursors from the bone marrow seed the thymus and differentiate into T cells. Mature T cells express a heterodimeric T-cell receptor that is either composed of one α chain and one β chain or one γ chain and one δ chain. When progressing through T-cell development, cells undergo lineage commitment, T-cell receptor gene rearrangements, proliferation and selection.

Using cell surface markers, several T-cell developmental stages can be distinguishes (Figure 5). Primarily, thymocytes are subdivided into double negative (DN), double positive (DP) and single positive (SP) populations, referring to the expression of the co-receptors CD4 and CD8. The most immature thymocytes lack expression of both CD4 and CD8 and are therefore called DN. In mouse and humans additional but different surface markers are used to further subdivide the DN stage. For mouse, the markers CD25 and CD44 are used: $CD44^{+}CD25^{-}$ cells are called DN1 cells, $CD44^{+}CD25^{+}$ cells are referred to as DN2, DN3 cells express CD25 but no CD44, and DN4 cells express neither CD25 nor CD44. The most immature human thymocyte population is characterized by the expression of CD34, but lacks CD1a and CD38 expression, and resembles the murine DN1 population. The next stage of differentiation is marked by the expression of both CD34 and CD38 and resembles the murine DN2 stage. The most mature human DN stage that can be discerned is made up of cells expressing CD34 and CD38 as well as CD1a. Thymocytes undergo a substantial number of cell divisions, 6 to 10, in the first DN stages.[41] The first round

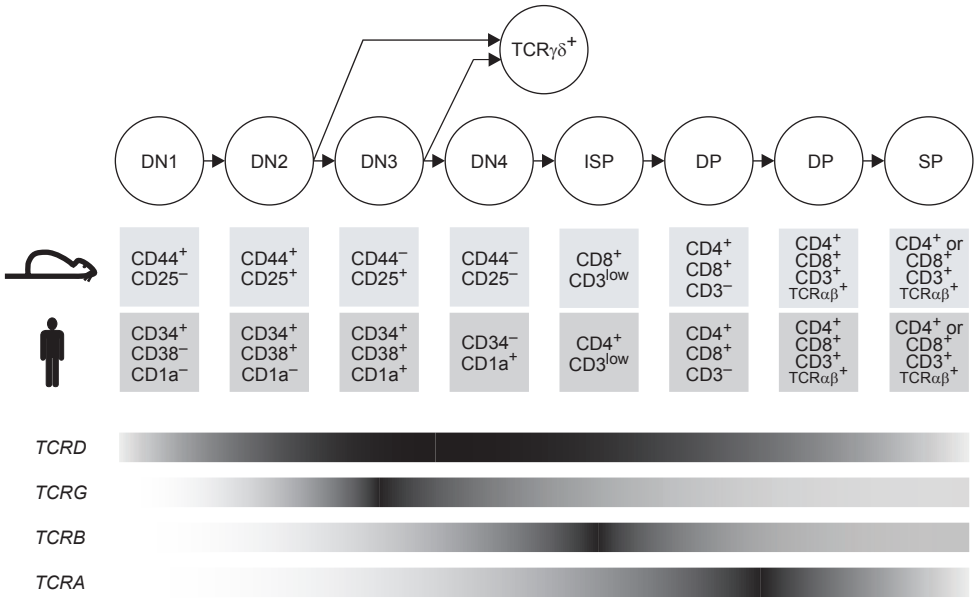

Figure 5. Schematic overview of T-cell development in the thymus.
Subsequent stages of development are described for mouse and human according to the surface markers that are expressed. Stages of development that are comparable between mouse and man are grouped together. In the lower part of the figure, rearrangement activity of the T-cell receptor loci is depicted. Light shades represent low rearrangement activity and dark shades represent high rearrangement activity. DN, double negative; ISP, immature single positive; DP, double positive; SP, single positive; TCR, T-cell receptor; CD, cluster of differentiation.

of gene rearrangement takes place in DN thymocytes and rearrangements are initially detected at the *TCRD* locus, mainly during DN2 and DN3 stages of development.[18, 42] Subsequently, *TCRG* rearrangements occur, mostly at the DN3 stage. When a TCR consisting of a γ and a δ chain is successfully formed, the developmental path of γδ$^+$ T cells diverges from the development of αβ$^+$ T-cells , most likely at the DN3 stage.[43] If rearrangements at the *TCRD* locus are not functional, rearrangement of the *TCRB* locus will proceed. Successful rearrangement of *TCRB* gene segments is tested by expression of the TCRβ chain on the cell surface paired with the invariant pre-Tα receptor in the pre-TCR complex. This process is also referred to as β-selection, a major checkpoint during T-cell development. Signaling through the pre-T-cell receptor will result in entry of the cell cycle. During proliferation cells go through immunophenotypic changes: immature single positive (ISP) cells arise when thymocytes express a co-receptor in the absence of high levels of CD3. In humans, ISP cells express co-receptor CD4, whereas in most strains of mice they express CD8. Subsequently, both CD4 and CD8 are expressed and therefore these cells are referred to as double positive. In the DP stage, *TCRA* gene rearrangements are initiated. The DP stage makes up approximately 85% of all

thymocytes. After *TCRA* rearrangement, a TCRαβ heterodimer is expressed on the cell surface. This unique TCR is then tested for the recognition of self-MHC molecules in a process called positive selection, while negative selection tests for the absence of self-reactivity. After failing the selection criteria, approximately 95% of thymocytes die through the induction of apoptosis.[44] After successfully undergoing positive and negative selection processes, thymocytes that express a functional T-cell receptor commit to either the $CD4^+$ T helper lineage or the $CD8^+$ cytotoxic T lineage, ready to migrate to the periphery.

B-cell development

Similar to the development of T cells, B-cell development progresses through several stages in a highly regulated fashion to generate mature, functional B cells capable of expressing immunoglobulins that can recognize a multitude of antigens. B-cell development from HSC initially takes place in specific niches of the bone marrow. Several cell surface and intracellular markers are used to characterize consecutive steps of B-cell development. Some distinct surface markers are used for the identification of mouse and human subsets (Figure 6), but highly similar processes take place in both species as detailed below.

The earliest stage with B-cell specific activity is the pro-B cell stage. These cells express TdT and undergo D to J rearrangements of the *IGH* locus. In the subsequent pre-B-I stage of B-cell development, V to DJ rearrangement takes place. A surrogate light chain as well as VpreB are expressed intracellularly. If no stop codons were introduced during the recombination process, an IgH chain can pair with the surrogate light chain and VpreB, forming a pre-B-cell receptor (BCR) complex. This complex is expressed on the cell surface in the pre-B-II large stage of development. Expression of a functional pre-BCR is an vital checkpoint during B-cell development[45] and signaling through this receptor instigates several processes. Pre-BCR signaling induces downregulation of RAG protein expression[46] and the accessibility of the *IGH* locus is limited. Through this process of allelic exclusion, only the Ig heavy chain that was positively selected for functionality is expressed. Signaling through the pre-B-cell receptor also induces proliferation and downregulation of surrogate light chain and VpreB expression.[45, 47] This results in downregulation of pre-BCR expression on the cell surface and consequent reduction of proliferation. Subsequently, RAG expression is upregulated and V to J rearrangement at the *IGK* locus is initiated. If rearrangement is productive, the Igκ chain pairs with the IgH chain and is expressed as IgM on the cell surface. If the first attempts at light chain rearrangement resultes in an autoreactive receptor, B cells can repeat V to J joining several times. If *IGK* gene rearrangements are not successful on either allele, rearrangement at the *IGL* locus can be initiated. If neither *IGK* nor *IGL* is productively rearranged, the cell undergoes apoptosis in the bone marrow. If the BCR is functional, the cell will be positively selected and become an immature B cell. After surviving the immature B-cell stage, cells start expressing IgD on the cell surface in addition to IgM. In the next stage, in transition from the bone marrow to the periphery, cells become IgM^+IgD^+: transitional B cells. In peripheral blood they

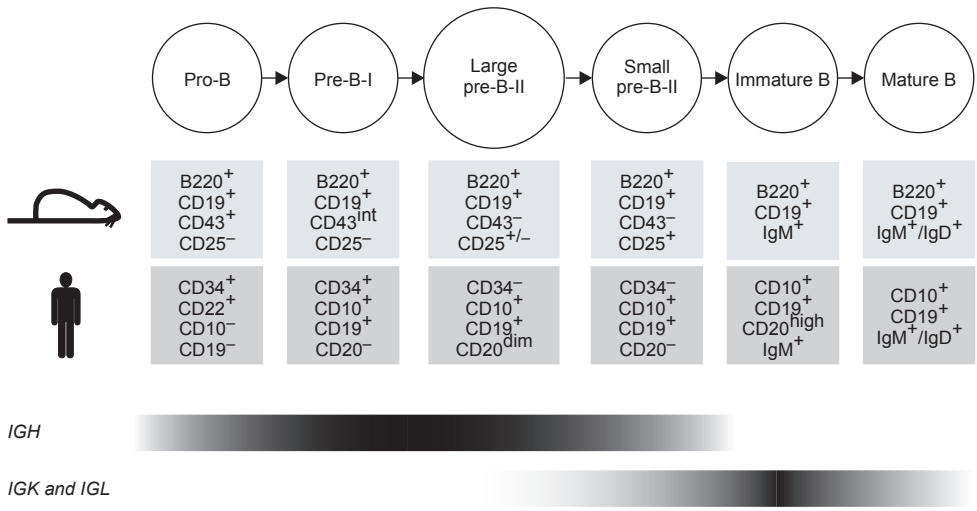

Figure 6. Schematic representation of B-cell development in the bone marrow.
Subsequent stages of mouse and human B-cell development are described according to the surface markers that are expressed. Stages of development that are comparable between mouse and man are grouped together. In the lower part of the figure, rearrangement activity of the immunoglobulin loci is depicted. Light shades represent low rearrangement activity and dark shades represent high rearrangement activity. Ig, immunoglobulin; CD, cluster of differentiation.

develop from transitional B cells to naive mature B cells. If naive mature B cells are not activated through antigen recognition, they will be short-lived. Upon encounter of antigen in the follicles of secondary lymphoid organs, B cells will undergo further modifications to their antigen receptor through a process called somatic hypermutation. During somatic hypermutation, the enzyme activation-induced cytidine deaminase introduces point-mutations throughout the sequence coding for the variable region. Mainly under the influence of cytokines, activated B cells can also undergo class-switch recombination. This results in the use of an alternate constant region of the antibody, which is recognized by different receptors, therefore resulting in a different effector function. During terminal B-cell differentiation, two kinds of cells are formed. Plasma cells produce and secrete large amounts of antibodies and reside in the bone marrow. Alternatively, activated B cells can form memory B cells which are long-lived cells, capable of a quick and strong response to secondary exposure to the same antigen.

NK-cell development

The third member of the lymphoid lineage, the NK cell, plays an important role in defense against virally infected cells and tumors as well as activation of the adaptive immune system. NK cells are large granular cells that make up 10-15% of all circulating lymphocytes that are immunophenotypically characterized as $CD3^{-}CD56^{+}$ in humans.

For murine NK cells, other markers are used, namely, DX5, NKG2D and NK1.1.[48]

In comparison to B- and T-cell development, relatively little is know about the development of NK cells. Unlike B and T cells, development of NK cells does not involve the process of V(D)J recombination. A significant part of NK-cell development takes place in the bone marrow and IL-15 has been identified as a critical mediator of NK-cell development, since mice that lack either IL-15 or IL-15R are NK-cell deficient.[49, 50] In early phases of NK-cell development, factors produced by stromal cells in the bone marrow mediate the generation of NK-precursor cells. These precursors are receptive for IL-15 and are able to develop in mature NK cells. The final NK-cell differentiation step can occur in the bone marrow, but other sites, such as lymph nodes, could also provide factors necessary for terminal NK-cell differentiation. Other cytokines than IL-15 have also been described to contribute to NK-cell development. The only cytokines that can support the development of NK cells *in vitro*, are IL-2, IL-15 and IL-7, although resulting NK cells are functionally and phenotypically immature.[51-53] The mature NK-cell population can be subdivided in $CD3^-CD56^{dim}$ and $CD3^-CD56^{bright}$ cells.[54] $CD3^-CD56^{dim}$ cells express high levels of CD16 and killer immunoglobulin-like receptor (KIR). The highly cytotoxic nature of these cells is underscored by their ability to mediate direct cytotoxicity, antibody-dependent cellular cytotoxicity as well as lymphokine-activated killing. In contrast, the expression of CD16 and KIR is low or absent on $CD3^-CD56^{bright}$ cells and these cells have a low toxicity potential. $CD3^-CD56^{bright}$ cells act as immunoregulatory cells and are capable of producing large amounts of interferon-γ and IL-10. A distinct feature of, mainly $CD56^{dim}$, NK cells is their ability to quickly mediate cellular toxicity. NK-cell responses are regulated by a balance of signals from activating and inhibiting receptors. In the absence of activating receptor ligation, effector function is inhibited as long as the KIR molecules to HLA class I molecules on the membrane of a target cell. As soon as the activating receptor-ligand interaction dominates the inhibitory KIR signals, NK cells are activated. Once NK cells are activated, granules that hold several type of cytotoxic proteins are released from their cytoplasm which results in the destruction of the target cell.

SEVERE COMBINED IMMUNODEFICIENCY: SCID

The significance of our immune system is emphasized by the fact that immunodeficiency can lead to severe illness and death.[55-57] Severe combined immunodeficiency (SCID) is a class of primary, inherited, immunodeficiencies that are characterized by severely reduced numbers or total absence of functional T cells. In addition, the number and function of B cells and NK cells can be affected in certain types of SCID. Patients with SCID, all of them being infants, suffer from persistent diarrhea, opportunistic infections and a failure to thrive.[55-57] Infections that are often found in SCID patients include oral candidiasis, Gram-negative sepsis, respiratory virus, adenovirus infection, and *Pneumocystis carinii* pneumonia. Clinical symptoms of SCID usually present within the first few months

of live and pose a medical emergency. If SCID is left untreated the infant will die. Different phenotypes of SCID can be distinguished when considering the absence or presence of B and NK cells. Finding the genetic defects underlying the different SCID-phenotype has revealed a lot about the crucial role of various proteins in lymphoid development. The blocks in lymphoid development caused by different genetic defects are depicted in Figure 7. Recently, a new classification has been proposed, restricting the number of genes considered to be involved in SCID.[58] These genes are described below, including genes that were recently published to have mutations that caused clinical SCID. Figure 8 shows the frequencies of mutations found in 'SCID genes'.

$T^-B^+NK^+$ SCID patients

Several genetic aberrations have been found to underlie $T^-B^+NK^+$ SCID. One of the defects is found in the interleukin-7 receptor α (IL-7Rα) chain.[59, 60] IL-7Rα deficiency is an autosomal recessive form of SCID. Mutations in the *IL7RA* gene include splice-site alterations, nonsense mutations, missense mutations and two-nucleotide deletions and all occur in the first five exons of the gene that code for the extracellular domain.[61]

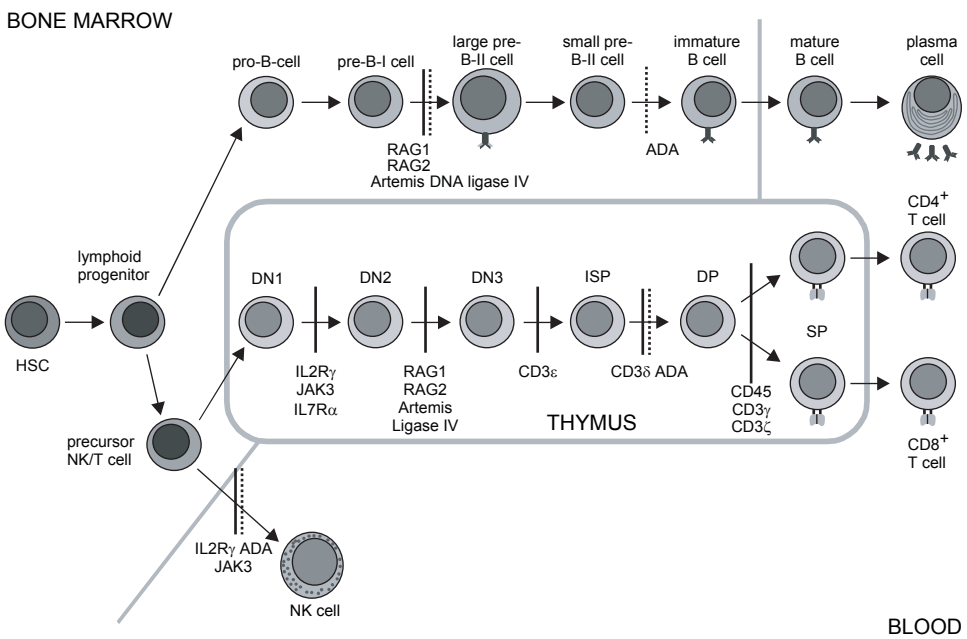

Figure 7. Schematic overview of developmental blocks in human T-, B-, and NK-cell development in SCID.
For deficiencies in the 13 genes that can bear SCID-causing mutations, the blocks in lymphoid development are indicated. Blocks in B-cell development are derived from immunophenotyping studies on bone marrow samples from SCID patients. Developmental blocks in the T-cell lineage largely rely on data from targeted mutation studies in the mouse.

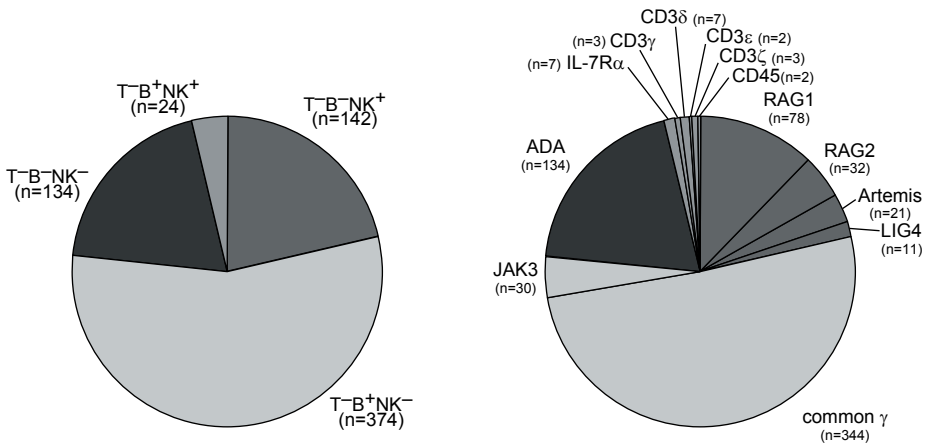

Figure 8. Mutation frequency in genes involved in SCID.
Relative distribution of mutations in genes known to be affected in SCID. In the left chart, genes are grouped according to the resulting absence and presence of B and NK cells. On the right, the mutation frequency of individual genes are plotted. Data obtained from: http://bioinf.uta.fi/IDbases/

The phenotype of IL-7Rα-SCID patients differs from IL-7R-deficient mice, in which the T-lineage as well as the B lineage is affected.[62]

Isolated cases of SCID-causing mutations in genes coding for all CD3 subunits have also been described.[63-66] CD3 chains form a complex with the T-cell receptor heterodimer[67, 68] and play a crucial role in TCR signaling. All CD3 chains carry immunoreceptor tyrosine-based activation motifs (ITAMs).[69, 70] Upon ligation of the TCR, ITAMs get phosphorylated and signaling events downstream of the TCR can occur. Besides playing an important role in the function of mature T lymphocytes, CD3 subunits also play a crucial role during T-cell development.[71] Each of the CD3δ, CD3ε and CD3ζ subunits is required for assembly, surface expression and function of the TCR and their deficiency results in a block in T-development.[63, 65, 66] CD3γ deficiency shows a more heterogeneous clinical picture as some patients have a complete SCID phenotype while other individuals present with a milder immunodeficiency.[64, 72-74] Not all targeted *Cd3* gene disruptions in mice completely emulate the human phenotype. The developmental block in *Cd3*δ$^{-/-}$ mice is not as severe as in human CD3δ-deficiency,[75, 76] while in *Cd3*γ$^{-/-}$ mice the block is stronger than in the human counterparts.[77]

Deficiency of the common leukocyte antigen CD45 also results in a T^{-}B^{+}NK^{+} phenotype.[78-80] The transmembrane protein tyrosine kinase phosphatase is critical for efficient antigen receptor signal transduction.[81] The vital importance of CD45 phosphatase activity is conserved between human and mouse since CD45-deficient mice also have a T-cell deficiency, caused by a block at the DP stage of development.[82, 83]

$T^-B^+NK^-$ SCID patients

The most frequent form of SCID is X-linked SCID, which is caused by mutations in the gene coding for the common cytokine receptor γ chain (γ_c) or common γ chain.[84, 85] No T or NK lymphocytes are found in these patients. B cells are present in normal or elevated numbers, but are not fully functional since serum Ig levels are decreased. Additionally, the lack of T cell help prevents Ig molecules to switch to different isotypes The common γ chain was initially identified as a component of the receptor for IL-2 and is therefore also known as interleukin-2 receptor gamma (IL-2Rγ) chain.[86] However, the common γ chain is a component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 and is important for the intracellular signaling events.[87-92] Lack of signaling through the IL-7 receptor causes the block in T-cell development,[59] whereas the absence of IL-15 receptor signaling is thought to block NK-cell development.[49, 50] In addition to the lack of T-cell help, the absence of signaling through the IL-4R and IL-21R could also lead to diminished immunoglobulin production.[93-95] The phenotype of mice that lack the common γ chain do not resemble the $T^-B^+NK^-$ phenotype of X-linked SCID patients. In addition to a block in T- and NK-cell development, the B-cell compartment is also reduced in *Il2rg*$^{-/-}$ mice,[96, 97] reflecting the requirement of IL-7 for murine B-cell development.

A genetic defect in the Janus kinase 3 (*JAK3*) gene can also cause a $T^-B^+NK^-$ phenotype.[98, 99] JAK3 is a tyrosine kinase that is part of the Janus family of kinases and plays a critical role in cytokine signaling. JAK3 associates with the common γ chain.[100, 101] Following cytokine binding, JAK3 becomes activated by transphosphorylation. Subsequently, the cytoplasmic tyrosine residue of the common γ chain gets phosphorylated, which allows recruitment and activation of proteins for downstream signaling. Because of the close physical interaction of JAK3 with the common γ chain, the phenotype of JAK3-deficient patients is identical to that of X-linked SCID patients and mice with a JAK3-deficiency were found to mirror the $T^-B^-NK^-$phenotype of *Il2rg*$^{-/-}$ mice.[102, 103]

$T^-B^-NK^-$ SCID patients

The adenosine deaminase (*ADA*) gene was the first SCID-causing gene to be identified.[104] The ADA enzyme is part of the purine salvage pathway and catalyzes deamination of deoxydenosine and adenosine.[105] ADA-deficiency results in accumulation of deoxydenosine and adenosine with causes systemic toxicity, particularly affecting cells and tissues that undergo rapid proliferation. Besides the severe consequences for lymphocyte development, many other abnormalities are a result of this purine metabolic defect, such as deafness, neuro-developmental deficits and skeletal abnormalities.[106-108] Straight ADA-deficient mice do not mimic the human ADA-SCID phenotype, since ADA-deficiency in mice results in perinatal mortality.[109, 110] Early postnatal mortality is prevented when expression of the ADA protein is restored in trophoblast cells.[111] Severe lymphopenia and combined immunodeficiency can be observed in these mice that indeed emulate the human ADA-SCID phenotype.

Patients with reticular dysgenesis, a very rare disease, also lack B, T and NK cells.

In addition, monocytes and granulocytes are absent from the blood.[112] To date, the genetic defect that causes reticular dysgenesis has not been identified.

$T^-B^-NK^+$ SCID patients

The first genetic defects causing a $T^-B^-NK^+$ SCID phenotype were found in *RAG1* and *RAG2.*[113] RAG proteins play a crucial role in the initiation of V(D)J recombination of Ig and TCR gene segments. Mutations found in RAG-SCID patients result in severely reduced recombinase activity. The inability to rearrange Ig and TCR gene segments results in a developmental block at early checkpoints in B- and T-cell development. When recombinase activity is absent, the B-cell developmental block manifests at the pre-B-I stage in humans.[114] The phenotype of RAG1- and RAG2-deficient mice is completely identical to the human RAG-SCID situation.[115, 116] The block in B-cell development in RAG-deficient mice occurs in the pro-B-cell stage. Murine thymocytes deficient in RAG-protein accumulate in the DN3 stage of development.

Patients with a $T^-B^-NK^+$ SCID phenotype can also suffer from Artemis-deficiency.[117-119] Artemis associates with the DNA-dependent protein kinase and functions in hairpin opening during the V(D)J-recombination process.[24, 120] The immunological phenotype of *Artemis* knock-out mice closely resembles that of Artemis-SCID patients, with the exception that $CD4^+$ T cells can be found in low numbers in some mice.[120]

Recently, a DNA ligase IV-deficient patient with a $T^-B^-NK^+$ SCID phenotype has been described.[121] Ligase IV plays a role in non-homologous end joining during repair of double-strand breaks.[122] DNA ligase IV-deficiency caused a severe disturbance of the V(D)J recombination process resulting in a block in T- and B-cell development.[121] DNA ligase IV-deficiency in mice leads to embryonic lethality,[123, 124] signifying a key role for DNA ligase IV in murine embryogenesis that is not apparent in humans.

Omenn syndrome

Similar to SCID, patients with Omenn syndrome present with increased susceptibility to opportunistic infections and protracted diarrhea. Additionally, patients with Omenn syndrome suffer from autoimmune responses resulting in squamous erythrodermia, alopecia, lymphadenopathy and hepatosplenomegaly. Omenn syndrome is a genetically heterogeneous disease.[125] Mutations, mostly hypomorphic, have been found in *Artemis*, *IL7RA,* and most frequently in *RAG1* and *RAG2*. The number of T cells is usually high, but the TCR repertoire in Omenn syndrome patients is restricted. A limited number of recombination events occurs in these patients, caused by low levels of Artemis protein or reduced recombination capacity of RAG proteins.

THERAPY FOR SCID

Conventional treatment for SCID

Discovery of the human leukocyte antigen (HLA) system[126, 127] opened the door for

therapeutic applications utilizing hematopoietic stem cells. In 1968, the first SCID patient received a bone marrow transplant from a related, HLA-identical donor.[128] For the majority of SCID patients, a related identical donor was (and is) not available and mismatched donors were used as an alternative. Initially, the use of mismatched donors often resulted in the manifestation of graft-versus-host disease (GVHD). To remove GVHD-causing T cells, several methods were developed, such as soybean lectin agglutination and depletion with monoclonal antibodies.[129-131] Retrospective studies have revealed that treatment with mismatched related transplants results in delayed or incomplete reconstitution of immune function.[132, 133] In stead, transplantation using a matched unrelated donor results in better engraftment, immune reconstitution and survival.[15, 132, 133] Furthermore, in patients receiving a transplant from mismatched donors, B-cell function is often so poor that patients need treatment with intravenous Ig (IVIG).[134] One study revealed that the immunological reconstitution for B-cell negative SCID patients was poorer then for B-cell positive SCID patients.[132] Due to improved transplantation protocols, the survival rate of SCID patients receiving a HLA-matched or HLA-identical stem cell transplantation is about 80%.[15, 132, 134] Newborn screening could increase the chances of survival, since SCID patients receiving a transplant in the first 28 days of life show a higher survival rate and lower morbidity than patients that are diagnosed later.[135] In absence of a suitable hematopoietic stem cell donor, ADA-SCID patients regularly receive enzyme replacement therapy in the form of polyethylene glycol-ADA (PEG-ADA).[136] Since a suitable donor is often not available, gene therapy is explored as an alternative therapy for SCID.

Developing gene therapy

Until a few years ago, hematopoietic stem cell transplantation was the only curative therapy for SCID. Several clinical trials have recently proven successful in the application of gene therapy for SCID. Using gene therapy, the correct copy of an affected gene is introduced in the target tissue. In gene therapy for SCID, the target cells are HSC isolated from the patients' bone marrow into which the correct gene is introduced using a virus-based vector. The process of viral gene transfer is also called transduction. It took several decades to develop gene therapy technology to a point where it could be applied effectively in patients.

After the DNA double helix structure was determined by Watson, Crick, Wilkins, and Franklin in 1953,[137-139] understanding of the structure, regulation and expression of genes quickly grew. In 1966, E.L. Tatum discussed the possibilities of genetic engineering and predicted that this kind of technology could be applied in humans within ten or twenty years.[140] Early attempts at correcting a genetic disease by way of gene therapy failed. Two girls, suffering from a form of cerebral palsy characterized by low levels of arginase, were treated with Shope papilloma virus that naturally contains the arginase-gene. Injection of the Shope virus turned out not to be a cure for the metabolic disease.[141] A better understanding of the processes occurring during the viral life cycle was needed for effective therapy.

SCID as candidate for gene therapy

From the early discussions about genetic manipulation it became clear that diseases caused by mutation of a single gene would be primary candidates for gene therapy.

In addition, the target cells for gene therapy should be easy to manipulate *ex vivo* to prevent expression of the therapeutic gene in other tissues. Because bone marrow cells were the easiest cells to manipulate *ex vivo*, several genes known to be involved in blood disorders were nominated for gene therapy. Abnormalities in hemoglobin that cause β-thalassemia were thought to be the first genetic diseases for which gene therapy would be a successful treatment. The complicated regulation of globin synthesis slowed down therapy development. At the time, other candidate genes included purine nucleoside phosphorylase (*PNP*), hypoxanthine-guanine phosphoribosyl transferase (*HPRT*), and *ADA*.[142] Deficiency in all three enzymes can be established in bone marrow cells but all three deficiencies also affect the central nervous system, which is a lot more difficult to treat than bone marrow. Severe neurologic disorders are seen in HPRT-deficiency and PNP-deficiency whereas neurological manifestation in ADA-SCID patients is less pronounced. Gene therapy might cure the immunodeficiency, but does not resolve the neurological disorder.

Since 1991, more SCID-causing genetic defects were discovered.[113] The majority of patients diagnosed with SCID carry a mutation in known genes. Therefore, gene therapy could in principle be developed for these defects. HSC can sustain production of all blood lineages throughout life and are present in bone marrow. In addition, HSC do well in a transplantation setting and can be manipulated *in vitro* for a couple of days with minimal loss of their characteristics. These features make them ideal target cells for gene therapy for SCID. Since development of lymphocytes is blocked in SCID patients, addition of the correct gene is thought to give cells a selective advantage. In the absence of a hematopoietic stem cell transplantation, SCID is a lethal disease. However, even if a matching donor is found, immunological function is often not completely restored after hematopoietic stem cell transplantation. When a suitable donor is not available, gene therapy could possibly provide curative treatment for SCID. The principle of gene therapy is depicted in Figure 9.

Vectors for SCID gene therapy

As part of their life cycle, viruses deliver their genetic material to cells. Different viruses employ different strategies to complete their life cycle. Depending on the therapeutic goal and the target cell, viruses with the desired properties have been modified to serve as tools for gene transfer. Some viruses insert their genetic content into the host genome. Such viruses use the transcription and translation machinery of the host to replicate their genome and produce proteins needed for assembly of new viruses. Prior to cell division, the integrated viral genome is replicated along with the host genome. Since HSC and their progeny have to undergo many cell divisions, stable integration in the host genome is one of the features required for sustained gene therapy in SCID patients. This feature is found in retroviruses.

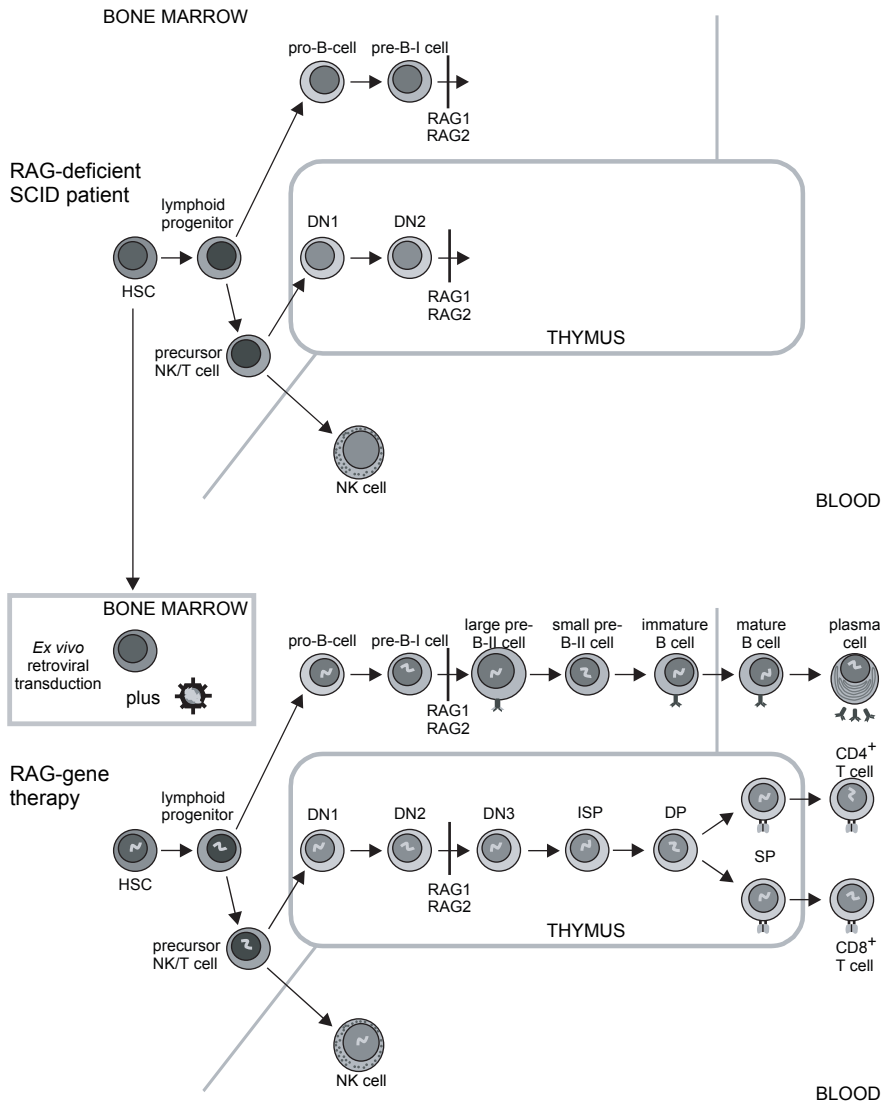

Figure 9. Principle of gene therapy.
B- and T-cell development is blocked in RAG-SCID patients. During gene therapy, hematopoietic stem cells are taken from the RAG-SCID patient and transduced with a viral vector carrying the correct copy of the *RAG* gene, resulting in the stable insertion of the transgene in the genome of HSC. Treated cells are given back to the patient, where HSC can give rise to several different hematopoietic lineages. The progeny of HSC will also carry the correct copy of the therapeutic gene and cells are no longer blocked in B- and T-cell development.

Retroviruses

Gene therapeutic applications are most frequently based upon the Moloney murine leukemia virus (Mo-MLV) or the human immunodeficiency virus (HIV). Both viruses belonging to the viral family *Retroviridae* (retroviruses). Retroviruses are enveloped viruses whose genome consists of two identical positive strands of single-stranded RNA. The hallmark of *Retroviridae* is their replication strategy. This strategy includes reverse transcription of the viral RNA into linear double-stranded DNA and the subsequent integration of this DNA into the genome of the cell. Retroviral genomes can range from 7 to 12 kilo bases in size and code for proteins that are essential for retroviral replication. A schematic representation of a prototypical retrovirus is given in Figure 10. All retroviral genomes contain the transcriptional units *gag*, *pol, pro,* and *env*. The coding region of the retroviral genome is flanked by long terminal repeats (LTR). LTR sequences play an important role in initiation of viral DNA synthesis, integration of the viral genome and transcriptional regulation of viral genes. In most retroviruses, the 5' non-coding region contains the packaging signal; ψ, that ensures packaging of the viral genome during assembly of viral particles. The retroviral genome in DNA-form is referred to as the provirus. Outside the host cell, viral particles are called virions. Retroviral virions are made up of RNA, protein and lipids and are about 100 nm in diameter.

A prototypic retroviral virion envelope is made up of host-derived plasma membrane phospholipids and viral envelope glycoproteins, coded by the *env* gene. Envelope proteins mediate attachment of virions to the host cells by binding to specific cell surface proteins. The inside of the envelope is lined with matrix protein, one of the proteins coded by *gag*. Another protein encoded by *gag* is the capsid protein which forms a protective structure around the core of the virion. The retroviral virion core contains two single-stranded RNA molecules, reverse transcriptase and integrase molecules (both encoded by *pol*) and protease molecules (encoded by *pro*). The core also contains nucleocapsid proteins, encoded by *gag*, that tightly bind viral genomic RNA forming a ribonucleoprotein complex.

Vectors based on Moloney murine leukemia virus

During the 1970s and early 1980s, researchers started modifying viruses so they could serve as gene transfer tools. The genome of the murine leukemia virus is fairly simple and the understanding of its life cycle made this virus a good candidate for recombinant viral techniques. The murine sarcoma virus (MSV) was the first retrovirus to be used as a replication-deficient viral vector.[143] The retroviral transfer vector carried a *neo*^{r} gene in place of viral genes and contained the packaging signal. A Mo-MLV was constructed as a helper virus; it lacked the packaging sequence, but the viral *gag*, *pol* and *env* genes were still present. Subsequently, a cell line was transduced with the Mo-MLV helper virus. Stable integration of the helper virus in the genome of the cell line resulted in constant production of viral proteins.[144] After transfecting this producer cell line with the MSV-based retroviral neo-resistance transfer vector, viral particles containing only the retroviral neo-

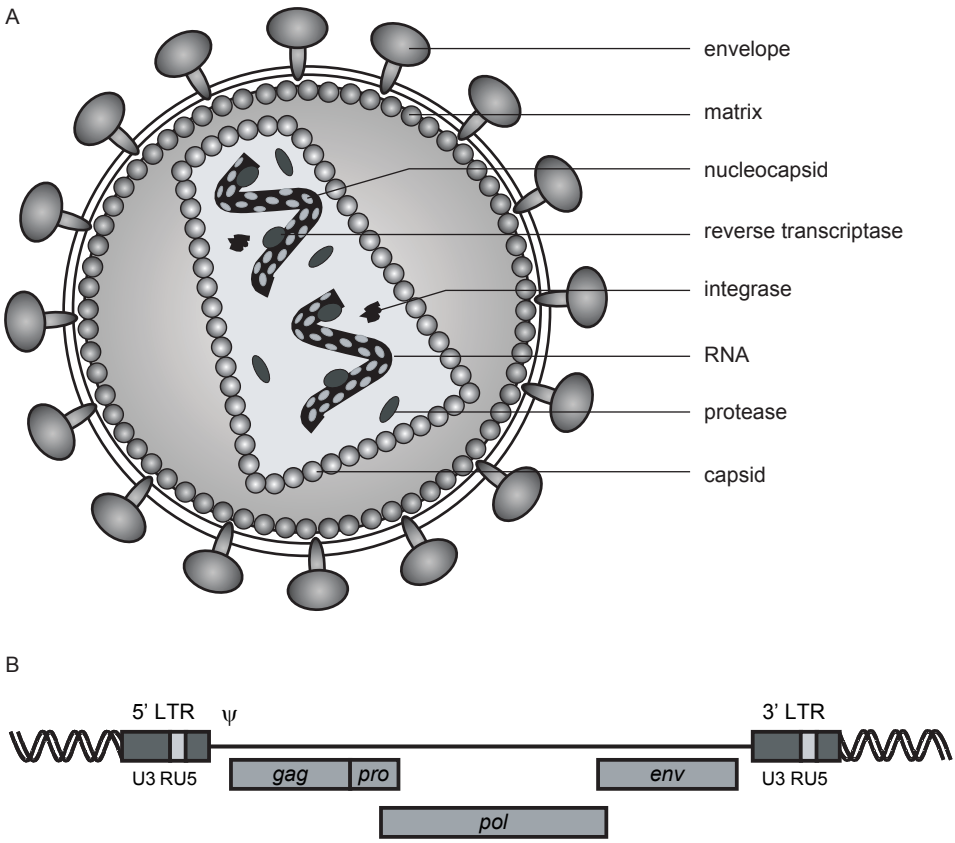

Figure 10. Prototypical retroviral virion and genome.
A. Schematic representation of a prototypical retroviral virion and its components. B. The genome of a simple retrovirus is schematically shown as integrated provirus. U3, unique 3′ regulatory sequences; R, repeat sequence; U5, unique 5′ regulatory sequences; Ψ, packaging signal.

resistance construct were produced. These replication deficient particles were capable of transferring resistance to neomycin to mouse bone marrow cells.[143] Based on this system, various gene transfer vectors have been constructed for gene therapeutic applications.

In early transfer vector systems, the viral LTR was used for driving transgene expression. Viral particles with Mo-MLV envelope proteins are capable of infecting murine and rat cells; these particles are ecotropic. A common modification of these vectors is the so called pseudotyping. Cell lines were made that contain Mo-MLV-derived *gag* and *pol* genes, into which genes coding for env derived from alternative viruses were introduced.[145-147] After transfection of a transfer vector, viral particles with an alternative envelope are produced by these cell lines. Because the envelope determines the host range,

viral vectors capable of infecting cells from different species can be produced. Commonly used envelopes are derived from amphotropic virus clone 4070A, gibbon ape leukemia virus (GALV), RD114 endogenous cat retrovirus (RD114) and vesicular stomatitis virus G protein (VSVG). Viral particles pseudotyped with an amphotropic or VSVG envelope have a wide host range. GALV-pseudotyped particles can infect most mammalian cells,[148] except murine cells, which lack the appropriate receptor. Viral particles bearing a RD114 envelope can infect human, but not murine cells.

MLV-derived vectors for treating SCID

Initial clinical trials for retroviral gene transfer were not entirely successful. The first gene therapy trial for SCID was done in 1990 in two ADA-SCID patients.[149] T cells of patients were expanded and transduced *in vitro* and injected back into the patients. After gene therapy, normal T cells were detected, but a proper evaluation could not take place because both patients still received PEG-ADA. The first attempt at HSC gene therapy for ADA-SCID did not result in correction of the disease, most likely because of low transduction efficiency.[150] Improvements to the transduction protocols eventually resulted in successful hematopoietic stem cell gene therapy trials for ADA-SCID. Autologous HSC were transduced using MLV-based ADA transfer vectors pseudotyped with either an amphotropic or a GALV envelope.[151, 152] Prior to reinfusion of the cells, patients underwent mild chemotherapy to facilitate engraftment. All children are healthy and most of them could be taken of IVIG treatment or prophylactic antibiotic therapy.[136] For the most common form of SCID, X-linked SCID, hematopoietic stem cell gene therapy has also been successful.[153-155] As of 2007, 20 X-linked SCID patients have been treated with HSC gene therapy and the majority of children showed immune reconstitution. Unfortunately, 4 patients treated under the Paris protocol developed a T-cell acute lymphoblastic leukemia (T-ALL)-like disease[156] (The third case was described at the 10th annual AGST meeting, in Seattle, WA, 2007. The fourth case was reported at the 33rd Annual Meeting of the European Group for Blood and Marrow Transplantation in Lyon, France, 2007). In the leukemic cells of at least two patients retroviral integration occurred near the T-ALL oncogene LIM-only protein 2 (*LMO2*).[156] In the third case, insertions near *LYL1*, *c-Jun* and *BMI1* were found, all known for their involvement in cancerous processes. The fourth patient was reported to have an insertion near *CCND2*, a known T-ALL oncogene. These cases of T-ALL emphasized the potential risk of integrating vectors and the need for additional safety studies.

Vectors based on human immunodeficiency virus

The discovery that the therapeutic vector used for X-linked SCID gene therapy trial resulted in lympho-proliferations resembling T-ALL, intensified discussions about the safety of gene therapy. More effort was put into improving safety of gene transfer procedures. Despite being a more complex virus, HIV-based vector systems might be more suitable for gene therapy. MLV and HIV have been shown to integrate in distinct regions within

genes.[157-159] The tendency of MLV to integrate toward transcription start sites was seen as less favorable then the integration pattern of HIV, which does not display this partiality. Combined with built-in safety features, HIV-based vectors are thought to be a good alternative to MLV based vectors for gene transfer.

Gene transfer vectors based on HIV-1 have been extensively researched since the early 1990s.[160, 161] Unlike MLV-based vectors, HIV-based vectors are capable of infecting quiescent cells such as HCS.[162, 163] Besides *gag*, *pol* and *env* genes, the HIV-1 genome consists of 6 other genes. The genome of wild type HIV-1 is shown in Figure 11A. The protein coded by *tat* regulates viral promoter activity and is essential for transcription from the 5' LTR. The *rev* gene codes for a protein that binds to the rev-responsive element, permitting unspliced RNA to be transported out of the nucleus and is needed for efficient *gag* and *pol* expression. *Tat* and *rev* are regulatory genes, whereas *vpr*, *vpu*, *vif*, and *nef* code for proteins that promote HIV virulence. Vpr protein aids transport of the double stranded DNA into the nucleus. Release of virions is promoted by Vpu protein, while Vif and Nef enhance virion infectivity. Modifications to the wild type HIV-1 genome are mainly aimed at avoiding replication-competent lentivirus and increasing gene transfer efficiency.

The first lentivirus-based vectors were made using a three-plasmid system.[164] The gene transfer plasmid contained wild type LTRs, the packaging signal and an internal promoter controlling expression of the transgene. A separate plasmid provided the envelope gene and a third plasmid contained *gag*, *pol*, *tat*, *rev*, *vif*, *vpr*, and *nef*. Co-transfection of the three plasmids into 293T cells yielded viral stocks of high titer. In the second generation of lentiviral vectors, virulence genes *vpr*, *nef*, and *vif* were removed without resulting in a lower viral titer.[165] The third generation lentiviral vectors is a four-plasmid system.[166] *Rev* was removed from the transfer vector and expressed from a separate plasmid. In addition, *tat* was deleted from the transfer plasmid. This was shown not to influence viral titers when the U3 region of the 5' LTR was replaced by a cytomegalovirus (CMV) or a Rous sarcoma virus (RSV) promoter, making the 5' LTR activity independent of tat protein.[167] This four-plasmid system minimizes the risk of producing a replication competent lentivirus.

In conjunction with the third generation lentiviral vector system, an additional modification to the transfer vector is often used. Deleting part of the U3 region from the transfer vector results in self-inactivating (SIN) viruses. When the enhancer of the U3 region is removed from the 3' LTR, reverse transcription of the transfer vector leaves the LTR transcriptionaly inactive.[166] This modification contributes significantly to the safety of gene transfer vectors, since the LTR can no longer transcriptionally activate genomic areas surrounding the insertion site. Additional improvements to the lentiviral transfer vector design include the addition of a central polypurine tract (PPT) making reverse transcription more efficient,[168] and a modified woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) for improved viral titer and transgene expression.[169] The plasmids used in a typical third generation lentiviral transfer system are depicted in Figure 11B, while Figure 11C shows

the proviral vector integrated in the host genome. Currently, lentiviral transfer vectors for several types of SCID are being developed, including ADA-SCID,[170] Artemis-SCID,[171] and RAG-SCID (this thesis).

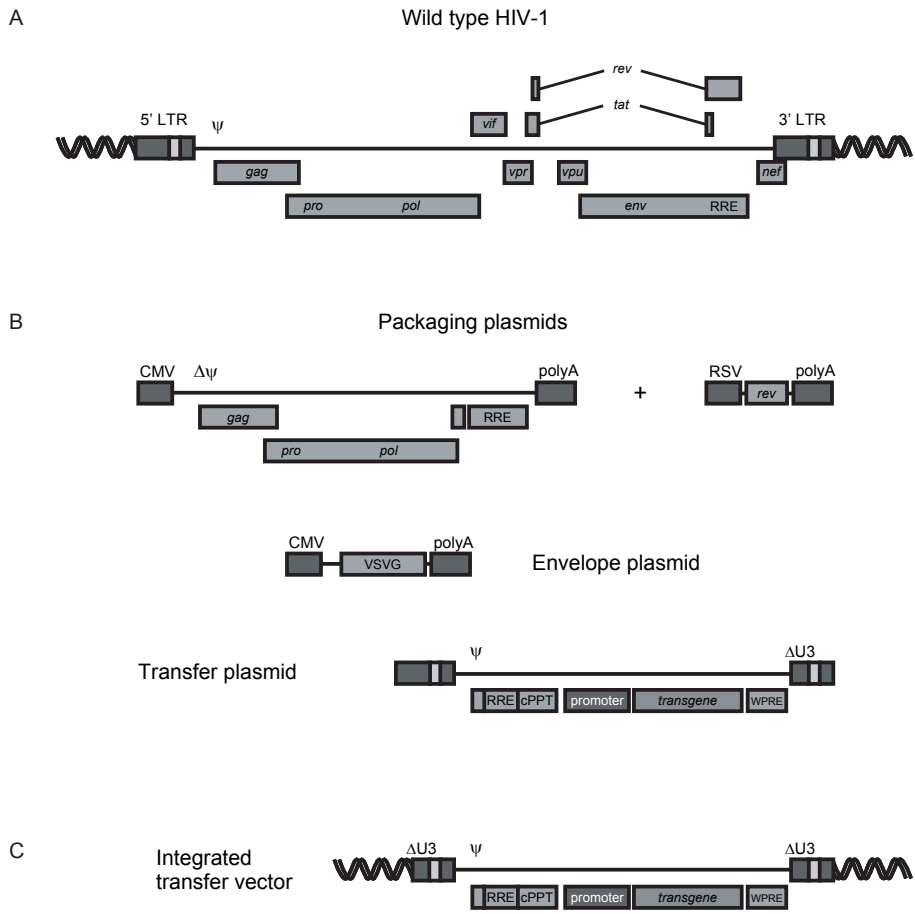

Figure 11. Wild type HIV-1 and lentivirus based gene transfer system.
A. Schematic representation of the wild type HIV-1 provirus, integrated in the host genome. B. The 4-plasmid system used for gene transfer consists of one envelope plasmid, two packaging plasmids and one transfer plasmid. C. Transfer vector, depicted as provirus integrated in the host genome. LTR, long terminal repeat; Ψ, packaging signal; CMV, cytomegalovirus, RSV, Rous sarcoma virus; ΔΨ, packaging signal deleted; ΔU3, unique 3′ regulatory sequences with viral enhancer and promoter sequences deleted; RRE, Rev-responsive element; cPPT, central polypurine tract sequence; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

OUTLINE OF THIS THESIS

SCID is a disease centered around defective T-cell development. Gene therapy is one of the treatment options for SCID. In this thesis, several aspects of T-cell development and gene therapy will be researched. In Chapter 2 we set out to gain a better understanding of normal human T-cell development. By combining detailed immunophenotyping with high-speed cell sorting we purified all known subsets of T-cell development. The mRNA expression profiles of these subpopulations were determined. In addition, TCR gene rearrangements were quantified. By combining these two datasets, we want to identify potential factors that regulate and/or initiate TCR rearrangements. In one of the gene therapy trials using hematopoietic stem cells, lymphoproliferations were observed in some patients. In 2 cases, insertion of the therapeutic vector was found near the T-ALL oncogene *LMO2*. The manuscripts in Chapter 3 explore the role of *LMO2* as well as the therapeutic *IL2RG* gene during human T-cell development. This is done by combining *in vitro* lymphoid development assays with mRNA expression data obtained in Chapter 2. In Chapter 4 and 5 we want to test the feasibility of *RAG* gene therapy using HIV-based transfer vectors. We will evaluate *RAG1* and *RAG2* gene transfer into hematopoietic stem cells using murine models for RAG-SCID. Reconstitution of B and T lymphocytes in peripheral blood will be monitored, primary and secondary lymphoid organs will be analyzed by immunophenotyping and Ig and TCR repertoire will be determined if B and T cells are present after gene therapy. In the General Discussion, Chapter 6, implications of the results obtained in this thesis are discussed and reflects on future research.

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CHAPTER 2

NEW INSIGHTS ON HUMAN T-CELL DEVELOPMENT BY QUANTITATIVE T-CELL RECEPTOR GENE REARRANGEMENT STUDIES AND GENE EXPRESSION PROFILING

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ABSTRACT

To gain more insight into initiation and regulation of T-cell receptor (TCR) gene rearrangement during human T-cell development, we analyzed TCR gene rearrangements by quantitative PCR analysis in nine consecutive T-cell developmental stages, including $CD34^+$ lin^- cord blood cells as a reference. The same stages were used for gene expression profiling using DNA microarrays. We show that TCR loci rearrange in a highly ordered way (*TCRD-TCRG-TCRB-TCRA*) and that the initiating Dδ2-Dδ3 rearrangement occurs at the most immature $CD34^+CD38^-CD1a^-$ stage. *TCRB* rearrangement starts at the $CD34^+CD38^+CD1a^-$ stage and complete in-frame *TCRB* rearrangements were first detected in the ISP stage. *TCRB* rearrangement data together with the *PTCRA* (pTα) expression pattern show that human TCRβ-selection occurs at the $CD34^+CD38^+CD1a^+$ stage. By combining the TCR rearrangement data with gene expression data, we identified candidate factors for the initiation/regulation of TCR recombination. Our data demonstrate that a number of key events occur earlier than previously assumed and that human T-cell development is therefore much more similar to murine T-cell development than reported before.

INTRODUCTION

T cells develop from progenitors that migrate from the bone marrow into the thymus.[1] Thymocytes are roughly subdivided as either being double negative (DN), double positive (DP) or single positive (SP), based on the expression of the CD4 and CD8 co-receptors.[1] The DN stage is heterogeneous and can be subdivided into four distinct subsets in mice based on the expression of CD44 and CD25. In human, three clearly distinct DN stages can be recognized: a $CD34^+CD38^-CD1a^-$ stage, representing the most immature thymic subset and the consecutive $CD34^+CD38^+CD1a^-$ and $CD34^+CD38^+CD1a^+$ stages. Human DN thymocytes mature via an immature single positive (ISP $CD4^+$) and a DP stage into either $CD4^+$ or $CD8^+$ SP T cells that express functional T-cell receptors (TCR) and that exit the thymus.[1]

A hallmark of T-cell development is the generation of T cells that express a functional TCR, either being TCRαβ or TCRγδ. During T-cell development, the variable domains of *TCRA, TCRB, TCRG,* and *TCRD* (located within *TCRA*) genes are assembled following rearrangement of variable (V), diversity (D), and joining (J) gene segments by a process called V(D)J recombination.[2] V(D)J recombination uses the RAG1 and RAG2 enzymes that selectively target recombination signal sequences (RSS) that flank V, D and J segments.[2]

Studies in T-cell acute lymphoblastic leukemias (T-ALL) suggest that recombinations of TCR genes are sequential between the different genes (*TCRD* > *TCRG* > *TCRB* > *TCRA*) as well as within a particular gene (e.g. *TCRD*: Dδ2-Dδ3, Dδ2-Jδ1, Vδ-Jδ1),[2, 3] which is

supported by limited data obtained from normal human T-cell subsets.[4] Therefore, the timing and efficiency of rearrangement of various TCR genes must be determined by the accessibility of gene segments to RAG enzymes. Evidence suggests that promoter and enhancer activity controlled by transcription factors regulate V(D)J recombination by modulating chromatin structures and rendering gene segments accessible to RAG cleavage.[5, 6]

T-cell development is for obvious reasons mainly studied in the mouse. Real-time quantitative PCR (RQ-PCR) and DNA microarray techniques allow careful analysis of small cell numbers. In this study we assessed the precise TCR gene configuration and the gene expression profiles of thymic subsets by RQ-PCR and Affymetrix DNA microarrays. By combining these two techniques we aimed for the identification of factors that play a role in regulating human TCR gene recombination.

RESULTS AND DISCUSSION

Definition of T-cell populations

From umbilical cord blood (UCB) $CD34^+$ lineage negative "stem cell-like" cells and from thymi $CD34^+CD38^-CD1a^-$, $CD34^+CD38^+CD1a^-$, $CD34^+CD38^+CD1a^+$, ISP $CD4^+$, DP $CD3^-$, DP $CD3^+$ and SP $CD4^+$ and SP $CD8^+$ subpopulations were obtained, representing consecutive stages of T-cell development (Figure 1). TCRαβ and TCRγδ expressing cells were obtained from thymic samples and peripheral blood. Cells from 5 donors were pooled to reduce intra-sample variation and all subsets were isolated twice from different donor pools.

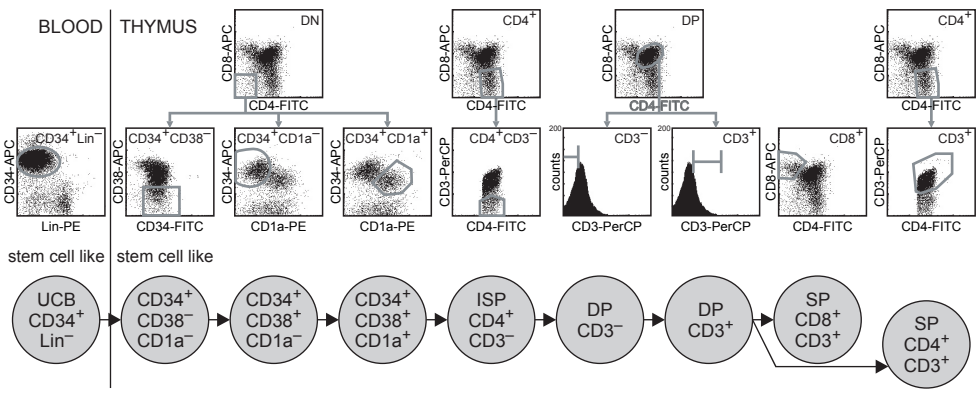

Figure 1. T-cell differentiation stages and gate settings for sorting.
The differentiation stages of human T-cell development are depicted schematically. FACS plots show the gating strategy used for sorting various populations.

Determination of TCR gene rearrangements by RQ-PCR and GeneScan analysis

TCR gene rearrangement analysis was performed in duplicate and on the two independently purified subsets (average is shown). The primers and TaqMan probes (listed at http://www.jem.org/cgi/content/full/jem.20042524/DC1) do not amplify germline DNA.

TCRD

The Dδ2-Dδ3 rearrangement was first detected in the earliest $CD34^+CD38^-CD1a^-$ thymic subset, much earlier during development than found in our previous Southern blotting-based report.[4] Dδ2-Dδ3 rearrangements reached maximum levels in the subsequent $CD34^+CD38^+CD1a^-$ stage and thereafter declined to low levels in the following subsets (Figure 2B). The initial Dδ2-Dδ3 wave was followed by Dδ2-Jδ1 rearrangements, which were detectable at low levels in $CD34^+CD38^-CD1a^-$ cells, increased in $CD34^+CD38^+CD1a^-$ and peaked in $CD34^+CD38^+CD1a^+$ cells (Figure 2C). Complete Vδ1-Jδ1 or Vδ2-Jδ1 rearrangements were first detected in $CD34^+CD38^+CD1a^-$ cells, increasing to peak levels in $CD4^+$ ISP cells after which they declined when the majority of thymocytes further differentiated into the TCRαβ lineage (Figure 2D). During TCRαβ T-cell lineage development, the *TCRD* gene is deleted from the *TCRA/D* gene complex[2, 7] which results in the formation of a T-cell receptor excision circle (TREC) that may contain Vδ-Jδ1 rearrangements. TRECs do not replicate on cell division; consequently, they are diluted out in proliferating, developing T cells.[2] Ki67 staining demonstrated high percentages of proliferating cells within the human ISP and DP $CD3^-$ thymic subsets (data not shown). This proliferation likely accounts for the observed decline in Vδ-Jδ1 rearrangements after the ISP stage. The Vδ1 gene segment appeared to be used preferentially in postnatal $TCR\gamma\delta^+$ thymocytes, which is in sharp contrast to the well-described preferential Vδ2 usage by peripheral $TCR\gamma\delta^+$ T cells (Figure 2D).[8] We detected very low Vδ-Dδ3 levels in the thymic subsets, indicating that this represents a minor pathway to initiate *TCRD* gene rearrangement in postnatal thymus (Figure 2E).

TCRG

The first *TCRG* rearrangements (Vγ-Jγ1.1/2.1) were detected in the $CD34^+CD38^+CD1a^-$ thymic subset, earlier than we previously reported,[4] and one stage after the initiation of *TCRD* rearrangement. These rearrangements peaked in $CD34^+CD38^+CD1a^+$ thymocytes, after which levels decreased to a relative constant level in subsequent subsets (Figure 3B). Vγ-Jγ1.3/2.3 rearrangements were first observed in $CD34^+CD38^+CD1a^-$ cells after which they rapidly increased and by far exceeded Vγ-Jγ1.1/2.1 rearrangements from the ISP stage onwards (Figure 3C). Peripheral $TCR\gamma\delta^+$ and $TCR\alpha\beta^+$ T cells revealed lower Jγ1.3/2.3 usage than then their thymic counter parts (Figure 3C). In thymocytes Jγ1.2 was used at very low frequency, but massive positive selection for the Jγ1.2 segment occurred in peripheral $TCR\gamma\delta^+$ T cells (Figure 3D).[8]

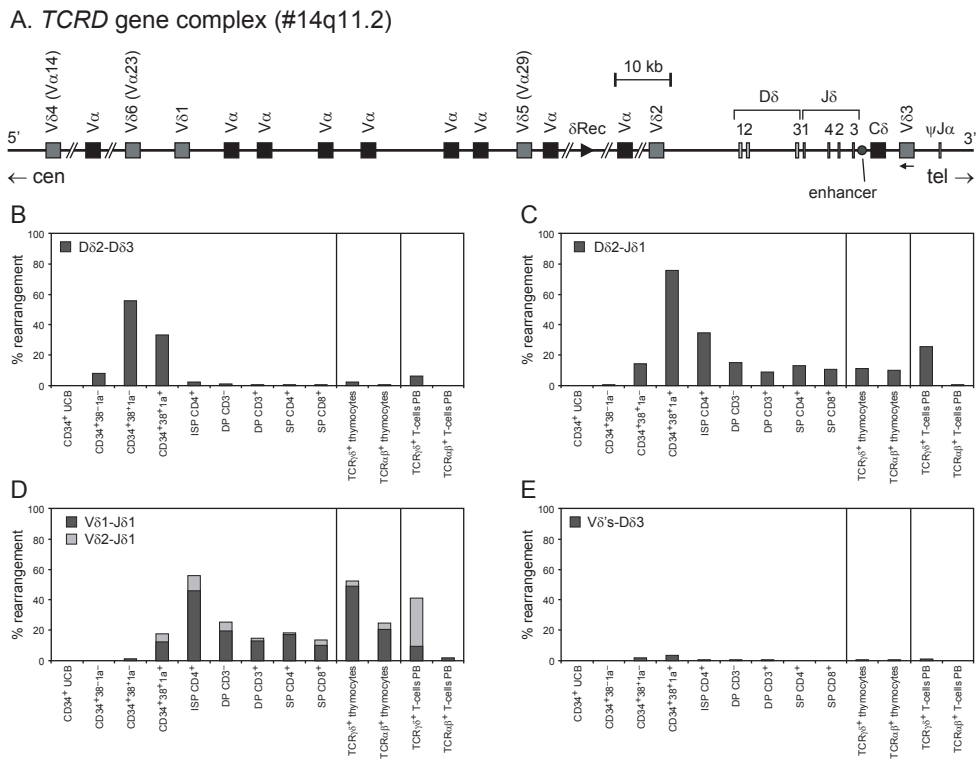

Figure 2. RQ-PCR analysis of *TCRD* gene rearrangements in human T-cell development and mature T cells.

A. Schematic diagram of the human *TCRD* gene complex. The six 'classical' Vδ gene segments are scattered between Vα gene segments. As Vδ4, Vδ5, and Vδ6 are also recognized as Vα gene segments, their Vα gene code is given in parentheses (adapted from reference 9). B. Analysis of Dδ2-Dδ3 rearrangement. C. Analysis of Dδ2-Jδ1 rearrangement. D. Analysis of Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements. E. Analysis of Vδ-Dδ3 rearrangements.

TCRB

Dβ1-Jβ1 rearrangements were first detected at low levels in the $CD34^+CD38^+CD1a^-$ population and increased thereafter from $CD34^+CD38^+CD1a^+$ to $CD4^+$ ISP cells (Figure 4B). Dβ2-Jβ2 rearrangements were first detected at low levels in $CD34^+CD38^+CD1a^+$ cells, one differentiation stage after Dβ1-Jβ1 rearrangements (Figure 4B). The seemingly lower levels of Dβ-Jβ in DP $CD3^+$ are probably caused by variation within the lower range of detection of our assay and likely do not represent a true decrease.

Due to the complexity of the *TCRB* locus, Vβ-Jβ rearrangements were determined using non-quantitative GeneScan analysis.[9] The $CD34^+CD38^+CD1a^+$ thymocytes contained low levels of the first Vβ-Jβ rearrangements in which exclusively Jβ2 gene segments were used (Figure 4D), much earlier than described before.[4] From the ISP

subset onwards Vβ-Jβ rearrangements were in-frame as shown by the triplet peaks (Figure 4E) and were retained throughout all subsequent stages of development (Figure 4E-H).

Previous reports suggested that TCRβ-selection in humans is initiated at the ISP/DP stages of T-cell development,[4, 10, 11] but the 3 nucleotide spacing of the peaks in our GeneScan analysis suggest that selection for in-frame *TCRB* already occurs at the transition from $CD34^+CD38^+CD1a^+$ to ISP. From the DP $CD3^-$ fraction Vβ-Jβ1 rearrangements were also present, although less abundant than Vβ-Jβ2 rearrangements (Figure 4F; tube A). For Vβ-Jβ rearrangements usage of Jβ2 gene segments was preferred over Jβ1 (Figure 4D-H). TCRγδ$^+$ thymocytes contained no Vβ-Jβ1 rearrangements but did contain Vβ-Jβ2 rearrangements (Figure 4H).

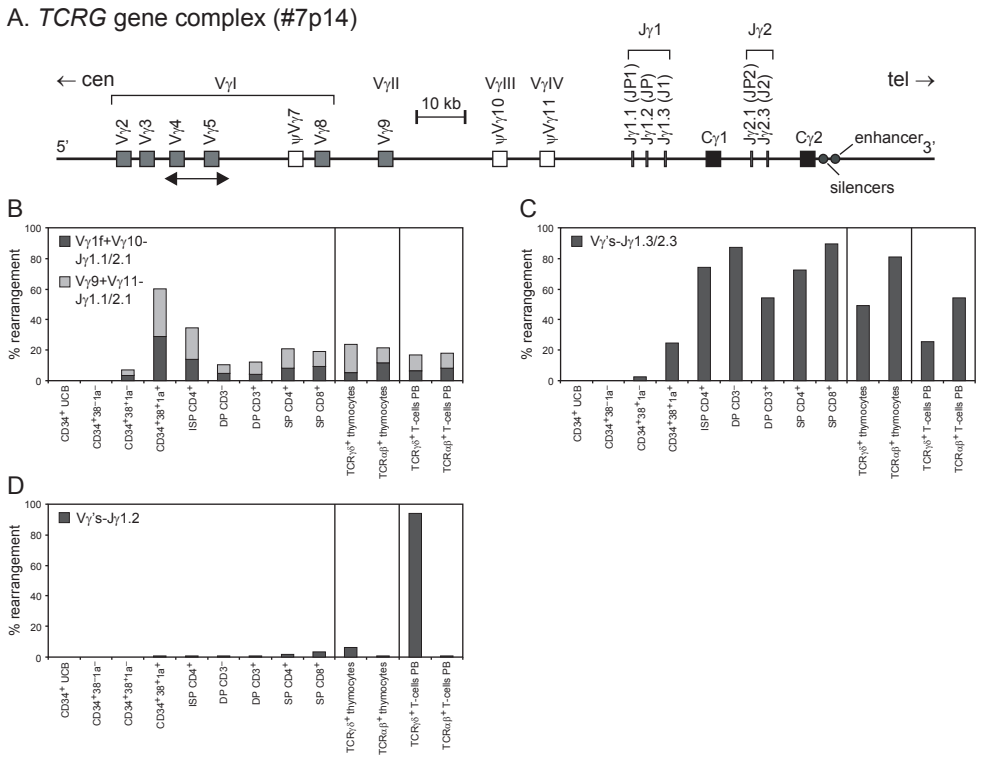

Figure 3. RQ-PCR analysis of *TCRG* gene rearrangements in human T-cell development and mature T cells.

A. Schematic diagram of the human *TCRG* gene complex. Only the rearrangeable Vγ gene segments are depicted in black (functional Vγ) or in gray (non-functional Vγ). For the Jγ gene segments both nomenclatures are used (adapted from reference 9). B. Analysis of Vγ to Jγ1.1 and Jγ2.1 rearrangements. C. Analysis of Vγ to Jγ1.3 and Jγ2.3 rearrangements. D. Analysis of Vγ to Jγ1.2 rearrangement.

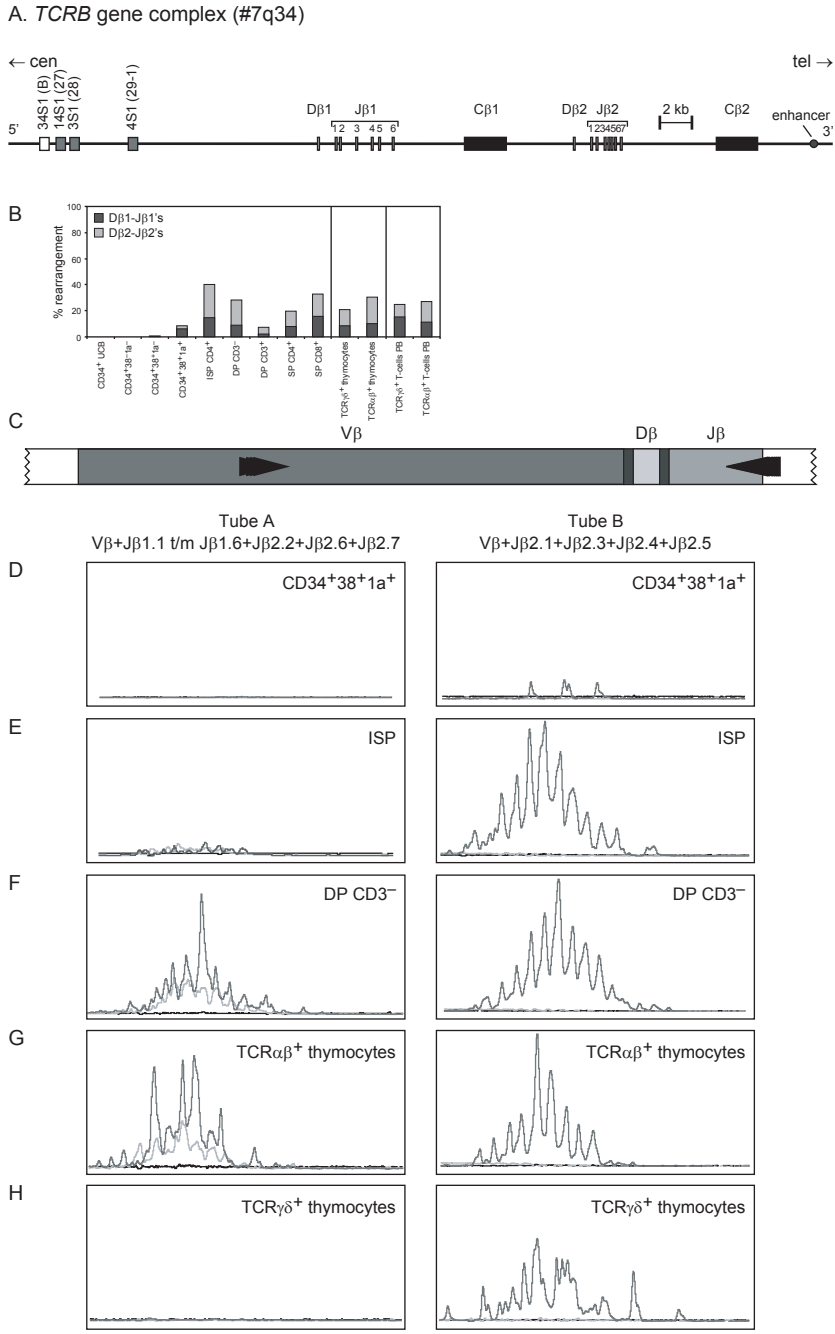
A. TCRB gene complex (#7q34)
← cen
tel →
5'
3'
34S1 (B)
14S1 (27)
3S1 (28)
4S1 (29-1)
Dβ1
Jβ1
1 2 3 4 5 6
Cβ1
Dβ2
Jβ2
1 2 3 4 5 6 7
2 kb
Cβ2
enhancer
B
% rearrangement
Dβ1-Jβ1's
Dβ2-Jβ2's
CD34+ UCB
CD34+38- 1a-
CD34+38+ 1a-
CD34+38+ 1a+
ISP CD4+
DP CD3-
DP CD3+
SP CD4+
SP CD8+
TCRγδ+ thymocytes
TCRαβ+ thymocytes
TCRαβ+ T-cells PB
TCRαβ+ T-cells PB
C
Vβ
Dβ
Jβ
Tube A
Vβ+Jβ1.1 t/m Jβ1.6+Jβ2.2+Jβ2.6+Jβ2.7
Tube B
Vβ+Jβ2.1+Jβ2.3+Jβ2.4+Jβ2.5
D
CD34+38+1a+
E
ISP
F
DP CD3−
G
TCRαβ+ thymocytes
H
TCRγδ+ thymocytes

Figure 4 (facing page). RQ-PCR and GeneScan analysis of *TCRB* rearrangements in human T-cell development and in mature T cells.
A. Schematic diagram of the human *TCRB* gene complex. B. Analysis of Dβ to Jβ rearrangements. C. Schematic diagram of Vβ-Jβ rearrangements as determined by GeneScan analysis. The primers for the Jβ1 cluster were HEX-labeled (solid lines), while primers for the Jβ2 cluster were FAM-labeled (dotted lines). D-H. Analysis of Vβ-Jβ rearrangements in $CD34^+CD38^+CD1a^+$ cells (D), ISP cells (E), DP $CD3^-$ cells (F), $TCR\alpha\beta^+$ thymocytes (G) and $TCR\gamma\delta^+$ thymocytes (H).

TCRA

Due to large numbers of rearrangable Vα (~54) and Jα (61) gene segments,[2] we could not design a multiplex RQ-PCR for reliable quantification of all Vα-Jα rearrangements. Instead, we aimed for an alternative approach in which we used different indirect measures to study *TCRA* recombination. *TCRA* recombination is initiated by the transcription of T-early alpha (TEA) in order to open the 5' site of the Jα cluster, which is followed by *TCRD* deleting rearrangements, particularly the δREC-ψJα rearrangement. These initiating events are then followed by multiple, consecutive Vα-Jα rearrangements.[7, 12]

To study initiation of *TCRA* rearrangement we determined the level of TEA-Cα transcripts as well as the occurrence of δREC-ψJα rearrangements. TEA-Cα transcripts started to increase in $CD34^+CD38^+CD1a^+$ cells and reached peak levels in ISP and DP cells after which they declined again (Figure 5B). δREC-ψJα rearrangements were first detected in ISP cells and reached peak levels in SP and $TCR\alpha\beta^+$ thymocytes (Figure 5C). These data show that *TCRA* rearrangement has already started in the ISP cell population, but that there are still cells within the $CD3^+$ DP population that start rearrangement of the (most likely) second *TCRA* allele. Although TEA-Cα transcripts and δREC-ψJα rearrangements are good measures for initiation of *TCRA* rearrangement they cannot be used for quantification of the actual *TCRA* rearrangements. TEA-Cα is an mRNA product that cannot simply be extrapolated to the actual level of *TCRA* rearrangements. Quantification of δREC-ψJα is complex because it is strongly influenced by ongoing Vα-Jα rearrangements and the consequently produced TRECs (containing δREC-ψJα), whereas the amount of TRECs (and their dilution) is heavily dependent on the fraction of proliferating cells within specific subsets. Therefore, extra accumulation of TRECs may explain the relatively high δREC-ψJα levels in non-proliferating SP cells as compared to the preceding proliferating stages.

In an attempt to quantify *TCRA* recombination, we determined loss of germline *TCRA* DNA based on the disappearance of germline ψJα as indirect measure for *TCRA* rearrangements. For this approach we used DNA from $CD34^+lin^-$ UCB cells as 100% germline reference. Germline *TCRA* clearly declined from the $CD34^+CD38^+CD1a^+$ stage onwards with a major decline when ISP cells progress towards DP cells (Figure 5D). The low levels of germline *TCRA* in DP $CD3^-$ cells indicate that extensive *TCRA* rearrangement has occurred at this stage. The seemingly inconsistence with the relatively low levels of δREC-ψJα in these DP $CD3^-$ cells can be explained by the fact that TRECs containing δREC-ψJα are rapidly diluted-out in these heavily proliferating cells and that

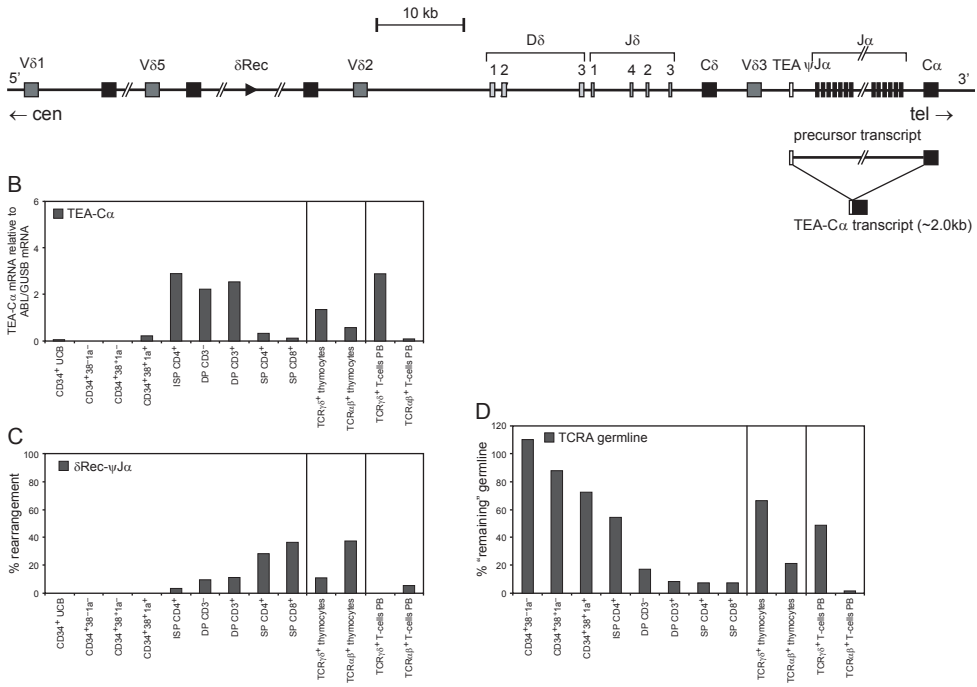

Figure 5. RQ-PCR analysis of initiating events around *TCRA* rearrangement in human T-cell development and mature T cells.
A. Schematic diagram of the human *TCRA* gene complex. The TEA element forms a sterile mRNA with Cα sequences. The *TCRD* deleting elements δRec and ψJα are also indicated. B. Analysis of TEA-Cα mRNA expression. Due to shortage of material, TEA expression was only determined once. C. Analysis of the *TCRD* deleting rearrangement δRec-ψJα. D. Analysis of "remaining" *TCRA* germline DNA.

other *TCRD* deleting rearrangements can occur as well, such as δREC-Jα58 and Vδ-ψJα[7]. It should be noted that our approach is only suitable for quantification of major decreases in germline *TCRA*, which occur at early stages of T-cell development (ISP/CD3$^-$ DP), but that it is much more difficult to accurately measure additional *TCRA* rearrangements at the later stages of T-cell development. The latter is because germline ψJα (for instance due to δREC-Jα58 rearrangements) remains detectable on TRECs and the difficulty of detecting further decreases within the 5-10% germline *TCRA* present in these stages.

We conclude that *TCRA* rearrangements are initiated when thymocytes progress from CD34$^+$CD38$^+$CD1a$^+$ towards the ISP stage, which is much earlier than previously reported,[11] and is apparently ongoing up until the CD3$^+$ DP stage.

Microarray analysis

A total of 3848 probe sets underwent a significant change between any two successive stages of differentiation. The expression levels of these probe sets were used to calculate a correlation coefficient between all possible pairs of microarrays and revealed high correlation between biological repeats (Figure 6). This allowed us to use the average expression values of the two arrays performed per subset (obtained from 5 pooled thymi) for further analysis.

Expression data of the 3848 probe sets were used to perform hierarchical clustering using Genlab. Expression patterns of the 3848 probe sets were subdivided in k=15 different clusters (A to O; Figure 7). This hierarchical clustering immediately revealed that the thymocyte subsets isolated based on the expression of surface markers have distinct gene expression profiles, although both SP subsets show highly similar expression profiles. A table with the members of each cluster including the expression data is provided at

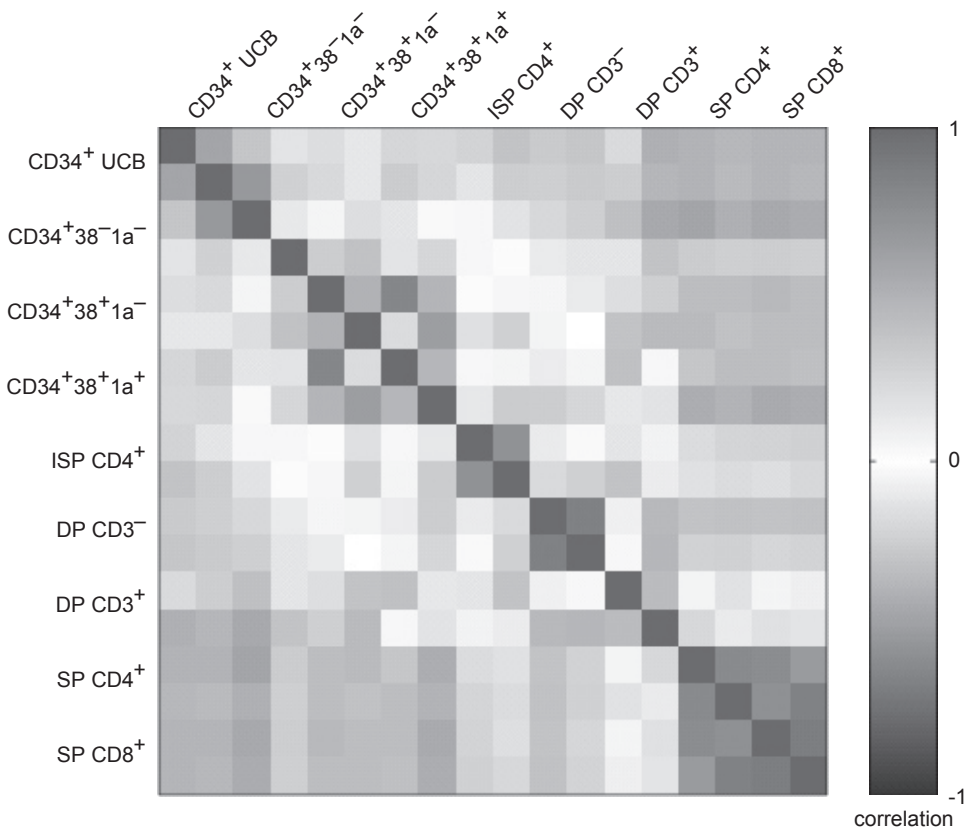

Figure 6. Plot of correlation coefficients calculated in Omniviz.
Grey shades correlate with the degree of correlation between a pair of microarrays.

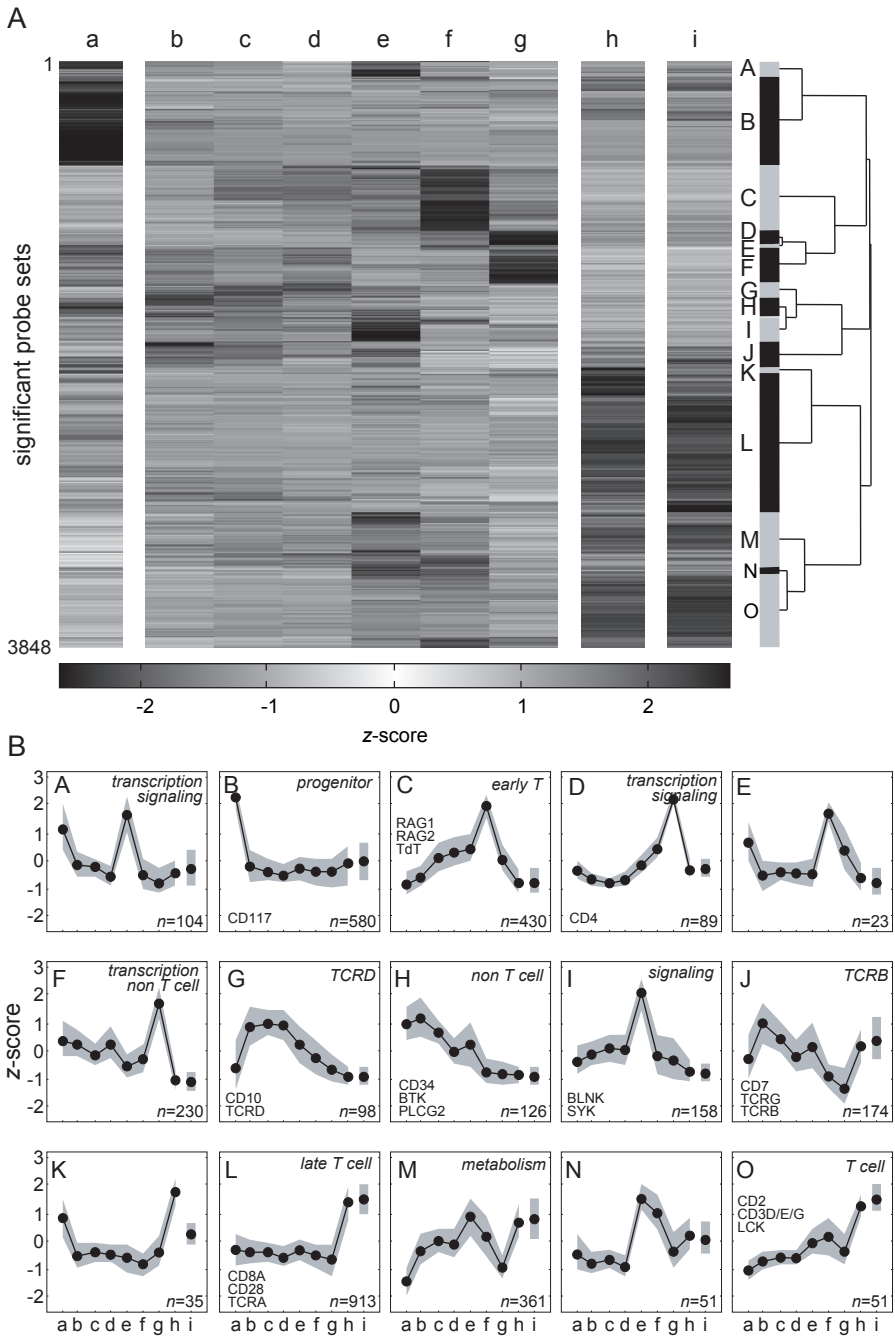
A
a b c d e f g h i
1
3848
significant probe sets
A B C D E F G H I J K L M N O
-2 -1 0 1 2
z-score
B
A transcription signaling n=104
B progenitor CD117 n=580
C early T RAG1 RAG2 TdT n=430
D transcription signaling CD4 n=89
E n=23
F transcription non T cell n=230
G TCRD CD10 TCRD n=98
H non T cell CD34 BTK PLCG2 n=126
I signaling BLNK SYK n=158
J TCRB CD7 TCRG TCRB n=174
K n=35
L late T cell CD8A CD28 TCRA n=913
M metabolism n=361
N n=51
O T cell CD2 CD3D/E/G LCK n=51
z-score
a b c d e f g h i

Figure 7 (facing page). Hierarchical clustering of the 3848 differentially expressed probe sets into 15 clusters.
A. Probe sets shown in rows. Analyzed subsets are shown in columns (a: $CD34^+lin^-$ UCB, b: $CD34^+CD38^-CD1a^-$, c: $CD34^+CD38^+CD1a^-$, d: $CD34^+CD38^+CD1a^+$, e: ISP, f: DP $CD3^-$, g: DP $CD3^+$, h: SP $CD4^+$, i: SP $CD8^+$). Grey values correlate with the value of the z-score. On the right side of the figure the 15 clusters are depicted (A-O). B. z-score trend representation shown for each cluster. Clusters codes A-O are displayed in the top left corner of each graph. The z-score is shown on the y-axis, the thymic subsets (labels are the same as in A) on the x-axis. The gray area represents 1 SD. In the bottom left corner of the graphs some representative cluster members are mentioned. *n* (bottom right corner) indicates the number of probe sets per cluster.

http://www.jem.org/cgi/content/full/jem.20042524/DC1. A large group of probe sets (580 in cluster B) is specifically expressed in $CD34^+lin^-$ UCB cells and shows a dramatic decrease in expression in the first thymic subset (e.g. *KIT* (CD117)). Other non-T cell markers such as *BTK* and *PLCG2* show a slower decrease starting in the second and third thymic population (cluster H and F, respectively). One of the first probe sets directly related to T-cell differentiation to show increased expression represents *TCRD* (cluster G), followed shortly thereafter by *TCRB*, *TCRG* and *CD7* (cluster J). Increased expression of *RAG1*, *RAG2*, *DNTT* (TdT) and *PTCRA* (pTα) was detected immediately after T-cell commitment (cluster C). This particular combination of genes is specific for the T-cell lineage.

Of special interest for TCRβ-selection and initiation of *TCRA* rearrangement is the fact that *PTCRA* (pTα) expression increased in the $CD34^+CD38^+CD1a^-$ stage and peaked in the $CD34^+CD38^+CD1a^+$ and ISP stages, after which it declined. Mouse microarray data[13] have shown a similar expression pattern of pTα, with a minor peak at DN3 and a larger peak at the DP $CD3^-$ stage. Experiments with pTα mutant mice indicate that TCRβ-selection in the mouse occurs at DN3,[14] and that *TCRA* recombination is initiated after TCRβ-selection has occurred.[14] Here we show that initiation of *TCRA* recombination starts in $CD34^+CD38^+CD1a^+$ cells. The analogous pTα expression between mice and men, together with our *TCRB* GeneScan and our *TCRA* recombination data, indicates that human TCRβ-selection occurs at the $CD34^+CD38^+CD1a^+$ stage instead of the previously suggested ISP/DP stage.[4,10,11]

Some probe sets that represent genes involved in double strand (ds)DNA break repair, such as *G22P1* (*KU70*), *XRCC5* (*KU80*), *PRKDC* (*DNAPK*), *DCLRE1C* (*ARTEMIS*), *XRCC4* and *LIG4* (*DNA ligase IV*), showed no significant change in expression between any of the subsequent developmental stages. Probe sets representing genes that take part in the TCR complex, such as *CD3D*, *CD3E*, *CD3G* and *ZAP70* are relatively over-represented starting in the ISP $CD4^+$ population (Figure 7; cluster O), although expression of *CD3D*, *CD3E* and *ZAP70* mRNA is already detectable in the first thymic subset that we analyzed. Transcription of the *TCRA* locus (cluster L) is first seen in the DP $CD3^-$ stage, after TCRβ-selection has already occurred. Surface markers such as CD5, CD8α and β1, CD27 and CD28 (cluster L) that are generally seen on more mature T cells, were indeed found to be upregulated in the latest stages of T-cell development. Clusters A and I, harboring genes involved in signaling, showed a peak in expression in the ISP stage of T-cell development.

Identification of factors regulating TCR gene rearrangements

To identify candidate transcription factors involved in regulating TCR recombination the list of 3848 probe sets was filtered based on Gene Ontology (GO) annotation (transcriptional activity and DNA binding) yielding a final list of 446 probe sets encoding a total of 361 genes (available at http://www.jem.org/cgi/content/full/jem.2004 2524/DC1). Expression of genes associated with T-cell commitment/differentiation and/or V(D)J recombination, such as *NOTCH1*, *HES1*, *GATA3*, *BCL11B*, *RAG1*, *RAG2*, and *DNTT* (TdT) strongly increased in early T-cell differentiation.

To determine which genes may have a role in regulating TCR rearrangement, hierarchical cluster analysis was performed (Figure 8A). The 446 probe sets were divided in 15 clusters, of which the prototypic expression patterns are depicted in Figure 8B. Clusters 3, 5, 6, 7, 14 and 15 (indicated by * in Figure 8B) contain genes of which expression levels increase concomitantly with episodes of active TCR gene rearrangement, as determined above, and which may therefore encode factors that initiate and/or regulate TCR rearrangements. Genes present in clusters 3, 5, 6, 7, 14 and 15 are presented in Table I.

We propose that clusters 3, 5 and 6 contain genes common for the rearrangement of all four TCR genes. Cluster 5 likely contains genes important for the regulation of *TCRD*, *TCRG* and *TCRB* rearrangements while cluster 3 likely contains genes that need to be expressed at a higher level when *TCRA* rearranges. These clusters include *DNTT* (cluster 6), *RAG1*/*RAG2* (cluster 3) as well as genes encoding factors such as NOTCH1 (cluster 6), RORC (cluster 3), SMARCA4 (BRG1) (clusters 6 and 3), H2AFX (cluster 3) that have previously been linked to regulation of *TCRG*, *TCRB* or *TCRA* rearrangements.[15-18]

Genes in cluster 7, 14 and 15 such as *SPIB*, *ICSBP1*, *TCF4*, *CREB1*, *ETS1* and *LEF1*, with high expression in ISP and DP subsets, may encode factors involved in regulating *TCRA* rearrangements, as well as allelic exclusion of the *TCRB* locus. CREB1 in conjunction with factors such as Ets1 and Lef1 activates the *TCRA* enhancer.[19] Recently, Ets1 has been identified as a critical factor for allelic exclusion at the *TCRB* locus.[20] Genes present in cluster 7 show a specific expression peak in the ISP stage. Among these genes *ICSBP1* (IRF8) and *SPIB* are likely candidates for initiating *TCRA* rearrangements, as they encode factors that have been implicated in the regulation of Ig light-chain transcription and recombination in developing B cells,[21, 22] while involvement of *TCF4* (E2-2) is supported by the finding that E2-2 deficient mice exhibit reduced transition from the DN to the DP stage.[23]

Figure 8 (facing page). Analysis of probe sets related to transcriptional regulation.
A. Hierarchical clustering of 446 probe sets differentially expressed over the various stages and related to transcriptional regulation and DNA binding. Labels of the thymocyte subsets are the same as in Figure 6. B. z-score trend representation for each cluster deduced from the 446 probe sets. Cluster codes (1-15) are shown in the top right corner, z-score on the y-axis, subsets on the x-axis. The gray area represents 1 SD. The asterisk in the upper right corner indicates clusters that contain genes that are potentially important in regulating TCR rearrangements. n indicates the number of probe sets in a cluster.

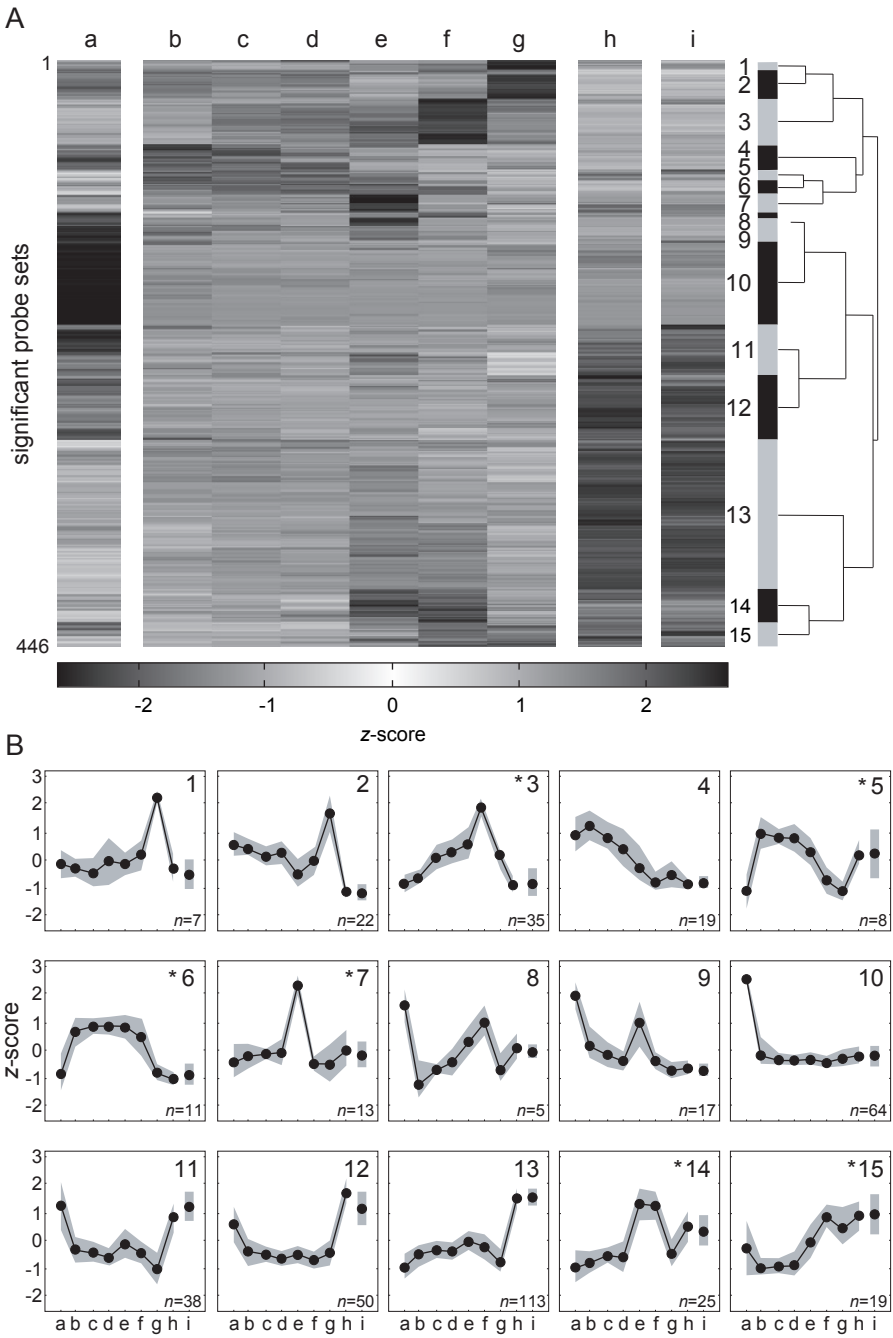
A
a
b
c
d
e
f
g
h
i
1
446
significant probe sets
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
-2
-1
0
1
2
z-score
B
z-score
1
2
*3
4
*5
*6
*7
8
9
10
11
12
13
*14
*15
n=7
n=22
n=35
n=19
n=8
n=11
n=13
n=5
n=17
n=64
n=38
n=50
n=113
n=25
n=19
a b c d e f g h i

Table I. Transcription and DNA binding related genes with expression profiles that correlate with TCR gene rearrangements in human T-cell development.

	common *TCR*	**common *TCR* *D,G,B>A***	**common *TCR* *A>B,G,D***	***TCRA* / allelic exclusion *TCRB***	***TCRA* / allelic exclusion *TCRB***	***TCRA* / allelic exclusion *TCRB***
catergory	cluster 6	cluster 5	cluster 3	cluster 7	cluster 14	cluster 15
transcription factor/ coregulator of transcription	*BAZ1B*	*BAZ2B*	*AEBP1*	*ACYP2*	*BRD8*	*ABCA7*
	ETS2	*FOS*	*CBFA2T3*	*BCL11A*	*CNOT3*	*ARNTL*
	HMGB3	*HES1*	*E2F1*	*CD86*	*CREB1*	*BCL6*
	KLF4	*IRF1*	*FOXM1*	*CUTL2*	*FALZ*	*CENTB1*
	MYB	*STAT4*	*GFI1*	*ICSBP1* (IRF8)	*IFI16*	*ELF1*
	NOTCH1	*TCEAL1*	*MYBL1*	*IRF7*	*ILF3*	*ETS1*
		TCFL1	*MYBL2*	*MNDA*	*LEF1*	*GATA3*
			NFATC3	*MYCL1*	*LEREPO4*	*GMEB2*
			NOTCH3	*SPIB*	*M96*	*HTATIP*
			ORC6L	*TCF4*	*NFYA*	*LEF1*
			RORC		*NR2C1*	*LRRFIP1*
			SAP30		*PWP1*	*MXD4*
			TFDP2		*SOLH*	*TCF7*
			TMPO		*TAF5*	*ZNF297B*
			TRIP13		*TBDN100*	
			TTF1		*TCF12* (HEB)	
			WHSC1		*TCF8*	
			ZNF423		*TFAM*	
					ZNFN1A1 (IKAROS)	
recombination	*DNTT* (TdT)		*RAG1*			
			RAG2			
chromatin-remodelling	*H2AFY*		*CHAF1A*		*HDAC4*	*HDAC7A*
	SMARCA4		*EZH2*		*YY1*	*SATB1*
			H2AFX			
			HMGB2			
			HMGN2			
			SMARCA4			
DNA repair	*MCM2*		*BRCA1*		*BTG2*	*BTG2*
	MCM6		*MCM7*			
			PTTG1			

Novel insights into human T-cell development

We confirm that TCR loci rearrange in a highly ordered way (*TCRD-TCRG-TCRB-TCRA*) and defined sequential rearrangement steps of *TCRD*, *TCRG*, *TCRB*, and initiation of *TCRA* recombination to specific human thymic subsets. Importantly, our data show that recombination of the TCR genes occurs earlier during human T-cell development than previously reported.[4, 11] Given that in mice *TCRD* rearrangement starts at DN1, followed by *TCRG* in DN2 and *TCRB* in DN2 but especially DN3,[1, 24] the human $CD34^+CD38^-CD1a^-$, $CD34^+CD38^+CD1a^-$ and $CD34^+CD38^+CD1a^+$ subsets resemble murine early DN1 ($CD44^+CD25^-CD117^-$), late DN1+DN2 ($CD117^+$ DN1; $CD44^+CD25^+$), and DN3 ($CD44^-CD25^+$) stages, respectively. However, the relative frequency of DN1 cells in mice is higher than that of the corresponding human subset ($CD34^+CD38^-CD1a$). We also demonstrate that TCRβ-selection and initiation of *TCRA* rearrangements do not occur at the ISP/DP stages of human T-cell development,[4, 10, 11] but instead already at the $CD34^+CD38^+CD1a^+$ stage. This is similar to the mouse, in which TCRβ-selection occurs in DN3 thymocytes.[14]

Based on the TCR rearrangement data and the expression of key recombination and differentiation genes (e.g., *RAG1, RAG2* and *PTCRA*), we show that human and mouse T-cell development are much more similar than previously assumed. In addition candidate factors for regulation of TCR recombination are identified. We propose an up-dated human T-cell differentiation model, as shown in the Discussion of this thesis. These novel data help to bridge gaps in our understanding of human T-cell development, and should provide insight into the development of T-cell acute lymphoblastic leukemia and SCID.

MATERIALS AND METHODS

Isolation of cell samples from thymus, umbilical cord blood and peripheral blood

Thymi were obtained as surgical tissue discards from children aged 7 weeks to 3 years (median of 6 month) undergoing cardiac surgery at the Erasmus MC Rotterdam, with informed consent from the parents. The children did not have immunological abnormalities. Thymocytes were isolated by cutting the thymic lobes into small pieces and squeezing them through a metal mesh, and stored at -80ºC until further analyses. Mononuclear cells were isolated by Ficoll-Paque (Amersham Biosciences) density centrifugation from human umbilical cord blood (UCB) obtained from full-term normal deliveries and from peripheral blood of healthy volunteers. All samples were obtained according to the guidelines of the Medical Ethical Committee of the Erasmus MC that also approved the human studies.

Purification of subpopulations

Total mononuclear cells or thymocytes from 5 donors were pooled to reduce intra-sample variation. After thawing, pooling and Ficoll density separation, thymocytes were labeled with fluorochrome-conjugated monoclonal antibodies. In order to isolate the $CD34^+$ lineage $(lin)^-$ UCB cells, $CD34^+CD38^-CD1a^-$, $CD34^+CD38^+CD1a^-$ and $CD34^+CD38^+CD1a^+$ thymocytes, magnetic beads (Myltenyi Biotech) were used to enrich for $CD34^+$ cells. For isolation of the ISP population, thymocytes were depleted of CD3-expressing cells using magnetic beads. For isolation of $TCR\gamma\delta^+$ thymocytes, magnetic beads were used to enrich for $TCR\gamma\delta^+$ cells. All magnetic beads were used according to the manufacturers protocol. After MACS isolation, the enriched cells were labeled with fluorochrome-conjugated monoclonal antibodies for further purification by high speed cell sorting. In order to isolate mature $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$

T-cells, peripheral blood mononuclear cells were labeled with fluorochrome-conjugated monoclonal antibodies for cell sorting. All cell sorting was performed on a FACS DiVa cell sorter (BD Biosciences). Monoclonal antibodies used, with the clone in brackets: CD4-FITC (SK3), CD38-FITC (HB7), TCRαβ-FITC (WT31), CD1a-RDI (T6), CD3-PE (SK7), CD16-PE (B73.1), CD19-PE (4G7), CD56-PE (M431), TCRγδ-PE (11F2), CD3-PerCP (SK7), CD8-PerCP (SK1), CD19-PerCP (SJ25C1), CD3-APC (SK7), CD8-APC (SK1) and CD34-APC (8G12) (all from BD Biosciences), CD13-RDI (MY7) and CD33-RDI (906) (from Beckman Coulter). Purity of sorted population was determined on the FACS Calibur (BD Biosciences) and shown to be over 95% for all populations. All populations were sorted twice using different donors for each sort.

DNA isolation, and RQ-PCR analyses of TCR gene rearrangements

DNA from thymic subsets, $CD34^+$ lin^- UCB cells and peripheral $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T cells was extracted with the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich), according to the manufacturers protocol. DNA was extracted from control cell lines using the QIAamp Blood Midi kit (Qiagen) according to the manufactures protocol. RQ-PCR for quantification of different TCR recombinations was essentially performed as described previously using ABI Prism 7700 equipment (Applied Biosystems).[25] The following *TCRD* recombinations were determined. Dδ2-Dδ3; using Dδ2 and Dδ3 specific primers, Dδ2-Jδ1; using Dδ2 and Jδ1 specific primers, Vδ1-Jδ1 and Vδ2-Jδ1; were performed in two independent PCR reactions using either a Vδ1or a Vδ2 primer in combination with a Jδ1 primer, Vδ-Dδ3; using six specific Vδ primers (Vδ1-Vδ6) in combination with a Dδ3 primer. The cell lines/diagnostic samples that were selected as clonal control DNA for *TCRD* rearrangements are the following: Dδ2-Dδ3 (Nalm 16), Dδ2-Jδ1 (Loucy), Vδ1-Jδ1 (Peer), Vδ2-Jδ1 (T-ALL sample), all Vδ's-Dδ3 (Nalm 16 with Vδ2-Dδ3). *TCRG* rearrangements were determined as follows: Vγ to Jγ1.1 and Jγ2.1 rearrangements were determined in two independent PCR reactions using a Jγ1.1/2.1 consensus primer (recognizing Jγ1.1 and Jγ2.1) in combination with either a VγI family primer (VγIf; recognizing Vγ2, Vγ3, Vγ4, Vγ5, ψVγ7 and Vγ8) and a Vγ10 primer or in combination with a Vγ9 and a Vγ11 primer. Analysis of Vγ to Jγ1.3 and Jγ2.3 rearrangement which was performed using a Jγ1.3/2.3 consensus primer (recognizing Jγ1.3 and Jγ2.3) in combination with VγIf, Vγ9, Vγ10 and Vγ11 primers. Analysis of Vγ to Jγ1.2 rearrangement which was performed using a Jγ1.2 primer in combination with VγIf, Vγ9, Vγ10 and Vγ11 primers. The cell lines/diagnostic samples that were selected as clonal control DNA for *TCRG* rearrangements are the following: Vγ1f+Vγ10-Jγ1.1/2.1 (HUT 78 with Vγ8-Jγ1.1), Vγ9+Vγ11-Jγ1.1/2.1 (ARR with Vγ9-Jγ1.1), all Vγ's-Jγ1.3/2.3 (RPMI 8402 with Vγ10-Jγ2.3 and Vγ4-Jγ2.3), all Vγ's-Jγ1.2 (T-ALL sample with Vγ9-Jγ1.2). *TCRB* Dβ to Jβ rearrangements were performed in two independent PCR reactions containing either a Dβ1 primer in combination with six primers for the Jβ1 cluster (one specific primer for each Jβ1 gene segment) or a Dβ2 primer in combination with seven primers for the Jβ2 cluster (one specific primer for each Jβ2 gene segment). As a clonal control for Dβ1-Jβ1 and Dβ2-Jβ2 gene rearrangements we used HUT78 which contains a Dβ1-Jβ1.2 and Dβ2-Jβ2.3 rearrangement. Furthermore, in order to examine initiation of *TCRA* rearrangement, we determined the TCRD deleting δREC-ψJα rearrangement using a δRec primer in combination with a ψJα specific primer and for which we used H-SB2 as clonal control. In addition we determined the decrease of *TCRA* germline DNA in all thymic subsets and peripheral $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T cells by using a primer located just upstream of ψJα in combination with a primer located just downstream of ψJα. For this purpose $CD34^+lin^-$ UCB cells were used as a hundred percent *TCRA* germline control. All applied forward/reverse primers and TaqMan probes are available at http://www.jem.org/cgi/content/full/jem.2004 2524/DC1; most have been described previously.[5, 9, 26, 27]

Control cell lines and T-ALL controls were serially diluted in a background of un-rearranged (germline) DNA in order to generate a standard curve ranging from 100% to 0.1% of specific template DNA (specific rearrangement) in a non-template background. An albumin RQ-PCR was performed to normalize the amount of input DNA of non-diluted control DNA and DNA from thymocyte subsets, $CD34^+lin^-$ UCB cells and peripheral T-cells, using serially diluted reference DNA. A typical RQ-PCR mixture of 25 µl contained TaqMan Universal MasterMix (Applied Biosystems), 540-900 nM of each primer, 100 nM of FAM-TAMRA labeled probe, 0.4 µg of bovine serum albumin and 50 ng of genomic DNA. Positive controls were considered to be clonal and to contain hundred percent of the specific rearrangement. C_T values of thymic subsets, $CD34^+lin^-$ UCB cells and peripheral T cell DNA for a particular TCR RQ-PCR were subsequently plotted on the corresponding standard curve, and expressed as relative rearrangement levels as compared to the

corresponding clonal control DNA. The sensitivity of each assay was at least 1%. All RQ-PCR experiments were performed in duplicate on two independently purified sets of all isolated subsets.

GeneScan analysis for complete and in-frame Vβ-Jβ gene rearrangements

In order to analyze Vβ-Jβ rearrangements and selection of the TCRβ chain (i.e. in-frame *TCRB* gene rearrangements) in the isolated populations, complete *TCRB* V-J rearrangements were amplified with HEX-labeled primers for the Jβ1 cluster and FAM-labeled primers for the Jβ2 cluster. This was done by two multiplex PCR reactions as described by the BIOMED-2 Concerted Action BMH4 CT98-3936: tube A containing primers for each Jβ1 segment as well as Jβ2.2, Jβ2.6, and Jβ2.7 primers in combination with Vβ primers and tube B containing Jβ2.1, Jβ2.3, Jβ2.4, and Jβ2.5 primers in combination with Vβ primers.[9] The obtained PCR products were subsequently subjected to GeneScan analysis as described before.[9]

TEA expression by real time quantitative (RQ)-PCR analyses

In order to determine the expression of the sterile T-early alpha (TEA) transcript in the isolated populations, RNA was reverse transcribed into cDNA as described previously.[28] Subsequently the cDNA was diluted by adding 80 µl of milli Q water yielding a final concentration of 10 ng/µl. As positive control for TEA expression the immature T-cell line SUPT3 was used and as a negative control the immature T-cell line H-SB2, which has both *TCRD* alleles deleted and therefore expected to be TEA negative, was used as well as the non-hematopoietic cell line HeLa. RQ-PCR was performed as previously,[29] and the expression of TEA was defined by calculating the ratio of TEA to the average value of the control genes Abelson (*ABL*) and beta-glucuronidase (*GUSB*).[29]

Microarray analysis

Microarray analysis was essentially done as described previously[30] and according to MIAME guidelines.[31] RNA was isolated using RNeasy columns (Qiagen) according to the manufacturers protocol and RNA quality was assessed using the Agilent 2100 BioAnalyzer. Two to five µg of total RNA from the $CD34^+CD38^+CD1a^-$, $CD34^+CD38^+CD1a^+$, ISP, DP $CD3^-$, DP $CD3^+$, SP $CD4^+$ and SP $CD8^+$ populations was used to generate double-stranded cDNA using SuperScript reverse transcriptase and a T7-oligo(dT) primer. An ENZO kit (ENZO-Life Sciences Inc) was used to convert cDNA into biotinylated cRNA utilizing T7 RNA polymerase and biotinylated ribonucleotides. Biotinylated cRNA was separated from enzymes and unincorporated nucleotides using RNeasy 'clean-up' spin columns (Qiagen). For the smaller, earlier populations, $CD34^+lin^-$ UCB cells, $CD34^+CD38^-CD1a^-$ thymocytes and for control purposes the $CD34^+CD38^+CD1a^+$ thymocytes, the GeneChip Eukaryotic Small Sample Target Labeling Assay Version II (Affymetrix) was used to generate biotinylated cRNA. Eleven µg of cRNA was fragmented for 35 min at 95˚C. 10 µg of fragmented cRNA was then hybridized to HG-U133A human genome microarrays for 16 hours at 45˚C followed by washing, staining and scanning at 570 nm (Affymetrix). All raw microarray data are freely available at http://franklin.et.tudelft.nl/.

Statistical analysis

Probe intensity background was removed using robust multichip analysis (RMA).[32] The intensity levels were then quantile normalised.[33] Array groups (two biologically independent arrays per group) corresponding to the development stages were compared based on the perfect match (PM) probe intensity levels only,[32] by performing a per-probe set two-way analysis of variance (ANOVA, with factors "probe" and "stage"). This resulted in average expression levels over the two biological repeats for each probe set in each stage, as well as a *p*-value for the significance of the difference between the stages. As data resulting from the standard protocol ("non-amplified") are not directly comparable to those from the small sample protocol ("amplified"), the expression levels measured for probe sets under the small sample protocol were scaled, according to the results in the $CD34^+CD38^+CD1a^-$ population, which was analyzed by both protocols. For each individual probe set, the scaling factor used was the ratio between the average expression level measured for that probe set in the $CD34^+CD38^+CD1a^-$ population, under the standard protocol and the small sample protocol.

The *p*-values were adjusted for multiple testing using Šidák step-down adjustment[34] and all differences with adjusted *p*-values <0.05 were considered significant. All probe sets that underwent a significant change

between any two successive stages of differentiation ($CD34^+lin^-$ UCB vs $CD34^+CD38^-CD1a^-$, $CD34^+CD38^-CD1a^-$ vs $CD34^+CD38^+CD1a^-$, $CD34^+CD38^+CD1a^-$ vs $CD34^+CD38^+CD1a^+$, $CD34^+CD38^+CD1a^+$ vs ISP, ISP vs DP $CD3^-$, DP $CD3^-$ vs DP $CD3^+$, DP $CD3^+$ vs SP $CD4^+$ and DP $CD3^+$ vs SP $CD8^+$) were selected.

The OmniViz software package was used to construct and display a Pearson correlation matrix between the individual microarrays, based on the expression values of significant probe sets. Before calculating correlations, all expression values lower than 30 were set to 30. Expression values were then $\log_2$-transformed and the per probe set geometric mean was subtracted. Further analysis was performed using the Genlab software toolbox, running in the Matlab programming environment (http://www.genlab.tudelft.nl). After per-probe set normalization to zero mean and unit standard deviation (*z*-score), a hierarchical clustering (complete linkage) was calculated based on Pearson correlation. The number of clusters *k* was determined by looking for a local minimum of the Davies-Bouldin index calculated for $k=1, \ldots, 30$.[35]

For further analysis the list of significant probe sets was filtered, using the annotation found in the Affymetrix NetAffx analysis database (http://www.affymetrix.com/), for probe sets related to transcriptional activity based on the following Gene Ontology (GO) Consortium designations: nucleic acid binding (GO: 3676), DNA binding (GO: 3677), transcription factor activity (GO: 3700), transcription cofactor activity (GO: 3712), transcription co-activator activity (GO: 3713), transcription co-repressor activity (GO: 3714), transcription (GO: 6350), transcription, DNA dependent (GO: 6351), regulation of transcription, DNA dependent (GO: 6355), transcription from Pol II promoter (GO: 6366), transcription initiation from Pol II promoter (GO: 6367), transcription factor complex (GO: 5667), negative regulation of transcription (GO: 16481), transcriptional activator activity (GO: 16563), transcriptional repressor activity (GO: 16564) and positive regulation of transcription (GO: 45941). Less relevant genes obtained from these categories (e.g., RNA editing enzymes/polymerases, nucleosome assembly proteins, histones, etc) where manually removed from this list. The resulting 446 probe sets were then hierarchically clustered as described above.

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CHAPTER 3

SEVERE ADVERSE EFFECTS IN GENE THERAPY FOR SCID

CHAPTER 3.1

ECTOPIC RETROVIRAL EXPRESSION OF LMO2, BUT NOT IL2Rγ, BLOCKS HUMAN T-CELL DEVELOPMENT FROM CD34$^+$ CELLS: IMPLICATIONS FOR LEUKEMOGENESIS IN GENE THERAPY

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ABSTRACT

The occurrence of leukemia in a gene therapy trial for SCID-XI has highlighted insertional mutagenesis as an adverse effect. Although retroviral integration near the T-cell acute lymphoblastic leukemia (T-ALL) oncogene LIM-only protein 2 (*LMO2*) appears to be a common event, it is unclear why *LMO2* was preferentially targeted. We show that of classical T-ALL oncogenes, *LMO2* is most highly transcribed in CD34+ progenitor cells. Upon stimulation with growth factors typically used in gene therapy protocols transcription of *LMO2, LYL1, TAL1* and *TAN1* is most prominent. Therefore, these oncogenes may be susceptible to viral integration. The interleukin-2 receptor gamma chain (*IL2RG*), which is mutated in SCID-XI, has been proposed as a cooperating oncogene to *LMO2*. However, we found that over-expressing *IL2RG* had no effect on T-cell development. In contrast, retroviral over-expression of *LMO2* in CD34+ cells caused severe abnormalities in T-cell development, but B-cell and myeloid development remained unaffected. Our data help explain why *LMO2* was preferentially targeted over many of the other known T-ALL oncogenes. Furthermore, during T-cell development retrovirus-mediated expression of *IL2RG* may not be directly oncogenic. Instead, restoration of normal IL-7 receptor signaling may allow progression of T-cell development to stages where ectopic LMO2 expression causes aberrant thymocyte growth.

INTRODUCTION

Clinical gene therapy trials have shown marked progress during the last decade, in particular for treatment of hereditary diseases such as the primary immunodeficiency diseases (PID)[1-4]. Indeed, clinical benefit has been demonstrated in two types of severe combined immunodeficiencies, namely those caused by *ADA* and *IL2RG* gene defects.[1-4] The *ADA* gene has been considered a suitable target for many years, but only recently improvements in vector technology, stem cell mobilization and conditioning have led to clinical success.[4] Similarly, two separate trials for SCID-XI have shown the clinical feasibility of introducing a therapeutic gene into stem cells. The deficiency was restored and hematopoietic development was no longer blocked.[1-3] The occurrence of leukemia in 3 patients in one of the gene therapy trials for SCID-XI[5-7] has emphasized insertional mutagenesis as an adverse effect. In the leukemic cells of these patients retroviral integration occurred near the T-cell acute lymphoblastic leukemia (T-ALL) oncogene LIM-only protein 2 (*LMO2*)[8] (Third case reported at the ESGT in Prague, October 1 to November 1, 2005).

The interleukin-2 receptor gamma chain (IL2Rγ), which is non-functional in SCID-XI, has been implicated as a cooperating oncogene to LMO2.[9, 10] Two recent short reports using murine models have suggested that the interleukin-2 receptor gamma chain (IL2Rγ) itself could possibly contribute to leukemic transformation.[9, 10] In the first report, Copeland and co-workers show that insertions near *Lmo2* and *Il2rg* were detected in a single

murine leukemia (tumor x031) represented in the Mouse Retroviral Tagged Cancer Gene Database, which has led to the notion that these genes may act as cooperating oncogenes. However, several other oncogenes were targeted in the same leukemic clone, a fairly common feature of replication competent Mo-MLV used for these kinds of experiments. It therefore is not always clear which of the genes hit are functionally most important and whether cooperation between target genes functionally exists. For instance, the tumor clone with insertions in *Lmo2* and *Il2rg* also contained insertions in *Bmi1*and *Rap1gds1* (query date: July 21, 2006). Both *Bmi1,* a repressor of *Arf,* and *Rap1gds1* are known oncogenes and could therefore also have contributed to oncogenesis in these mice.[11-13]

In the second report, a murine model system was used in which stem cells were transduced with very high levels of human *IL2RG* using a lentiviral vector. These transplanted mice developed tumors with a high incidence, higher than reported for classical T-cell oncogenes such as *LMO2* or *LYL1* and comparable to the most active oncogene in transgenic mice, *Tal1*. This was a surprising finding because in human T-ALL, *IL2RG* has never been reported to act as oncogene. However, the very high levels of *IL2RG* in these mice compared to the slightly lower than normal levels obtained with retroviral MFG vectors in SCID-XI trials may have contributed to development of T-cell lymphomas in these mice. Similarly, insertional mutagenesis may have been a significant contributory factor.[14] The report also did not indicate whether the tumors were clonal, expressed *Il2rg*, JAK3 was activated or the *Il2rg* gene might be mutated for instance due to errors in lentiviral reverse transcription.

Genetic predisposition for tumor development has been proposed as an additional risk factor. Sorrentino and co-workers have recently developed a mouse model that is more prone to develop T-cell malignancies. In this model, both the *Arf* tumor-suppressor gene and the *IL2rg* gene were ablated.[15] Retroviral transduction of *IL2RG* into the HSC of these mice emulates the high incidence of integration-dependent T-cell tumors than were found in the SCID-XI trial. In parallel, the third patient that developed leukemia in the SCID-XI trial had an insertion near *BMI1*, which represses *ARF*, while the family of the first leukemic patient had a history of juvenile tumor development.[5] However, such a genetic predisposition does not explain the consistent latency of 30-34 weeks in the three cases of T-cell leukemia in SCID-XI patients. All three patients did have a retroviral insertion near *LMO2.* The consensus in the field is that the viral LTR caused aberrant expression of the *LMO2* gene. Consequently, we have analyzed the mechanisms involved further by ectopic over-expression of *LMO2* and *IL2RG.*

We used functional hematopoietic assays inducing differentiation of human $CD34^+$ cells into myeloid, B-, T- and NK-cell lineages to assess the effect of *LMO2* and *IL2RG* over-expression. We demonstrate here that only the T-cell lineage is affected by *LMO2* over-expression, while *IL2RG* over-expression has no detectable effects on T-cell development. Because *IL2RG* was suggested to be oncogenic, we also assessed the expression of *Il2RG*, and its associated cytokine receptors, *LMO2* and all other classical T-ALL oncogenes in normal human HSC and thymocyte subpopulations in detail. We have

presented a small part of the thymocyte data (without methods or figures) in a brief report elsewhere, but without the essential gene expression data nor B-, NK- and myeloid cell developmental assays.[16] Our data on T-ALL oncogene expression shown here provide an explanation why *LMO2* was preferentially targeted over other known T-ALL oncogenes. In addition, *IL2RG* may not act as an oncogene but by restoring IL-7 receptor signaling, allows development of T cells to stages where ectopic *LMO2* expression hampers T-cell development in the thymus. This may create a pre-leukemic condition by accumulation of immature cells under intense proliferative pressure.

MATERIALS AND METHODS

Monoclonal antibodies

CD4-PE, CD16-PE, CD56-PE, CD8-APC, CD14-APC, CD19-APC and CD34-APC (BD Biosciences, Santa Clara, CA), CD1a-RDI, CD13-RDI and CD33-RDI (Beckman Coulter).

Isolation and purification subsets from thymus and umbilical cord blood (UCB) for gene expression profiling of normal human T-cell development

Material was handled and purified as described.[17] Briefly, all stages of human T-cell development as well as CD34+ UCB were isolated in duplicate. Thymi or cord blood cells from 5 individuals were pooled for each isolation. Purity of sorted population was determined on the FACS Calibur and shown to be >95% for all populations. When samples were less than 95% pure they were excluded from further experiments.

Purification and stimulation of CD34+ UCB and CD34+ PBSC for gene expression profiling

CD34+ UCB cells were obtained from placentas of full-term pregnancies after informed consent according to legal regulations in The Netherlands. Mononuclear cells were isolated by Ficoll density gradient centrifugation (1.077 g/cm^2, Nycomed Pharma AS, Oslo, Norway) and cryopreserved. After thawing, CD34+ cells from 3 to 5 individuals were isolated using magnetic beads (Miltenyi Biotech, Germany).

Purity of the cells that were used was >90% as determined by flow cytometric analysis. CD34+ UCB cells were either lysed immediately or cultured for 2 and 4 days before isolating RNA. Cells were cultured in stem cell medium supplemented with 100 ng/ml rhSCF, 50 ng/ml rhFLT3-L and 10 ng/ml rhTPO as previously described.[18, 19] Blood obtained from three individuals that were treated with cyclophosphamide and G-CSF was used as a source of PBSC. Informed consent was obtained. The donors had the following characteristics: one healthy male donor, aged 35; one male myeloma patient in stable plateau phase, aged 53; one female myeloma patient in stable plateau phase, aged 48. CD34+ PBSC were isolated from using CliniMACS magnetic beads. Isolated CD34+ PBSC were cultured as was done for the 2 clinical trials for SCID-XI and lysed for RNA isolation. Samples for all three individuals were stimulated and analysed independently. Stimulation 1 was done according to Cavazzana-Calvo *et al*.[20] Briefly, cells were cultured at $0.5 \cdot 10^6$ cells/ml in X-vivo 10 medium containing 4% FCS, SCF (300 ng/ml), PEG-MDF (100 ng/ml), IL-3 (60 ng/ml) and FLT3-L (300 ng/ml) for 24 hours. Then cells were cultured for an additional 3 days in the same media but with 50 ng/ml retronectin and 4 ng/ml protamine sulfate added. Stimulation 2 was done according to Gaspar *et al*.[1] Briefly, cells were cultured at $0.5 \cdot 10^6$ cells/ml in X-vivo 10 medium containing 1% human serum albumin, SCF (300 ng/ml), TPO (100 ng/ml), IL-3 (20 ng/ml) and FLT3-L (300 ng/ml) for 40 hours. After that cells were cultured for an additional 3 days in the same media but with 25 ng/ml retronectin added.

Gene expression profiling of CD34+ umbilical cord blood cells and thymocytes

Micro-array analysis was essentially done as described previously[17] and according to MIAME guidelines. For expression analysis of normal human T-cell development the HG-U133A Array from Affymetrix (22,283 probe sets) was used. For expression analysis of untreated (baseline) and stimulated

CD34+ UCB cells as well as stimulated CD34+ PBSC the HG-U133 Plus 2.0 Array from Affymetrix (54,675 probe sets) was used. For data analysis, probe intensity background was removed using robust multi-chip analysis.[21] The intensity levels were quantile normalized.[22, 23] Expression is considered absent when the arbitrary fluorescence value is below 80, corresponding to the cut-off given by MAS and GCOS software. The same criteria were used for making the absent-present calls for data extracted from UCB CD34+ cells, thymocytes as well as PBSC.

Production of MLV-based recombinant retrovirus

The retroviral plasmids LZRS-IRES-EGFP (control), LZRS-LMO2-IRES-EGFP and LZRS-IL2RG-IRES-EGFP were constructed and transfected into Phoenix amphotropic packaging cell lines using Fugene-6 transfection reagent. The LZRS vector has been described earlier.[24] Stable high-titer producer clones were selected using puromycin (1 µg/ml). Recombinant virus-containing supernatant was harvested, filtered and stored at -80˚C.

Transduction of CD34+ umbilical cord blood cells

CD34+ UCB cells from 3 to 5 individuals were pooled and pre-stimulated in IMDM medium supplemented with 10% heat inactivated fetal bovine serum, 50 ng/ml rhSCF, 50 ng/ml rhFLT3L and 10 ng/ml rhTPO for 24 hours. Recombinant retrovirus containing supernatant was incubated in Retronectin-coated 35mm Petri dishes for 2 to 3 hours at 37˚C. Transduction was done using 0.33 x10^6 to 0.5x10^6 cells per dish. Cells were cultured for two additional days in the medium as described above. Extra recombinant retrovirus-containing supernatant was added after 24 hours. The efficiency of retroviral transduction was evaluated after 48 h by flow cytometry and ranged between 25% and 39% (EGFP), 8% and 32% (LMO2-IRES-EGFP) and 7% and 12% (IL2RG-IRES-EGFP). We checked *LMO2* and *IL2RG* expression in CD34+ UCB cells transduced with LMO2-IRES-EGFP and IL2RG-IRES-EGFP, respectively by RQ-PCR. Sorted EGFP+ cells expressed 11 fold more *LMO2* RNA than sorted EGFP- cells, as determined by RQ-PCR (not shown), whereas for *IL2RG* the difference was 12 times (not shown).

Real-time quantitative-polymerase chain reaction (RQ-PCR)

For validation of microarray experiments on UCB samples, RQ-PCR was done for *LMO2*, *LYL1* and *TAL1* according to standard procedures. Primers and probes: *LMO2*: FW-LMO2-EMC: CAAACTGGGCCGGAAGC, RV-LMO2-EMC: ACCCGCATTGTCATCTCAT, probe (FAM) Tr-LMO2-EMC: CAAAAAGCCTGAGATAGTCTCTCCGGCAG. *LYL1*: FW-LYL1-EMC: CCCCTTCCTCAACAGTGTCTACA, RV-LYL1-EMC: CTCCCGGCTGTTGGTGAA, probe (FAM) Tr-LYL1-EMC: CTCACAGTGGC TTGGTCTCCGCTTC. *TAL1*: FW-TAL1-EMC: CCGGATGCCTTCCCTATGT, RV-TAL1-EMC: TCCCGGCTGTTGGTGAAGA, probe (FAM) T-TAL1-EMC: AGACCTTCCCCCTATGAGATGGAGATTACT GAT. For validation of *LMO2* expression using the LZRS-IRES-EGFP construct we used the primers and probes described above. Validation of *IL2RG* expression using the LZRS-IRES-EGFP construct was done with primers and probes obtained from Applied Biosytems (Assay on demand # Hs 00173950_m1). For each sample *ABL* was amplified as a control target: fw: TGGAGATAACACTCTAAGCATAACTAAAGGT, rev: GATGTAGTTGCTTGGGACCCA, probe (FAM-TAMRA): CCATTTTTGGTTTGGGCTTCACACCATT.

Co-culture assays

The co-culture assays were performed using confluent cultures of the murine bone marrow stromal cell line S17 plated in 12-well plates. The cell line was co-cultured with 10,000 CD34+ UCB cells per well. In some experiments, a part of the cells was taken from the co-culture after 1 week and tested for their ability to develop along the NK cell lineage in suspension culture in the presence of 100 units rhIL-2.

Fetal thymus organ cultures (FTOC)

Mouse-human hybrid FTOC were essentially done as described.[25] Fetal thymic lobes from littermates were obtained from C57Bl/6 mice on day 14 of gestation and endogenous thymocytes were depleted by gamma-irradiation (14.4 Gy). Hanging drops were created in Terasaki plates by adding one thymic lobe to a 25 µl cell suspension containing 30,000 to 40,000 transduced CD34+ UCB cells. Inverted Terasaki plates were incubated for 2 days in a humidified incubator (5% CO_2 in air, 37˚C) to facilitate the entry of UCB cells

into the thymic lobes. Subsequently, lobes were transferred to Nuclepore filters, which were layered over 1 ml medium in 12-well plates. Starting at day 7 10 ng/ml rhIL-7 was added to the culture medium twice a week. Cultures were incubated at 37°C in 5% CO_2 for 9 or 21 days after which lobes were mechanically disrupted into single cell suspensions and flow cytometric analysis was done.

RESULTS

We have previously reported global gene expression profiling of $CD34^+lin^-$ UCB cells and 8 consecutive stages of human thymic T-cell development.[17] We mined these data for the transcription levels of several well-known T-ALL oncogenes in $CD34^+$ progenitor cells. When normalizing expression against all probe sets on the array, in unstimulated $CD34^+$ progenitor cells from umbilical cord blood (UCB) *LMO2* was expressed at the highest level, followed by *LYL1, TAN1, HOX11* and *TAL1. LCK* was expressed at low, but detectable levels (Figure 1A). Other T-ALL oncogenes investigated were not transcribed at detectable levels. Expression was considered absent when the arbitrary fluorescence value fell below 80, corresponding to the cut-off given by MAS and GCOS software.

$CD34^+$ cells used in gene therapy protocols are routinely stimulated with growth factors before and during the gene transfer procedure to facilitate transduction of progenitor cells.[1, 4, 18-20] After stimulation with TPO, Flt3L and SCF, transcription of *LMO2, LYL1, TAL1* and *TAN1* was most pronounced, while the levels of *LCK* and *HOX11* decreased (Figure 1A). The array data were validated by RQ-PCR for the *LMO2*, *LYL1* and *TAL1* genes (Figure 1B, right panel). Average values of two independent experiments are given. Results of the RQ-PCR were very comparable to the array data, here normalized to *ABL* expression (Figure 1B, left panel). Recent studies by Bushman and co-workers indicate that transcriptional activity correlates with integration site selection.[26] This notion was confirmed by other studies from this laboratory showing that retroviral integration closely parallels the transcriptional expression as measured by ESTs (i.e., transcriptional activation).[27] Our studies in primary murine stem cells also demonstrated a strong correlation between the number of viral insertions and expression levels of immediately neighbouring genes (M. Brugman *et al*., Mol Ther, 2006, 13 (Supplement 1), page S37, abstract). While this correlation may not necessarily be valid for any given individual gene, it seems likely that genes that are actively transcribed (called present on the array) have a high probability of being located in euchromatin, making it more accessible for viral insertion. Therefore, these results predict that *LMO2* is a likely oncogene to be targeted by retroviral integration, along with *LYL1, TAL1* and *TAN1*.

$CD34^+$ UCB cells are often used in research settings for gene therapy. However, in clinical SCID-XI gene therapy, BM of very young infants is used. Because it is difficult to obtain age matched normal bone marrow aspirates for research purposes, we have also analyzed the expression of T-ALL oncogenes in G-CSF mobilized peripheral blood stem cells (PBSC) after *in vitro* stimulation to corroborate the UCB expression data. When $CD34^+$ PBSC were stimulated according to transduction protocols used for the two published clinical SCID-XI gene therapy trials,[1, 20] the same pattern of T-ALL oncogene

transcriptional activation was observed as was found in the primary as well as cytokine stimulated CD34⁺ UCB cells (Figure 1C). The average expression values of CD34⁺ cells from three individuals are shown. These results may indicate that CD34⁺ cells display a similar pattern of proto-oncogene expression irrespective of their source. Since the gene expression profiles of CD34⁺ cells from BM, PBSC and UCB are highly similar, with UCB being slightly more similar to BM, we would predict that the oncogenes *LMO2, LYL1, TAL1* and *TAN1* are also transcriptionally active in BM CD34⁺ cells.

Since all three adverse events in the SCID-XI trial presented as uncontrolled clonal T-cell proliferations, it was also of interest to study the expression of T-ALL oncogenes during normal human T-cell development. Compared to CD34⁺ UCB cells, the most

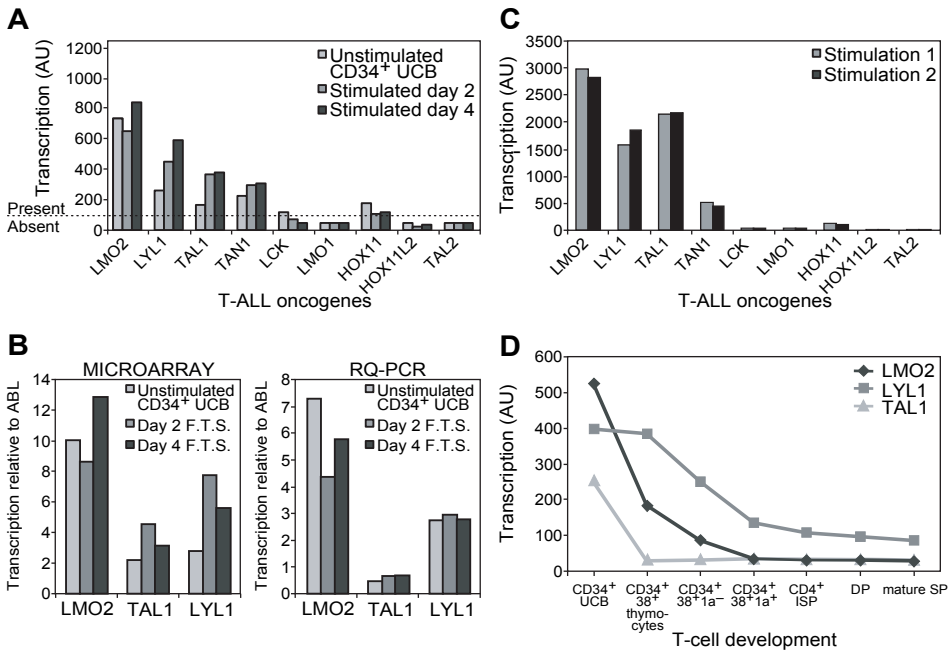

Figure 1. Oncogene expression in human UCB and thymocytes.
A. Expression of T-ALL oncogene transcripts in unstimulated (day 0) and stimulated (day 2 and day 4) UCB CD34⁺ cells analyzed on Affymetrix HG-U133 Plus 2.0 Arrays. Stimulated: growth factor stimulation using Flt3-L, TPO and SCF. Data were normalized against all probesets present on the array. Expression is considered absent when the arbitrary fluorescence value is below 80, corresponding to the cut-off given by MAS and GCOS software. B. Expression of *LMO2*, *TAL1* and *LYL1* relative to *ABL*. The left panel represents data extracted from the microarray analysis described in Figure 1A. The right panel shows the average expression of *LMO2*, *TAL1* and *LYL1* relative to ABL as determined by RQ-PCR in two independent experiments. C. Expression of T-ALL oncogene transcripts in CD34⁺ PBSC stimulated with Flt3-L, TPO, IL-3 and SCF analyzed on Affymetrix HG-U133 Plus 2.0 Arrays. Data were normalized against all probesets present on the array. Stimulation 1 was done according to Cavazzana-Calvo *et al.*[20] and stimulation 2 was done according to Gaspar *et al.*[1] D. Expression of T-ALL oncogene transcripts during normal human T-cell development and progenitor cells analysed on Affymetrix HG-U133A Arrays. Stages of T-cell development are plotted on the X-axis, from immature to mature thymocytes.

immature cells in the thymus ($CD34^+CD38^-$) showed a decreased expression of *LMO2*, similar to *TAL1*, but *LYL1* expression was maintained until the $CD4^+CD8^+$ DP stage (Figure 1D). Thus, *LMO2* expression is highest in $CD34^+$ progenitor cells and rapidly decreases during T-cell differentiation, suggesting that ectopic expression of *LMO2* at later stages of development may have adverse effects on T-cell development.

The effects of LMO2 over-expression have not been studied in human hematopoiesis. We utilized two culture systems to study ectopic LMO2 expression, schematically depicted in Figure 2. The murine bone marrow stroma cell line (S17), supplemented with IL-7 and SCF allows simultaneous development of human $CD34^+$ UCB into B lymphocytes and myeloid cells, and the mouse-human hybrid fetal thymus organ culture (FTOC) was used to study T-cell development. This assay allows for evaluation of human T-cell development *ex vivo*, but variability of individual human samples results in a relatively high inter-experiment variability, and quantitative assessments are most reliably done by intra-experiment comparisons, which is the approach chosen here. The *LMO2* transgene was expressed via an MFG-based retroviral vector, together with an IRES-EGFP cassette to allow easy detection of transduced cells.[24]

$CD34^+$ UCB cells were transduced with 8% to 32% efficiency. Expressing *LMO2* using the MFG-based LZRS vector resulted in an 11-fold increased level of *LMO2* mRNA as measured by RQ-PCR. When co-cultured with the stromal cell line S17, ectopic expression of *LMO2* did not interfere with the development of $CD34^+$ UCB cells into the B-cell lineage since the percentage of CD19 positive cells did not differ from the control-transduced cells (4% vs. 6% respectively, Figure 3A). Similarly, the development of the myelo-monocytic cells was not affected, as illustrated by staining for CD13/CD33 and CD14. About one-

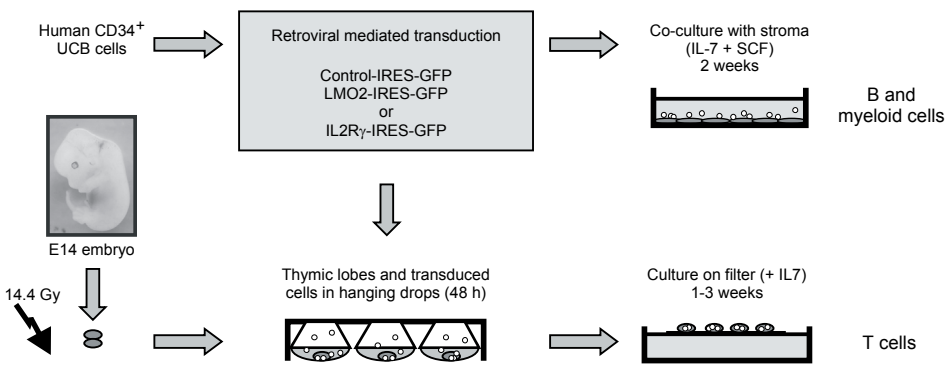

Figure 2. Schematic overview of differentiation assays.
Development of human $CD34^+$ UCB into B lymphocytes and myeloid cells was studied using the murine bone marrow stroma cell line (S17), supplemented with human recombinant IL-7 and SCF. The mouse-human hybrid FTOC was used to study T-cell development. Transduced $CD34^+$ UCB cells were used as input material for both assays. The *LMO2* or *IL2RG* transgene were expressed via an MFG-based retroviral vector, together with an IRES-EGFP cassette allowing for easy detection of transduced cells.

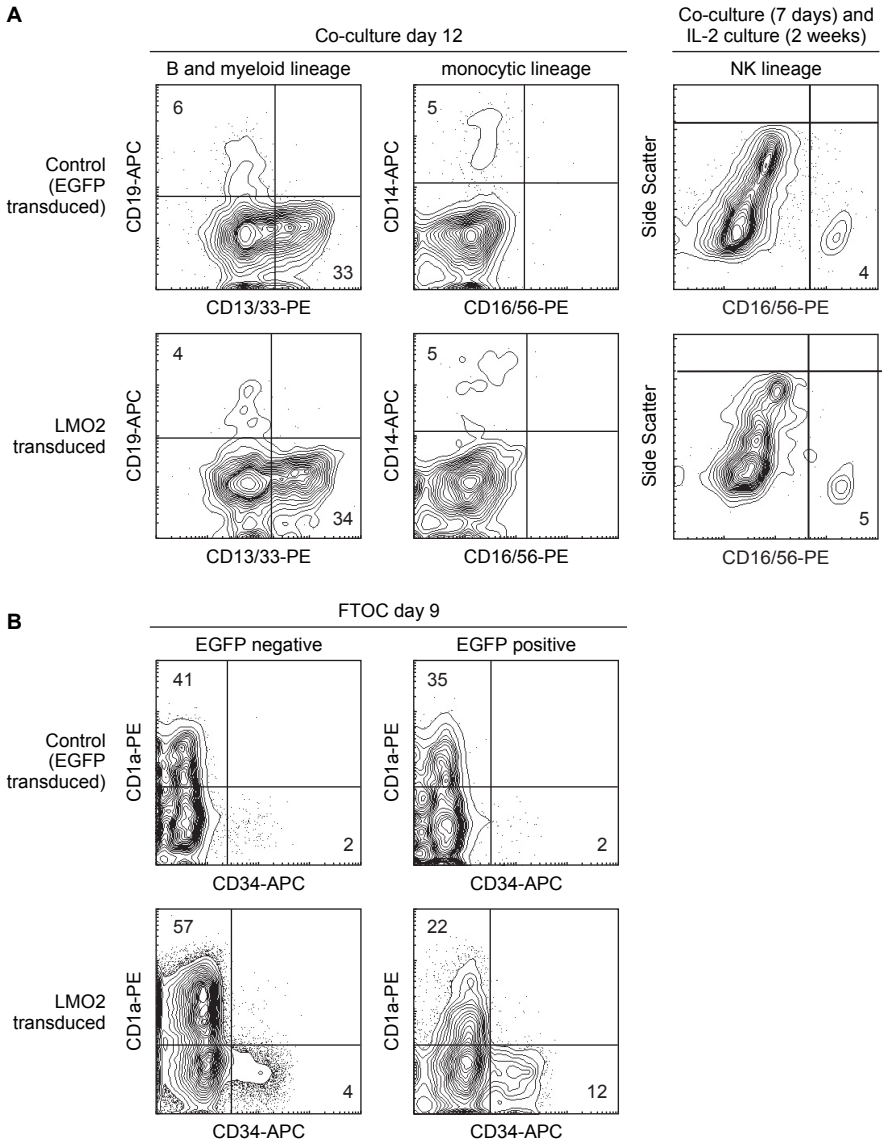

Figure 3. The effects of ectopic *LMO2* expression on B, myelo-monocytic, NK and T lineage development.

A. Flow cytometric analysis of co-culture experiments after 12 days of culture. The FACS-plots display CD45 and EGFP-positive cells in the lymphocyte gate. Numbers represent the percentage of cells within the quadrant. Representative results from 1 out of 3 co-culture experiments are shown. B-D. The FACS-plots show cells in the lymphocyte gate. Numbers represent the percentage of cells within the quadrant. Representative results for 1 or 2 out of 5 experiments are shown. B. Flow cytometric analysis of FTOC at day 9. C and D Flow cytometric analysis of FTOC at day 21.

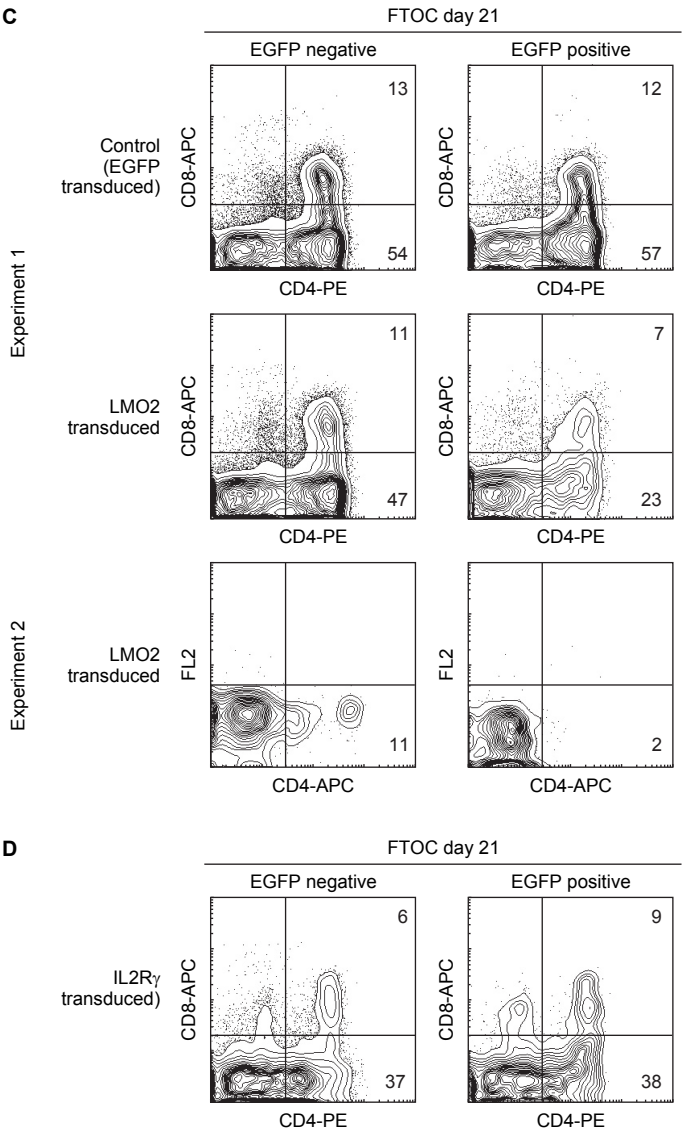

Figure 3. The effects of ectopic *LMO2* expression on B, myelo-monocytic, NK and T lineage development (continued).

third of the cells were positive for the myeloid markers CD13/CD33 (34% vs. 33%) in both cultures, while five percent of the cells were positive for CD14. In parallel, part of the transduced cells was removed from co-culture after 1 week and cultured in suspension for an additional 14 days in the presence of IL-2 to assay for the ability to develop into the NK

lineage. Both *LMO2* as well as control-transduced cells showed an equal percentage of CD16/56 positive cells indicating equal NK cell development. Thus, *LMO2* over-expression does not affect the development into B, NK and myelo-monocytic lineages.

A relatively high inter-experiment variability is seen in FTOC experiments due to variability of individual human samples. Quantitative assessments were therefore done using intra-experiment comparisons. In FTOC experiments at day 9 of culture, *LMO2*-expressing cells showed a lower percentage of the early thymocyte marker CD1a[28, 29] when compared to untransduced cells derived from the same culture (Figure 3B). In addition, in comparison to untransduced cells and control-transduced cells, a higher percentage of *LMO2*-transduced cells retained CD34 expression (12% vs. 2-4%, representative of 5 different FTOC experiments conducted). These data indicate that progenitor cells ectopically expressing *LMO2* are hampered in the initial stages of T-cell development.

Later in T-cell development (day 21 of culture), the total percentage of $CD4^+$ cells was drastically reduced in LMO2-transduced cells compared to untransduced cells (Figure 3C), mostly due to the reduced percentage of CD4 single positive cells (ISP), i.e., 57% in EGFP-transduced controls vs. 23% in LMO2-IRES-EGFP transduced cells. Despite differences in efficiency of development, other experiments also demonstrated this reduction in ISP (Figure 3C, bottom panel). These results are representative of 5 different FTOC experiments conducted. Control-transduced and untransduced ($EGFP^-$) cells displayed similar frequencies of $CD4^+$ and $CD8^+$ cells. Therefore, ectopic *LMO2* expression led to an incomplete inhibition of T-cell development in the $CD34^+$ DN stages and a severe block at the ISP stage. Apparently, as human T-cell development advances, differentiation is increasingly hampered by ectopic *LMO2* expression.

It has been suggested that *IL2RG,* the gene involved in SCID-XI, could possibly contribute to leukemic transformation.[9] We therefore investigated the effect of retrovirus-mediated expression of *IL2RG* on T-cell development. In parallel with the experiments described in Figure 3C, FTOC was done using $CD34^+$ cells that were transduced with the MFG-based virus containing the *IL2RG* gene together with IRES-EGFP, which resulted in 12-fold increased *IL2RG* mRNA as measured by RQ-PCR. Thymocytes derived from the cultures were analyzed for CD4 and CD8 expression (Figure 3D). The percentages of CD4 single positives (37% vs. 38%) as well as CD4/CD8 double positives (6% vs. 9%) were similar in untransduced and *IL2RG*-transduced cell populations: We therefore conclude that retroviral-mediated expression of *IL2RG* does not affect the development of T cells. We also investigated whether cells expressing *IL2RG* under a retroviral promoter have a higher proliferation rate. When comparing percentages of transduced cells after culture with percentages of EGFP-positive cells at the start of the FTOC, a proliferative advantage was not apparent (data not shown).

The IL-2Rγ chain pairs with other cytokine receptor chains (receptors for IL-2, IL-4, IL-7, IL-9, IL-15, IL-21) to form functional signal-transducing entities. We analyzed the transcription levels of *IL2RG* and its partner genes throughout T-cell development (Figure 4). The only chains expressed in developing T cells are *IL2RG* and *IL7RA*.

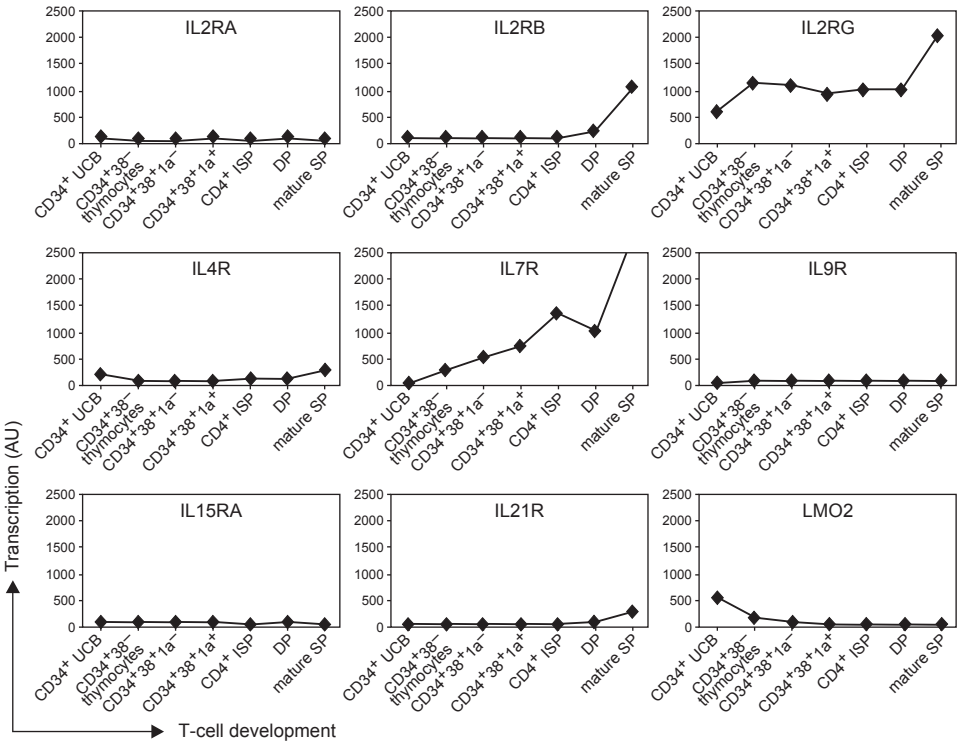

Figure 4. Expression of LMO2, IL2RG and associated IL receptor chains during normal human T-cell development.
Stages of T-cell development are plotted on the X-axis, from immature to mature. Transcription levels are plotted on the Y-axis in arbitrary units (AU).

Mature thymocytes also express *IL2RB,* but not *IL2RA,* the gene product that regulates responses via the full IL-2R complex. Judging from the expression pattern, the only signaling events in which IL-2Rγ could be involved during the course of T-cell development occurs through the IL-7R, in accordance with the known role of both IL-7 and IL-7Rα in T-cell development.[30,31] Therefore, we conclude that the main effect of *IL2RG* gene therapy is restoration of IL7-driven signals during thymic development.

DISCUSSION

Recently, the oncogenic risk of retrovirus mediated gene transfer has received major attention as a safety concern, and has been approached in various experimental model systems, including murine- and non-human primate studies.[32, 33] In the SCID-XI trial in which severe adverse effects were reported, the T-ALL oncogene *LMO2* was ectopically expressed due to insertional mutagenesis. In this study we investigated the expression of *LMO2* and other T-ALL oncogenes during hematopoiesis, in particular the various stages of human T-cell development. Since retroviral insertions predominantly occur in actively transcribed genes,[26, 27] high expression of *LMO2* in immature $CD34^+$ cells make this gene a likely target for deregulated expression through insertional mutagenesis, particularly since this high *LMO2* expression is maintained by cytokines that are used in gene therapy protocols. We show here that over-expression of *LMO2* is well tolerated in all major leukocyte lineages, with the exception of the T-cell lineage. Ectopic *LMO2* expression hampers T-cell development in the thymus, creating a likely preleukemic condition by accumulation of immature cells under intense proliferative pressure. We speculate that such an accumulation at an early stage of T-cell development of the IL2RG-restored cells may have contributed to the apparently uniform latency time of 30-34 weeks.

We have argued elsewhere that murine and human T-cell development is highly similar at the genome-wide level.[17, 34] However at the level of individual genes there are differences in expression and function. For instance defects in the two genes involved in T-negative SCID, *ZAP70* and *IL2RG*, have species-specific phenotypes when deficient. The *Il2rg* deficient mice lack T, B and NK cells, while the human SCID-XI patients usually have normal or increased numbers of B cells. Thus, murine studies should be interpreted with care when extrapolating to human diseases.

In the introduction of this manuscript we refered to two recent murine studies that have suggested that *Il2rg* may be a direct oncogene. We argued that both studies are hampered by an experimental design that makes extrapolation to the leukemias that arose in the SCID-XI trial problematic. It is important to note that the phenotype of the murine tumors found by Woods *et al*.[10] is very different ($B220^+CD3^+$) from that of the T-ALL like tumors that occurred in the patients enrolled in the SCID-XI trial. In our view, the retroviral-mediated expression of *IL2RG* in hematopoietic precursors does not represent a preleukemic or otherwise leukemogenic event because this chain is also highly expressed in normal hematopoietic $CD34^+$ precursors as well as in all subsets of T-cell development (Figure 4). In addition, signaling through IL-2Rγ can only occur when the chain is in close proximity to a partner chain (or multiple chains) bound to its specific ligand, resulting in trans phosphorylation of JAK proteins. Therefore, it is unclear how the extremely high expression of IL-2Rγ alone could lead to tumor development in the study by Woods *et al.* Furthermore, IL-2Rγ expression in treated SCID patients was within the normal range, although this was only measured in peripheral blood T cells.[8, 20] Persistent activation of JAK3 was also not detected in these patients indicating normal

function of IL-2Rγ in the setting of retroviral expression.[8] In addition, a brief report described *IL2RG*-transgenic mice that overexpress human *IL2RG* using the CD2 promoter. In 54 out of 54 mice analysed, no T-cell lymphomas were found.[35] Taken together, these observations argue against IL-2Rγ acting as a cooperative oncogene in the human gene therapy setting.

Most T-negative SCID have an early developmental block like the one found in IL-2Rγ deficiency. Thus we would predict that gene therapy aimed at other T-negative SCID, such as deficiencies in the *RAG1*, *RAG2*, *IL7RA* or *JAK3* genes, would also present with the risk of leukemia development as long as retroviral vectors with high incidence of insertional mutagenesis are used.

Since *LMO2* expression is normally completely down-regulated at the transition of the $CD34^+CD1a^-$ to $CD34^+CD1a^+$ stage (Figure 1B), ectopic *LMO2* expression is apparently tolerated to some extent in the early stages of T-cell development, although it hampers further development resulting in an incomplete developmental block. The block in early human T-cell development is in line with data from *LMO2*-transgenic mice.[36, 37] In mice the initial block is at the DN stage, similar to the first abnormalities in human development we report here, at the $CD34^+CD1a^+$ stage, the equivalent of murine DN3(34).

As the most immature thymocytes undergo extensive proliferation (up to 10,000 fold),[38] any restoration of proliferative signals will have a tremendous selective advantage. The LMO2-induced arrest at a stage of differentiation in which proliferative pressure is high may generate a pre-leukemic state in which additional genetic lesions could lead to full-blown T-ALL, for instance through mutations in the Notch1 gene.[39] Ironically, by restoring T-cell development, *IL2RG* retroviral gene therapy allows *LMO2* to act as an oncogene for T-ALL development. Of course, this scenario will only occur in those cases in which normal regulation of *LMO2* expression is disrupted by nearby retroviral insertion.

We have used DNA microarrays and quantitative PCR to survey expression of a large number of genes in various subpopulations and their quantitative expression levels are useful in predicting which genes are likely targets for insertional mutagenesis. Therefore, a corollary of our findings is that it may be useful to include gene expression profiling of the target cells in gene therapy to assess which oncogenes are transcribed and thereby represent potential targets for insertional oncogenesis. Current efforts to generate self-inactivating (SIN) vectors may overcome some of the problems associated with insertional mutagenesis[40, 41] as these vectors may not transactivate nearby cellular genes as strongly. Finally, we find no evidence for involvement of over-expression of the therapeutic *IL2RG* gene in abnormal T-cell development. Our results also predict that in other gammaretrovirus mediated gene therapy efforts directed at restoring T-cell development (*ADA, RAG1, RAG2*), cells with insertions near the *LMO2* gene might result in similar aberrant LMO2 expression.

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CHAPTER 3.2

GENE THERAPY: IS *IL2RG* ONCOGENIC IN T-CELL DEVELOPMENT?

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INTRODUCTION

The gene *IL2RG* encodes the γ chain of the interleukin-2 receptor and is mutated in patients with X-linked severe combined immune deficiency (X-SCID). Woods *et al*.[1] report the development of thymus tumours in a mouse model of X-SCID after correction by lentiviral overexpression of *IL2RG* and claim that these were caused by *IL2RG* itself. Here we find that retroviral overexpression of *IL2RG* in human $CD34^+$ cells has no effect on T-cell development, whereas overexpression of the T-cell acute lymphoblastic leukaemia (T-ALL) oncogene *LMO2* leads to severe abnormalities. Retroviral expression of *IL2RG* may therefore not be directly oncogenic - rather, the restoration of normal signalling by the interleukin-7 receptor to X-SCID precursor cells allows progression of T-cell development to stages that are permissive for the pro-leukaemic effects of ectopic LMO2.

The occurrence of leukaemia in three patients in a gene-therapy trial for X-SCID[2-4] has highlighted an adverse effect of insertional mutagenesis as a delivery technique for the therapeutic gene. In our investigation, we expressed both *LMO2* and *IL2RG* transgenes in the same retroviral vector that was used in the clinical gene-therapy trials; those trials found that retroviral integration of the corrective *IL2RG* occurred near the locus of the *LMO2* oncogene.[5] We concluded that this integration may have upregulated the expression of *LMO2* and triggered the leukaemia cases in the gene-therapy trials.

Woods *et al*. used a murine model in which stem cells from wild-type or mutant $IL2RG^{-/-}$ mice were transduced with very high amounts of human *IL2RG* by means of a lentiviral vector.[1] They found that these transplanted mice developed a high incidence of tumours. To the best of our knowledge, however, *IL2RG* has never been reported to act as an oncogene in human T-ALL, and murine studies should be interpreted with caution when extrapolating to humans. Although T-cell development is very similar in mice and humans at the genome-wide level,[6, 7] there are differences in individual genes: for instance, mutant $IL2RG^{-/-}$ mice lack T, B and natural killer (NK) cells, whereas human X-SCID patients have normal numbers of B cells.

Mouse-human hybrid fetal thymus organ culture (FTOC) is often used to study human T-cell development *ex vivo*. In FTOC experiments, we found that *LMO2*-transduced cells expressed the early thymocyte marker CD1a less frequently than untransduced cells derived from the same culture (22% compared with 57%), whereas a higher proportion of *LMO2*-transduced cells retained the progenitor marker CD34 (12% compared with 2-4%, representative of five different FTOC experiments). Later, during T-cell development, this block was even more pronounced (58% of cells in the subsequent immature CD4 single-positive stage, compared with 22% in controls). These results indicate that progenitor cells ectopically expressing *LMO2* are severely hampered in T-cell development.

The percentage of immature CD4 single-positive (37% compared with 38%) and the later $CD4^+CD8^+$ double-positive cells (6% compared with 9%) was similar in untransduced and *IL2RG*-transduced populations, indicating that retroviral-mediated expression of *IL2RG* does not affect T-cell development. *LMO2*-transduced cells developed normally

into B, NK and myeloid cells.

The very high expression of lentiviral transgenic *IL2RG* in the transplanted mice studied by Woods *et al*.[1] may have contributed to development of T-cell lymphomas.

In contrast, *IL2RG* concentrations were slightly lower than normal in the human X-SCID trials after treatment using retroviral vectors. The phenotype of the mouse tumours is very immature and different from that of the T-ALL that occurred in the three X-SCID patients. Unfortunately, Woods *et al*. do not indicate whether the tumours were clonal, whether they expressed *IL2RG*, or whether JAK3 was activated; it is possible that the *IL2RG* gene might itself be mutated — for example, as a result of errors in lentiviral reverse transcription, but details of the insertion sites recovered are not given.

In our view, the retroviral-mediated expression of *IL2RG* in haematopoietic precursors does not represent a leukaemogenic event. This γ chain is highly expressed in normal haematopoietic $CD34^+$ precursors and during all stages of T-cell development, whereas *LMO2* is only expressed in progenitor cells and the earliest thymocytes.[6] Furthermore, *IL2RG* expression in treated X-SCID patients is within the normal range.[5] Persistent activation of JAK3 was not detected in these patients, indicating that IL2Rγ function was normal in the setting of retroviral expression.[5]

Taken together, these observations argue against *IL2RG* acting as an oncogene in human gene therapy, although our experiments cannot rule out the possibility that a therapeutic transgene might provide an inappropriate growth stimulus when expressed at high dose or at an inappropriate time. Instead, it is likely that restored *IL2RG* expression allows T-cell development to progress to stages at which *LMO2* would normally be completely downregulated but might contribute to leukaemogenesis if ectopically expressed.

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CHAPTER 3.3

NEW INSIGHTS AND UNRESOLVED ISSUES REGARDING INSERTIONAL MUTAGENESIS IN X-LINKED SCID GENE THERAPY

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ABSTRACT

The oncogenic potential of retrovirus-mediated gene therapy has been re-emphasized because four patients developed T-cell acute lymphoblastic leukemia (T-ALL)-like disease from an otherwise successful gene therapy trial for X-linked severe combined immunodeficiency (X-linked SCID). X-linked SCID, a disease caused by inactivating mutations in the *IL2RG* gene, is part of a heterogeneous group of severe combined immunodeficiencies (SCID) characterized by the lack of T cells in conjunction with the absence of B and/or NK cells. Gene therapy approaches are being developed for this group of diseases. In this review we discuss the various forms of SCID in relation to normal T-cell development. In addition, we consider the possible role of *LMO2* and other T-ALL oncogenes in the development of adverse effects as seen in the X-linked SCID gene therapy trial. Furthermore, we debate whether the integration near the *LMO2* locus is sufficient to result in T-ALL-like proliferations or if the gammaretroviral viral expression of the therapeutic *IL2RG* gene contributes to leukemogenesis. Finally, we review some newly developed murine models that can have added value for gene therapy safety studies.

INTRODUCTION

Recently, the oncogenic risk of retrovirus-mediated gene transfer has received major attention as a safety concern, and has been addressed in various experimental model systems, including *in vitro*, murine, and non-human primate studies.[1-6] Such studies are increasingly recognized as a major pre-clinical prerequisite because of four patients who developed T-cell acute lymphoblastic leukemia (T-ALL)-like disease in an otherwise successful gene therapy trial for the most common form of human severe combined immunodeficiency (SCID), termed “SCID-XI”.[7-9] (A third patient’s case was described at The American Society of Gene Therapy meeting, 2007 in Seattle, WA, USA and a fourth was reported at the 33rd Annual Meeting of the European Group for Blood and Marrow Transplantation in Lyon, France, on March 25-28, 2007.) In this X-linked disease the IL2R γ chain is non-functional because of inactivating mutations. IL2Rγ is also called “common γ chain” because it functions as the signal-transducing component for a number of cytokine receptors. Together with specific α and/or β chains, the common γ chain forms receptors for interleukin 2 (IL2), IL4, IL7, IL9, IL15 and IL21. The common γ chain is an essential component of these receptors because it is needed for JAK3 tyrosine kinase-mediated signal transduction downstream of the receptor complexes.[10]

Two separate trials for SCID-XI have shown the clinical feasibility of introducing a therapeutic gene into hematopoietic stem cells. These experimental therapies are commonly referred to as the French (n=10 patients) and British (n=10 patients) SCID-XI trials and both have been highly successful.[11] The deficiency was restored and lymphocyte

development was no longer blocked,[12-14] but the occurrence of leukemia in 4 patients in the French trial has emphasized that insertional mutagenesis and its oncogenic consequence is an unexpectedly frequent adverse effect of the gammaretroviral gene transfer technology. Even so, 3 out of 4 patients have been successfully treated with conventional anti-leukemic chemotherapy and are currently doing well with sustained expression of the transgene and immune function. The integration site or sites found in the most recent case of leukemia have so far not been reported in literature. In the leukemic cells of the first two patients retroviral integration occurred near the T-ALL oncogene LIM-only protein 2 (*LMO2*).[15] While according to several investigators the integration near the *LMO2* gene suffices to explain the development of these T-ALL cases, there are other reports indicating that the gammaretroviral expression of the therapeutic *IL2RG* gene itself is oncogenic and contributes to leukemogenesis. Here we review the available evidence for both points of view, explain the models used to reach these conclusions, and come up with a risk assessment for other types of SCID in which T-lymphocyte development is deficient.

Normal human T-cell development

In order to understand the effects of ectopic expression of various genes that may affect T-cell development, it is imperative to first discuss normal T-cell development and the effects of mutations in key genes that lead to SCID. T-cell development takes place in the thymus, a primary lymphoid organ that provides a unique microenvironment for the development of hematopoietic progenitors into mature T cells. In adult mice and humans, these progenitor cells are derived from the bone marrow, while during fetal development the liver is the principle source. The exact nature of the progenitors is unclear, but some level of lineage multipotency is now commonly accepted.[16] Development into a mature T cell involves the ordered rearrangements of the different T-cell receptor (*TCR*) genes, mediated by the recombinase activating genes (*RAG*) 1 and 2 (Figure 1). Positive and negative selection events finally lead to the expression of mature TCRαβ molecules. These processes take place during consecutive developmental stages that can be recognized by expression of several key membrane molecules, most notably CD4 and CD8. It is not known at exactly what stage the TCRγδ lineage diverges from the TCRαβ lineage, but murine *in vitro* and *in vivo* indicate that γδ T cells are mainly derived from the double negative 2 (DN2) and DN3 subsets.[17, 18] In both human and mouse, thymocytes are consecutively $CD4^-CD8^-$ (DN), $CD4^+CD3^-$ (human immature single positive) or $CD8^+CD3^-$ (mouse immature single positive), $CD4^+CD8^+$ (double positive) and finally $CD4^+CD8^-CD3^+$ or $CD4^-CD8^+CD3^+$ (single positive). In the mouse, the DN subset can be further subdivided into four stages: $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3) and $CD44^-CD25^-$ (DN4). In humans, $CD34^+CD1a^-$ cells correspond to murine DN1 and DN2 thymocytes and $CD34^+CD1a^+$ cells are homologous to murine DN3 cells (Figure 1).[19] As the number of progenitors that enters the thymus is very limited, a vast expansion takes place during the DN1 and DN2 stages of development.[20] This initial

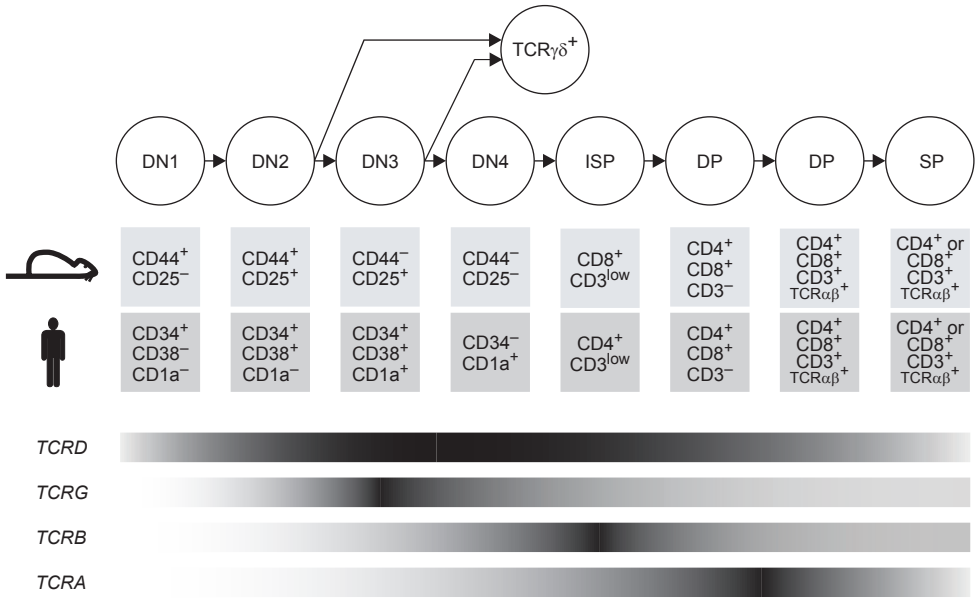


Figure 1. T-cell development.
Subsequent stages of development are described for mouse and human according to the surface markers that are expressed. Based on a previously presented model, stages of development that are comparable between mouse and man are grouped together. In the lower part of the figure, rearrangement activity of the T-cell receptor loci is depicted. Light shades represent low rearrangement activity and dark shades represent high rearrangement activity.

expansion is independent of *TCR* gene-expression and is facilitated by several cytokines, most notably IL7 and other molecules such as Wnt proteins.[21] During the DN3 stage cells stop proliferating and start rearranging the *TCRB* locus.[19] The RAG proteins that are responsible for rearranging the *TCR* gene segments also rearrange of the immunoglobulin gene segments in precursor-B cells in the bone marrow. Thus, mutations in the *RAG1* or *RAG2* genes can lead to a type of SCID in which both mature T and B cells are lacking, while whereas NK lymphocytes are present at a normal level ($T^-B^-NK^+$ SCID).[22-24] In the DN3 to immature single positive stages the TCRβ chain is expressed on the cell surface together with a pre-TCRα chain. During this process, called β-selection, the functionality of the TCRβ chain is tested. Signals emanating from this pre-TCR complex induce proliferation, followed by initiation of *TCRA* gene rearrangements. The thymocytes now become double positive cells. Productive rearrangements lead to the expression of a TCRαβ complex on the cell surface, which is then tested for the recognition of self-major histocompatibility complex molecules (positive selection) and absence of reactivity against self-antigens (negative selection). It has been estimated that 95% of developing thymocytes die by apoptosis during this selection process. The surviving cells become

single positive thymocytes that mature into either $CD4^+$ T helper cells or $CD8^+$ cytotoxic T cells, and migrate to the periphery as naïve mature T lymphocytes.

Deficient T-cell development causing SCID

The proliferation, differentiation and *TCR* gene rearrangement processes that occur in the thymus are tightly regulated. Any gene mutation leading to a blockage in the development of T cells in the thymus results in a deficiency or sometimes even a complete absence of mature T lymphocytes in peripheral blood and secondary lymphoid organs. When T cells are lacking a patient exhibits SCID, sometimes accompanied by lack of B- and/or NK cells. Patients with such a deficiency cannot mount an effective immune response. Even when they are present in these patients, B cells are barely functional and fail in immunoglobulin class switching because of the lack of T-cell help. At least 13 different underlying genetic defects have been identified to lead to SCID[25-28] (Figure 2A). One of the defects is located in the gene coding for adenosine deaminase (ADA). ADA deficiency leads to a $T^-B^-NK^-$ SCID caused by the accumulation of purine metabolites that are particularly toxic to more mature lymphocytes. Mutations in at least four genes coding for proteins involved in Ig- and TCR-rearrangement processes (RAG1, RAG2, Artemis and DNA Ligase IV) have been identified, all leading to a severe reduction of T and B cells ($T^-B^-NK^+$ SCID). Mutations in the genes encoding for the common gamma chain and its associated tyrosine kinase JAK3 lead to a $T^-B^+NK^-$ phenotype. A $T^-B^+NK^+$ (isolated T-cell) SCID phenotype can be caused by mutations in the genes coding for IL7Rα, CD45, CD3δ and CD3ε. More recently, also mutations in the genes coding for CD3γ and CD3ζ have been shown to lead to a $T^-B^+NK^+$ SCID phenotype.[27, 28]

Bone marrow aspirates provide an insight into the stage of B-cell development affected in T^-/B^- SCID.[24, 26, 29] Blockages in T-cell development are harder to figure out, because thymic biopsies are not usually taken from these patients. Blockages indicated in Figure 2A therefore rely largely on data from targeted mutation studies in the mouse and on gene expression profiling studies of normal human thymocyte subsets. It needs to be stressed that the differentiation blocks are indicated at the first stage in which a gene product is believed to be functionally required, which is not necessarily at the peak of its expression. In fact, some proteins crucial for human T-cell development are not expressed at very high levels (Figure 2B).

Murine and human T-cell development are generally very similar,[19] but there are differences in expression and function of individual genes, *e.g.*, inactivating mutations in *IL7RA* in humans only affects developing T cells, whereas in the mouse both T and B cells are severely diminished in numbers.[30, 31] Targeted *Ada* disruption in mice leads to early postnatal mortality due to severe liver damage, pointing toward a more important role for ADA in murine than human fetal development.[32, 33] Perinatal mortality is prevented only when expression of the ADA protein is restored in trophoblast cells.[34] In these mice severe lymphopenia and combined immunodeficiency can be observed, closely resembling human ADA-SCID. It is clear that mutations affecting early human T-cell development lead to a

more severe deficiency than mutations that act later in development. For instance, IL2Rγ deficiency leads to a full-blown SCID phenotype whereas ZAP70 deficiency mainly affects the development of $CD8^+$ single positive cells.[35] Not only does gene therapy for SCID-XI restore the early blockage in development and IL2R γ chain-dependent proliferation, but corrected cells also benefit from a later proliferative advantage, such as the proliferation driven by the pre-TCR signaling. This will lead to a further selective and proliferative advantage of *IL2RG* gene corrected thymocytes. In contrast, we anticipate that gene therapy for ZAP70 would probably not lead to such a strong proliferative advantage, because the ZAP70 block only affects the latest stages of thymocyte development.

LMO2 in T-cell development

LMO2 is known as an oncogene involved in human T-ALL. Overexpression of *LMO2* can be caused by chromosomal translocations,[36, 37] by unknown mechanisms that lead to aberrant expression on account of the disruption of the negative regulatory element,[38] or by retroviral insertional mutagenesis as underlined by the adverse effects reported in a gene therapy trial for SCID-XI.[15] The LMO2 protein functions as a bridging molecule for multi-protein complexes that affect cell differentiation during hematopoiesis. For a recent review on the multiple roles of LMO2 in hematopoiesis we refer to Nam *et al*.[39] Seminal work of Rabbits and co-workers has shown that ubiquitous expression of *LMO2* in mice exclusively leads to T-cell lymphomas, stressing its role as a T-cell-specific oncogene. During normal human T-cell development expression of *LMO2* is rapidly down regulated during the early DN1-DN3 thymocyte stages (Figure 2C). Using human $CD34^+$ cells, we recently demonstrated that retrovirus-mediated expression of *LMO2* hampers T-cell development alone, with differentiation into other blood lineages being unaffected.[40] We showed that overexpression of *LMO2* in human cells leads to a blockage in early T-cell development, and this was consistent with earlier findings in *Lmo2*-transgenic mice.[41, 42] Although this LMO2-induced differentiation arrest under high proliferative pressure is by itself insufficient to induce tumor development, it is likely to be an important early event, perhaps the initiating event, in leukemogenesis. Additional mutations to activate other oncogenes and/or inactivating tumor suppressor genes will then lead to full-scale leukemia.

Other T-ALL oncogenes in T-cell development

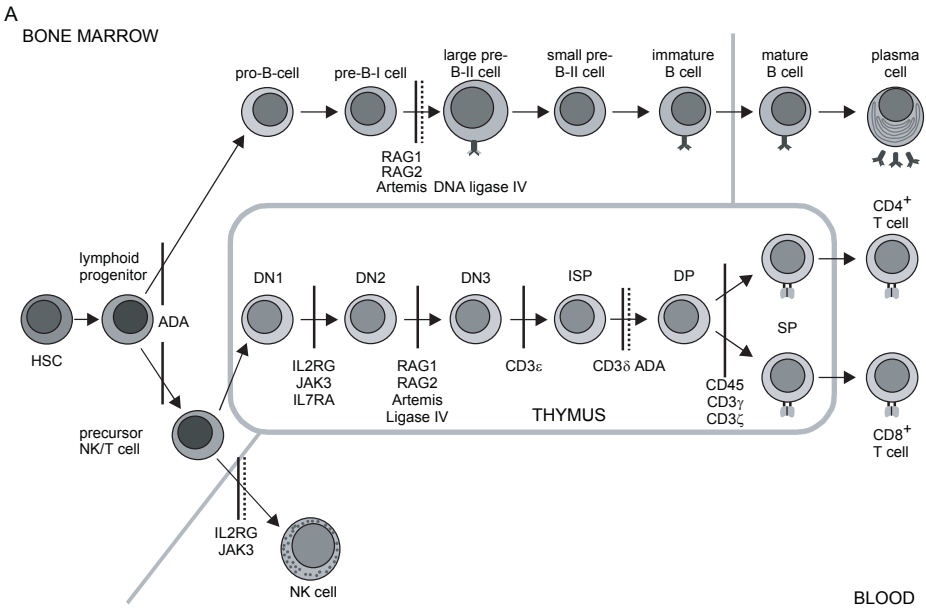
It is striking that, so far, proliferative problems have been confined to the T-cell lineage alone in the otherwise successful SCID-XI gene therapy trial. Other T-ALL oncogenes could pose a risk similar to that posed by *LMO2*. In addition, retroviral insertion could potentially also inactivate tumor suppressor genes, as the *Bmi1* gene product does by repressing expression of the tumor suppressor *Arf*. Activation of Bmi1 by retroviral insertion has been noted in several systems, including a tumor sample from a patient in the French study. Enforced BMI1 expression could potentially collaborate with LMO2 expression, again by suppressing ARF expression. ARF expression is usually increased in response

to proliferative mutations and acts by inducing senescence or apoptosis in the mutated clone. Some known T-ALL oncogenes, such as *LMO1*, *HOX11* and *HOX11L2*, are not expressed in hematopoietic stem cell-like cells or during T-cell development. Upregulation of expression during T-cell development could result in a T-ALL, but the likelihood of this being caused by provirus insertion during transduction of hematopoietic stem cells is low, because expression at that stage of development is extremely low, making an integration event less likely. The low expression level of *LCK* in hematopietic stem cells and the fact that its expression is strongly upregulated over the course of T-cell development does not make it a likely candidate for insertional mutagenesis either. *TAN1*, *c-MYB* and *c-MYC* are moderately expressed in hematopietic stem cells and therefore at risk for proviral integration. Like *LMO2*, other T-ALL oncogenes are expressed in hematopoietic stem cell-like cells and downregulated during T-cell development. *TAL1*, *LYL1* and to a lesser extent, *CCND2*, *HOXA9* and *HOXA10* fit this particular profile. Integrations near these loci could potentially activate their expression and ultimately lead to T-ALL-like lympho-proliferation. Recent data on the third and fourth patients who developed a T-ALL like disease in the French SCID-XI trial lend support to this prediction. Their insertion sites were not associated with *LMO2*, but most likely with the T-ALL oncogene *LYL1*, and the widely expressed oncogene *c-Jun* as well as the *Bmi1* gene in the third case and with *CCND2* (encoding CyclinD2) in the fourth patient (ASGT meeting 2007, Seattle, WA, USA, reported by Dr. Cavazzana-Calvo). As discussed earlier, *CCND2*, functions as an oncogene in the T-cell lineage, where it normally is not expressed. Unpublished data from our laboratory show that just as with LMO2, overexpression of LYL1 can lead to early arrest in human T-cell development in *in vitro* culture systems (F. Weerkamp and F.J.T. Staal, unpublished observations). It follows that, as a general concept, integrations near genes that normally require down regulation in expression during the immature stages of T-cell development but remain highly expressed through insertional mutagenesis at later stages, pose a high risk for leukemogenesis.

IL2Rγ (common gamma) chain in T-cell development using various model systems

The common gamma chain pairs with multiple cytokine receptor chains to form functional signal-transducing complexes.[10] Hematopoietic stem cell-like cells already express IL2Rγ at fairly high levels,[40] and it is expressed throughout T-cell development

Figure 2 (facing page). Expression of genes involved in SCID and T-ALL.
A. Schematic overview of development of human T, B, and natural killer (NK) cells. For deficiencies in the 13 known SCID genes the blocks in lymphoid development are indicated. B. Expression of SCID genes during human T-cell development. C. Expression of T-cell acute lymphoblastic leukemia oncogenes during human T-cell development. B and C. Light shades represent low transcriptional activity and dark shades represent high transcriptional activity. The figure is constructed by mining raw microarray data on human T-cell development that have been described earlier.[19] ADA, adenosine deaminase; DN, double negative; DP, double positive; HSC, hematopoietic stem cell; ISP, immature single positive; SP, single positive.


A
BONE MARROW
pro-B-cell
pre-B-I cell
large pre-B-II cell
small pre-B-II cell
immature B cell
mature B cell
plasma cell
RAG1
RAG2
Artemis
DNA ligase IV
lymphoid progenitor
HSC
ADA
DN1
DN2
DN3
ISP
DP
IL2RG
JAK3
IL7RA
RAG1
RAG2
Artemis
Ligase IV
CD3ε
CD3δ ADA
THYMUS
SP
CD45
CD3γ
CD3ζ
CD4+ T cell
CD8+ T cell
precursor NK/T cell
IL2RG
JAK3
NK cell
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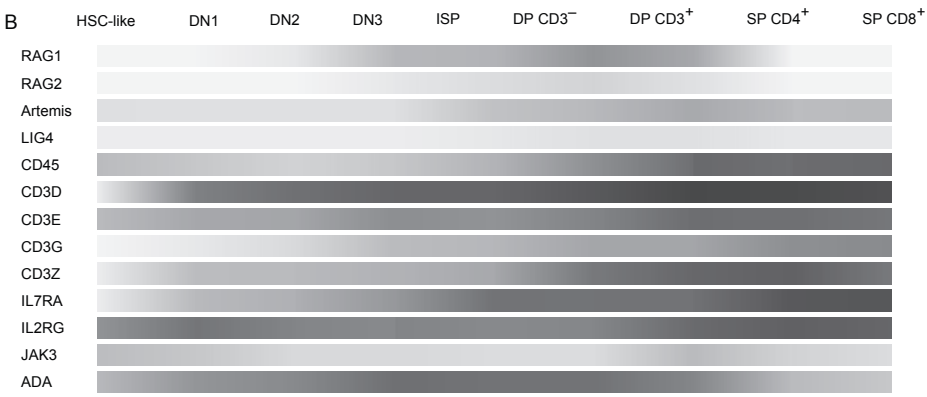
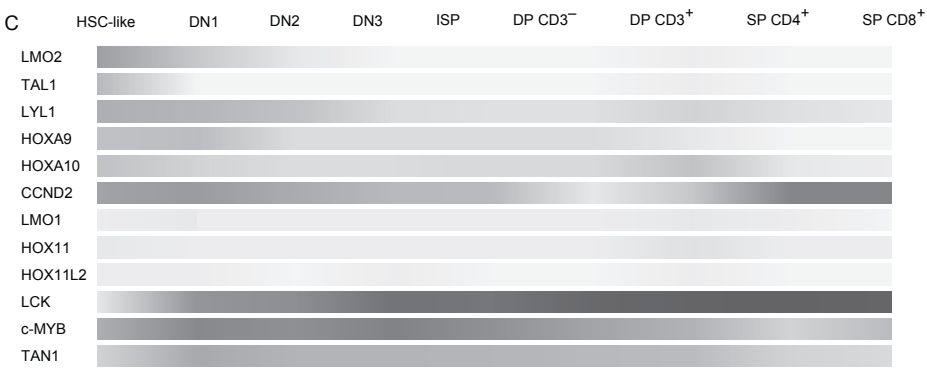

B
HSC-like
DN1
DN2
DN3
ISP
DP CD3−
DP CD3+
SP CD4+
SP CD8+
RAG1
RAG2
Artemis
LIG4
CD45
CD3D
CD3E
CD3G
CD3Z
IL7RA
IL2RG
JAK3
ADA


C
HSC-like
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TAL1
LYL1
HOXA9
HOXA10
CCND2
LMO1
HOX11
HOX11L2
LCK
c-MYB
TAN1

(Figure 2B). The significance of *IL2RG* messenger RNA expression in hematopoietic stem cell-like cells is not clear, but it does show that the *IL2RG* locus is actively transcribed and probably more accessible for virus integration. Signaling through the IL2Rγ chain can only occur when it is multimerized with one or multiple partner chains and bound to its specific ligand. Trans-phosphorylation of JAK proteins takes place only under these conditions. In developing thymocytes the major partner chain is IL7Rα, and the IL7Rα/IL2Rγ complex is responsible for transducing proliferative signals after binding of the IL7 cytokine. The potential role of the IL2Rγ chain as contributing factor to leukemogenesis has been the subject of intense scientific debate. Two recent short reports of studies using murine models have suggested that *IL2RG* itself could possibly contribute to leukemic transformation.[43, 44] Two other reports, one using human *in vitro* differentiation assays, the other using mouse models strongly argue against *IL2RG* having such a role.[45, 46]

In the first report on *IL2RG* as a putative oncogene, Copeland and co-workers show that insertions near *Lmo2* as well as *Il2rg* were detected in a single murine T-cell leukemia present in the Mouse Retroviral Tagged Cancer Gene Database, leading to the hypothesis that these genes may act as cooperating oncogenes.[43] However, it should be noted that several other oncogenes were targeted in the same leukemic clone as well, a fairly common characteristic of the replication-competent murine leukemia virus used. Which of the genetic hits are responsible for oncogenesis and whether cooperation between these insertion sites functionally exists is very difficult to demonstrate. It is noteworthy that of the two tumor clones with insertions in both *Lmo2* and *Il2rg* present in the database (query date: May 26, 2007) one also contained insertions in *Bmi1* and *Rap1gds1. Bmi1 is* a repressor of *Arf*, itself a known tumor suppressor, and *Rap1gds1* is a known oncogene. An insertion near both genes could therefore have equally well contributed to lymphoma development in these mice.[47-49] Another tumor clone (tumor 7107), of the B-cell phenotype, with insertions in *Lmo2* as well as *Il2rg* also contained insertions in *5830484A20Rik*, *Laptm5*, *Rere*, *Prdm16*, *Fgfr3*, *Ccnd1, Thoc4* and *Pacsin2*. The majority of the latter genes has known associations with various tumors; most notably *Laptm5* and *Ccnd1*, which are known for their involvement in tumors of the B-cell lineage.[50, 51]

In the second report, Wood *et al.* used a murine model system in which hematopoietic stem cells were transduced with the human *IL2RG* gene using a lentiviral vector.[44] In these experiments by Woods *et al.* very high transgene expression was reached and multiple copies of the transgene were introduced per target cell. Mice transplanted with these transduced hematopoietic stem cells developed tumors with a higher incidence than reported for classical cell oncogenes such as *LMO2* or *LYL1* and comparable to the most active oncogene in transgenic mice, *Tal1*. This was an unexpected observation because *IL2RG* has never been reported to act as oncogene in human T-ALL. Thymic lymphoma is the term commonly used for the murine equivalent of human clonal T-ALL, but clonality has not been addressed in this model, therefore these tumors are mainly referred to as “lympho-proliferations”. On comparing the high expression levels of IL2Rγ in these mice to the slightly lower-than-normal levels observed in SCID-XI trials, it

is evident that the expression level may have played a role in the development of T-cell lymphomas. Insertional mutagenesis may also have been a significant contributory factor to lymphoma development, especially because of the high copy number of integrated provirus that is usually associated with lentviral transduction at a high multiplicity of infection. Unfortunately, the short report did not specify the degree of tumor clonality, the level of *IL2RG* expression or the activation status of JAK3.[44] In addition, it was not reported whether the *IL2RG* gene might be mutated, for instance due to errors in lentiviral reverse transcription. It is also noteworthy that the phenotype of the murine tumors ($B220^+CD3^+$) is very different from that of the T-ALL-like tumors that occurred in the patients enrolled in the SCID-XI trial. The tumors found in the SCID-XI trial express T-cell specific molecules without co-expressing B-cell markers.

Conversely, reports from our laboratory and those of Drs. Thrasher, Baum, Fisher and Cavazzana-Calvo presented data arguing against a direct oncogenic role for *IL2RG*.[45, 46] In our view, the retroviral-mediated expression of *IL2RG* in hematopoietic precursors does not represent a pre-leukemic or otherwise leukemogenic event since this chain is also expressed at substantial levels in normal hematopoietic $CD34^+$ precursors as well as throughout T-cell development. Importantly, our findings show that retroviral overexpression of *IL2RG*, in contrast to *LMO2*, does not hamper human T-cell development.[40] In addition, IL2Rγ expression was within the normal range in SCID patients that underwent gene therapy. There was no indication for persistent activation of JAK3, which would expected if *IL2RG* were to act as oncogene. The results of this trial show that IL2Rγ is functioning properly in the setting of retroviral expression. Also, transgenic mice expressing human *IL2RG* driven by the CD2 promoter do not develop cell lymphomas. This was shown in 54 out of the 54 mice analysed.[46] Similarly, when retroviral vectors are used for inducing expression of *IL2RG* in bone marrow cells of C57Bl6/J mice, transplanted recipients develop leukemia only as a clonal disorder in conjunction with insertion near a proto-oncogene such as *Evi1* (Dr. C. Baum and Dr. U. Modlich, Hannover Medical School, personal communication, March 3, 2007). *Evi1* is the most frequently targeted oncogene in murine retroviral insertion experiments. Even when mouse bone marrow cells from *LMO2* transgenic mice were transduced with *IL2RG* vectors as a direct test for cooperation, there was evidence of increased development of tumors over *LMO2* alone, or of quicker tumor development (M. Cavazzana-Calvo, Hospital Necker, personal communication, August, 2007). Taken together, these observations argue against *IL2RG* acting as a cooperative oncogene in the human gene therapy setting.

We hypothesize that insertional mutagenesis near the *LMO2* locus provides a sufficient explanation for the development of leukemias, although a subtle role for *IL2RG* cannot fully be excluded at this point. It is somewhat ironic that, by restoring T-cell development, the therapeutic *IL2RG* gene creates a situation in which *LMO2* is inappropriately expressed (Figure 3). Extending this hypothesis, it is possible to postulate that gene therapy aimed at correcting other early T-cell defects (*e.g. RAG1*, *RAG2*, *JAK3*) could also result in a lymphoproliferative disorder. Just as in *IL2RG* gene therapy, the therapeutic virus could

integrate near and activate the *LMO2* locus giving the cells an inappropriate proliferative advantage in the early stages of T-cell development.

An important recent insight from the ADA-SCID trial is that not all insertions in the vicinity of the *LMO2* gene may be oncogenic. The highly successful Italian trial established that three patients have insertions near *LMO2* as detected by linear amplification-mediated polymerase chain reaction, but the integration of the retroviral long terminal repeat promoter/enhancer sequences did not lead to ectopic expression of LMO2.[52] Additional factors, such as the disruption of negative regulatory elements in the *LMO2* enhancer/promoter sequences, could play a role in activation of gene expression.[53] This idea is supported by a recent publication investigating T-ALL samples with an 11;14 translocation (*LMO2-TCRD*).[38] This study shows that uncoupling the negative regulatory element and the coding sequences through translocations results in over-expression of *LMO2*. Consequently, not only linear amplification-mediated polymerase chain reaction, but also expression studies, preferentially at the protein level, should be conducted when investigating gene therapy safety.

The power of high throughput molecular screening in gene therapy clinical research

The four cases of a T-ALL like disease in one trial of SCID-XI have given rise to a wealth of research into the molecular mechanisms underlying gammaretroviral insertional mutagenesis. Through a combination of linear amplification-mediated polymerase chain reaction techniques for identifying viral integration sites, and gene expression profiling using microarray technology several important findings have emerged. In three back-to-back reports in *The Journal of Clinical Investigation*, each report dealing with a different SCID gene therapy trial,[52, 54, 55] viral insertions sites in non-leukemic patients were compared with the gene expression profiles of the target cells. It is noteworthy that data from the three reports (French SCID-XI, British SCID-XI, Italian ADA-SCID) reach similar and important conclusions: the integrations certainly do not occur at random, with up to two-thirds of insertions being in or very near to genes that are highly expressed in $CD34^+$ cells. In all three trials a significant proportion of all integrations are clustered as common integration sites. This shows that viral integrations are correlated with the level of expression of genes in the $CD34^+$ stem/progenitor cells that are used as target cells. The viral integrations map to genes involved in stem cell biology, especially growth factor genes, and are highly expressed as common integration sites in circulating T cells. These genes may have conferred selective advantage on cell engraftment, survival and proliferation of stem cells and T lymphocytes. These type of analyses help not only to explain insertional mutagenesis, but also to elucidate the relationship between vector insertion and long term *in vivo* selection of transduced cells in gene therapy trials for SCID.

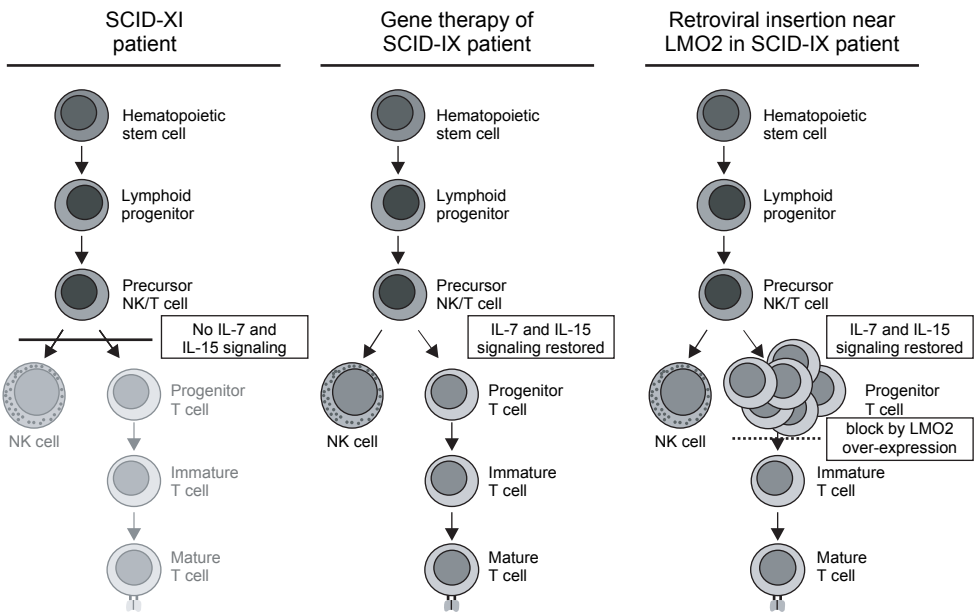

Figure 3. Model for leukemogenesis in X-linked severe combined immunodeficiency (SCID-XI) gene therapy.
Left panel: T and natural killer (NK) cells are lacking when IL2Rγ is absent or not working properly. Middle panel: T- and NK-cell development is restored after retroviral SCID-XI gene therapy. Right panel: Overexpression of *LMO2* causes an partial block in early T-cell development. IL, interleukin.

Towards better model systems to assess risk factors for gene therapy

The activation of *LMO2* by insertional mutagenesis is clearly an important oncogenic factor, but is by itself probably insufficient to cause full-blown leukemia. Additional genetic abnormalities are likely required. It is important to note that genetic predisposition for tumor-development has been proposed as an additional risk factor. One of the patients who developed T-ALL had a family history of juvenile tumors, while chromosomal translocations of unknown importance were found in another patient.

Sorrentino and co-workers have recently developed an elegant mouse model that is more prone to develop T-cell malignancies.[56] In this model, both the *Arf* tumor-suppressor gene and the *Il2rg* gene were ablated. Retroviral transduction of *IL2RG* into the hematopoietic stem cells of these mice emulates the high frequency of integration-dependent T-cell tumors than was found in the SCID-XI trial. The deletion of *Arf* leads to a more rapid development of T-cell lymphomas, allowing for a much easier readout of oncogenic events. Naldini and coworkers have developed a different tumor-prone mouse model.[57] They have used Cdkn2a$^{-/-}$ mice to demonstrate that lentiviral vectors have a more favorable safety profile when compared with murine leukemia virus vectors. However, it is

essential to emphasize the importance of disease-specific models for other types of SCID, such as those underlying the *RAG1* or *RAG2* deficiency, in order to evalute safety prior to a clinical trial. For the RAG1 deficiency, gammaretroviral gene therapy has been conducted in a mouse model, including follow-up for adverse effects through insertional mutagenesis.[58]

It would be desirable to test gene therapy constructs in human cells from the point of view of safety. The non-obese diabetic-SCID model is classically used for *in vivo* studies of human hematopoiesis. The disadvantage of this model is the lack of proper human T-cell development. Recently developed immunodeficient mice are being used for studying human hematopoiesis including T-cell development, and these may serve as good models for studying gene therapy vectors. The $Rag2^{-/-}\gamma c^{-/-}$ model, for example, allows for human T-cell development in the murine thymus. This model could be used for testing whether adverse effects, like those observed in gene therapy-treated patients, can be reproduced. Newly designed vectors could be tested for safety in this manner.

Most T-cell negative SCIDs have an early developmental block such as the one found in IL2Rγ deficiency. Consequently, one would predict that gene therapy aimed at most other T-negative SCID, such as RAG1, RAG2, IL7RA or JAK3-deficiencies would also present with the risk of leukemia-development as long as retroviral vectors with strong viral promoter/enhancer sequences are used. Therefore lentiviral self-inactivating vectors and retroviral self-inactivating vectors, which lack the strong viral promoter and use weaker internal promoters, may overcome some of these problems. Recently developed mouse models would be very useful tools to assess disease specific risks of insertional mutagenesis when novel types of vectors are used.

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CHAPTER 4

CORRECTION OF MURINE RAG2 DEFICIENCY BY LENTIVIRAL VECTOR-MEDIATED GENE TRANSFER

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To be submitted

ABSTRACT

Inactivating mutations in the recombination activating gene 1 or 2 (*RAG1* or *RAG2*) lead to severe combined immunodeficiency (SCID) with a T-negative B-negative phenotype. SCID can be cured by hematopoietic stem cell transplantation, but since an HLA-matched donor is not always available, effort has been put into developing alternative treatment options for SCID in the form of gene therapy. In light of safety concerns we sought to investigate the use of lentiviral self-inactivating (SIN) vectors for human *RAG2* gene transfer in a *Rag2*$^{-/-}$ murine model. Lineage-depleted *Rag2*$^{-/-}$ bone marrow cells were transduced with lentiviral SIN vectors that contained human *RAG2* cDNA under control of either the human elongation factor 1α (EFS) promoter or the MLV-derived enhancer-promoter from the spleen-focus-forming virus (SFFV) and transplanted into lethally irradiated *Rag2*$^{-/-}$ mice. Over the course of 3 months, peripheral B- and T-cell reconstitution was monitored. We were able to detect immunoglobulin(Ig)M^{+}IgD^{+} B cells as well as T-cell receptor(TCR)β^{+} T cells in peripheral blood of mice treated with SFFV-RAG2 virus but not in mice that received EFS-RAG2 transduced cells. Successful restoration of T- and B-cell development was confirmed by phenotypic analysis of central and peripheral lymphoid organs at the end of the experiment. Serum Ig levels had completely normalized in SFFV-RAG2 treated mice. In addition, T cells showed usage of all Vβ gene families analyzed and a polyclonal repertoire was found in B cells. The presence of Ig and TCR molecules confirms complementation of the recombinase deficiency in this model. The EFS-RAG2 construct did not result in B- or T-cell reconstitution, most likely due to a low transduction efficiency and subsequent low expression of the transgene. The B- and T-cell reconstitution upon SFFV-RAG2 transduction provides proof-of-principle that *RAG2* gene-transfer using a lentiviral SIN vector is feasible.

INTRODUCTION

Severe combined immunodeficiency (SCID) is a rare disease in which cells of the specific immune system are unable to develop. The phenotype of SCID depends on the underlying genetic defect, but all SCID patients lack T lymphocytes in their peripheral blood.[1-4] SCID patients with a mutation in the recombination activating gene 1 or 2 (*RAG1* or *RAG2*) lack both T and B lymphocytes because RAG proteins are crucial for the rearrangement of immunoglobulin (Ig) and T-cell receptor (TCR) genes. In the absence of these rearrangements, functional Ig and TCR chains will not be produced by the B- and T-cell progenitors in bone marrow and thymus. This leads to a developmental arrest which causes the T^{-}B^{-}SCID phenotype.[5-8] Allogeneic hematopoietic stem cell transplantation with an HLA-haploidentical donor is a curative treatment for SCID, but a HLA-matched donor is often not available. Over the past decades gene therapy using gammaretrovirus-based vectors has emerged as an alternative treatment option for SCID. Several clinical

trials have been successful in the treatment of ADA-SCID and X-linked SCID patients.[9-11] In addition, therapeutic gammaretroviral transfer vectors are being developed and tested for other SCID-causing genetic aberrations, such as *Artemis, IL7RA, RAG1* and *RAG2*.[12-15] Unfortunately, clonal T-cell proliferations were observed in 4 patients enrolled in the French X-linked SCID gene therapy trial[16-18] (the fourth case was reported at the 33rd Annual Meeting of the European Group for Blood and Marrow Transplantation in Lyon, France, March 25-28, 2007). In at least 2 patients insertions near the *LMO2* oncogene were found.[19] Aberrant expression of this oncogene hampers T-cell development in murine as well as human test systems.[20-22] The occurrence of clonal T-cell proliferations has lead to wide spread efforts to improve the safety of gene transfer methods. Using a different vector system is one of the options for improving safety. *In vitro* studies have shown that, as compared to LTR-driven gammaretroviral vectors, lentiviral vectors have a more favorable integration pattern.[23] Integrations of lentiviral vectors are spread out over the transcriptional part of genes, whereas gammaretroviral vectors tend to integrate more toward the transcription start site of genes,[23] a region that mostly contains regulatory elements. Over the past decade, lentiviral transfer systems underwent several rounds of modifications to improve their safety.[24-27] An additional modification resulted in lentiviral self-inactivating (SIN) vector system. Lentiviral SIN vectors contain a long terminal repeat (LTR) from which viral promoter-enhancer sequences have been deleted from the U3 region of the 3' LTR, leaving the LTR transcriptionally inactive when integrated in the host genome.[26, 28] This vector design makes it possible to incorporate more physiological promoters (e.g. elongation factor 1a promoter).

Besides the safety concerns in relation to provirus integration, persistent expression of RAG proteins could pose an additional safety threat. The *in vivo* gene therapy studies using gammaretroviral transfer vectors expressing *RAG* as well as observations in *Rag1* and *Rag2* single-transgenic mice suggest that constitutively overexpressing one of the RAG proteins does not lead to malignancies or other pathological changes.

Only when both RAG1 and RAG2 proteins are ubiquitously expressed, mice quickly develop lymphadenopathy, splenomegaly, and lymphocytic perivascular infiltrates.[29] This is in line with the fact that both RAG proteins are needed for recombinase activity and that mutations in one of the *RAG* genes can lead to total lack of recombinase activity.[7, 30, 31] Taking these observations into account, it is unlikely that lentiviral (over-) expression of *RAG1* or *RAG2* would be harmful, as expression of the unaffected, endogenous *RAG* gene would regulate the total RAG activity. Studying the efficacy of RAG2 gene transfer vectors is facilitated by the availability of a murine model that is equivalent to the situation in RAG-SCID patients.[31] In these mice the development of B cells is blocked at the $B220^{lo}CD43^{+}IgM^{-}$ pro B-cell stage and T-cell development cannot progress beyond the $CD4^{-}CD8^{-}$ double negative (DN), $CD25^{+}CD44^{-}$ (DN3) stage.

In our laboratories, we have set out to develop clinical lentivirus-based gene therapy for RAG1 and RAG2 deficiency. To this end the lentiviral vectors that are to be used in the clinic are tested in preclinical models. In the current study, we explored the possibility

of using lentiviral SIN vectors for human *RAG2* gene transfer. Two lentiviral SIN transfer vectors were constructed in which expression of human *RAG2* cDNA is driven by the human elongation factor 1α (EFS) promoter[32] or the MLV-derived enhancer–promoter from the spleen-focus-forming virus (SFFV). In addition, the transfer vectors contain a woodchuck hepatitis virus-derived post-transcriptional regulatory element (WPRE) which augments both viral titer and transgene expression.[33, 34] The WPRE present in our vectors does not have the X protein open reading frame and mutations were introduced in 4 possible ATGs.[35] Lineage-depleted hematopoietic precursor cells from *Rag2*$^{-/-}$ bone marrow were subjected to *ex vivo* gene transfer using EFS-RAG2 and SFFV-RAG2 lentiviral SIN vectors and transplanted into lethally irradiated *Rag2*$^{-/-}$ recipient mice. Reconstitution of peripheral and central lymphoid compartments as well as the Ig and TCR repertoire were evaluated.

MATERIAL AND METHODS

Mice

Rag2$^{-/-}$ animals were obtained from Balb/c *Rag2/Il2rg* double-knockout animals on a mixed background by outcrossing *Rag2*$^{-/-}$ to the Balb/c background. Balb/c *Rag2/Il2rg* double-knockout animals were a kind gift from Dr. E.J. Rombouts from the Department of Hematology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands. Balb/c wild-type mice were purchased from Charles River Nederland (Maastricht, The Netherlands).

Mice were maintained in the specified pathogen-free (SPF) breeding section of the Experimental Animal Center of the Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands. This study was approved by the institutional Animal Ethical Committee of the Erasmus MC.

Lentiviral vectors and vector production

Lentiviral gene transfer constructs were derived from pRRL.PPT.PGK.GFPpre.[26] The pRRL.PPT.EFS.RAG2.pre (hereafter: EFS-RAG2) and pRRL.PPT.SFFV.RAG2.pre (hereafter: SFFV-RAG2) transfer vectors were constructed by replacing the PGK promoter by a human elongation factor 1α promoter or a MLV-derived enhancer–promoter from the spleen-focus-forming virus. The *GFP* sequence was replaced by human *RAG2* cDNA. The WPRE used in these constructs is lacking the X protein ORF and 4 possible ATGs were mutated.[35] Gene transfer constructs were validated by full DNA sequencing of the transgene. Helper plasmids pMDLg/pRRE, pRSV-Rev and pMD2.VSVG[26] for lentivirus production were kindly provided by L. Naldini (San Raffaele Telethon Institute for Gene Therapy Milano, Italy). Large-scale helper-plasmid preparations were produced by PlasmidFactory (Bielefeld, Germany). 293T cells were transiently transfected with transfer and helper plasmids using the calcium-phosphate method. Cells received fresh media 16h after transfection. Lentivirus was harvested 24h, 38h and 48h after transfection. Cleared supernatant was filtered through 0.22 µm pore cellulose acetate filters and stored at -80°C. Pooled lentiviral supernatant was concentrated by ultracentrifugation for 16 hours at 18,000 x g and 4°C. Pellets were resuspended in StemSpan serum-free expansion medium (StemSpan-SFEM, Stemcell Technologies Inc, Vancouver, BC, Canada). Because suitable anti-RAG2 antibodies are not available, we determined the viral titer using RQ-PCR as described below.

Transduction and transplantation of bone marrow cells

To obtain bone marrow cells, femurs and tibias of BALB/c wild-type mice and Balb/c *Rag2*$^{-/-}$ mice were flushed, passed through a 0.7 µm pore nylon mesh and washed. Cells were viably frozen. After thawing, lineage negative precursor cells were isolated using a mouse lineage depletion kit and a magnetic cell sorter (Miltenyi Biotech, Germany). Cells were grown in StemSpan-SFEM supplemented with 100 ng/ml

recombinant mouse stem cell factor (rmSCF), 10 ng/ml recombinant mouse thrombopoietin (rmTPO) and 50 ng/ml recombinant human FMS-related tyrosine kinase 3 ligand (rhFLT 3-L) for 1 day. *Rag2*$^{-/-}$ cells were subsequently transduced by way of spin-occulation, in the presence of 4 µg/ml protamine sulphate (Sigma-Aldrich, Saint Louis, MO, USA) at 800 x g and 32°C for 1 hour. Using a lentiviral RAG2-IRES-EGFP construct, we found that viral titers as determined by RQ-PCR were approximately 65 times higher than transduction-based viral titers (data not shown), allowing us to make a rough estimation of the multiplicity of infection (MOI). Cells transduced with the EFS-*RAG2* vector received a viral dose of 2,000 WPRE per cell (approximate MOI of 31). Cells transduced with the SFFV-*RAG2* vector received a viral dose of 180 WPRE per cell (approximate MOI of 3). The difference in viral dosage was based on results obtained from preliminary experiments that tested the transduction efficiency of lentiviral batches. After transduction, cells were grown for another 24h in the presence of cytokines. Transduced cells (5 x 10^5/mouse) were mixed with *Rag2*$^{-/-}$ spleen cells (3 x 10^5/mouse) and injected into the tail vein of female *Rag2*$^{-/-}$ recipient mice (age 11 to 16 weeks) that were exposed to a single dose of 800 cGy total-body γ-irradiation. Two control groups were included in this study; *Rag2*$^{-/-}$ mice that received wild-type BALB/c lineage depleted bone marrow cells (referred to as WT control in the text) and *Rag2*$^{-/-}$ mice that received mock-transduced Rag2$^{-/-}$ lineage depleted BM cells (referred to as *Rag2*$^{-/-}$ in the text). Mice were given antibiotic-containing water and their welfare was monitored daily.

Real-time quantitative-polymerase chain reactions

Real-time quantitative polymerase chain reactions (RQ-PCR) were used for the quantitative analysis of genomic lentiviral RNA, proviral DNA copies, and transgene mRNA expression using *WPRE*, *RAG2* and Abelson (*ABL*) as targets. Total RNA from viral supernatant and cells was extracted using the RNeasy Total RNA isolation kit (Qiagen, Hilden, Germany). RNA was reverse transcribed into cDNA. Genomic DNA was extracted using the GeneElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich, Saint Louis, MO, USA). RQ-PCR reactions were performed on the ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The following primers and probes were used. *WPRE*: forward primer, 5'-CCGTTGTCCGTCAACGTG-3'; reverse primer, 5'-AGTTGACAGGTGGTGGCAAT-3'; probe, 5'-FAM-TGCTGACGCAACCCCCACTGGC-TAMRA-3'. *RAG2*: forward primer, 5'-ACTGAAGC CTACAATTTTCTCTAAGGA-3'; reverse primer, 5'- AAGTGGCTGGGTAGCGAAGA-3'; probe, 5'-FAM-TCCTGCTACCTCCCTC-TAMRA-3'. For copy number quantification of unknown samples, standard curves were created by preparing ten-fold serial dilutions of plasmid constructs of known concentration that contained the WPRE sequence. For samples derived from cellular RNA, the expression levels were normalized to the expression of the *ABL* gene: forward primer, 5'-TGGAGATAACACTCTAAGCATAACTA AAGGT-3'; reverse primer, 5'-GATGTAGTTGCTTGGGACCCA-3'; probe, 5'-FAM-CCATTTTTGGTTTGG GCTTCACACCATT-TAMRA-3'.

Flow cytometric studies

Peripheral blood, spleen, thymus and bone marrow were obtained from CO_2-euthanized mice and cell suspensions were prepared. Cells were counted and stained with the following anti-mouse antibodies: fluorescein isothiocyanate (FITC)-conjugated CD3ε (145-2C11), IgD (11-26c.2a) and T cell receptor β (TCRβ) (H57-597); R-phycoerythrin (PE)-conjugated CD19 (ID3), IgM (R6-60.2) and TCR γδ (GL3); peridinin chlorophyll protein (PerCP)-conjugated CD8α (53-6.7), allophycocyanin (APC)-conjugated CD3ε (145-2C11) and CD19 (ID3); PE-Cy7-conjugated CD4 (L3T4) and CD45R/B220 (RA-6B2); biotin-conjugated CD11b (M1/70), CD43 (S7), CD45R/B220 (RA-6B2), GR-1 (RB6-8C5), NK1.1 (PK136) and TER-119 (Ly-76) (all antibodies BD Biosciences, Santa Clara, CA, USA). When necessary, streptavidin-conjugated APC-Cy7 (BD Biosciences) was added as a second step. Data were acquired on a FACSAria (BD Biosciences). Analyses were performed using FlowJo software (Treestar, Ashland, OR, USA).

Serum immunoglobulin quantification

A sandwich enzyme-linked immunosorbent assay (ELISA) was done to determine IgM and IgG1 serum levels using unlabeled and peroxidase-labeled anti-mouse IgM (1B4B1) and IgG1 (H143.225.8) antibodies (SouthernBiotech, Birmingham, AL, USA) for capture and detection, respectively. Serially diluted sera were incubated at room temperature for 3 h, and azino-bis-ethylbenzthiazoline sulfonic acid (ABTS) was used as a substrate. Antibody concentrations were calculated by using purified IgM and IgG1 proteins as standards.

Ig and TCRβ repertoire analysis

Total RNA was extracted from spleen cells using the RNeasy Total RNA isolation kit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA. For TCRβ repertoire analysis, cDNA was amplified using a FAM-labeled C gene segment-specific primer (5'-FAM-CTTGGGTGGAGTCACATTTCTC-3') along with 24 TCR Vβ-specific primers.[36] For Ig repertoire analysis, cDNA was amplified using a FAM-labeled constant $(C)_{\mu}$ or C_{γ} gene segment-specific primer (C_{μ}: 5'-FAM-TCTCTGCGACAGCTGGAATGG-3' and C_{γ}: 5'-FAM-GGA CAG GGA TCC AGA GTT CCA-3') and a consensus V_H primer (5'- AGG TCA AAC TGC AGC AGT CTG G-3'). Labeled PCR-products were size-separated on an ABI Prism Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The fluorescent intensity of each band was recorded and analyzed using GeneScan Analysis Software, version 3.7.1 (Applied Biosystems).

RESULTS

Lentiviral *RAG2* gene transfer

To evaluate the possibility of gene therapy for RAG2-SCID using lentiviral SIN vectors, two transfer vectors were constructed (schematically depicted in Figure 1). The pRRL.PPT.PGK.GFPpre construct[26] was adapted to contain a modified WPRE[35] and the *GFP* sequence was replaced by *huRAG2* cDNA. Either the EFS or the SFFV promoter was used to control the expression of *RAG2*. Lineage depleted precursor cells from *Rag2*$^{-/-}$ bone marrow underwent one cycle of transduction with EFS-RAG2 or SFFV-RAG2 lentiviral particles and were transplanted into lethally irradiated *Rag2*$^{-/-}$ recipients. A small part of the transduced cells was kept in culture for an additional 6 days after which transgene expression and the number of integrations was determined by RQ-PCR. For cells transduced with EFS-RAG2 we found on average 0.29 integrations per cell and *RAG2* expression levels were 1.14 relative to *ABL*. SFFV-RAG2-transduced cells had 8.70 integrations per cell and a *RAG2* expression-level of 13.69 relative to *ABL*.

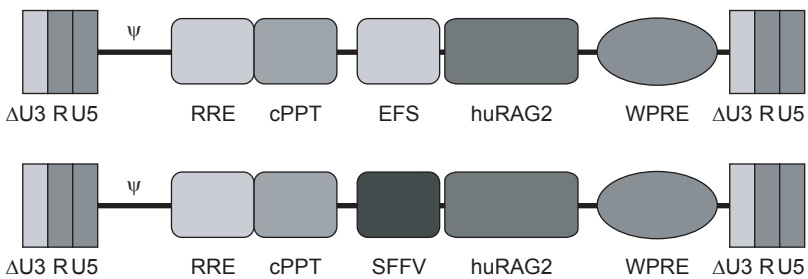

Figure 1. Schematic design of lentiviral vector constructs.
The figure shows the relevant elements that are present on the transfer vectors used in this study. Vectors are depicted as proviruses and are not to scale. ΔU3, unique 3′ regulatory sequences with viral enhancer and promoter sequences deleted; R, repeat sequence; U5, unique 5′ regulatory sequences; Ψ, packaging signal; RRE, Rev-responsive element; cPPT, central polypurine tract sequence; EFS, elongation factor 1α promoter; SFFV, spleen-focus-forming virus promoter- enhancer; huRAG2, human recombination activating gene 2 ; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

Because a suitable anti-RAG2 antibody is currently not available, transgene expression levels were not determined at the protein level.

Peripheral lymphoid reconstitution in *RAG2*-transduced mice

Three, 6 and 15 weeks after transplantation peripheral blood was analyzed for the presence of B and T lymphocytes. In animals that received untransduced *Rag2*$^{-/-}$ cells (referred to as the *Rag2*$^{-/-}$ group) or EFS-RAG2 transduced cells neither B nor T lymphocytes could be detected at any of these time points (Figure 2A and C). Three weeks

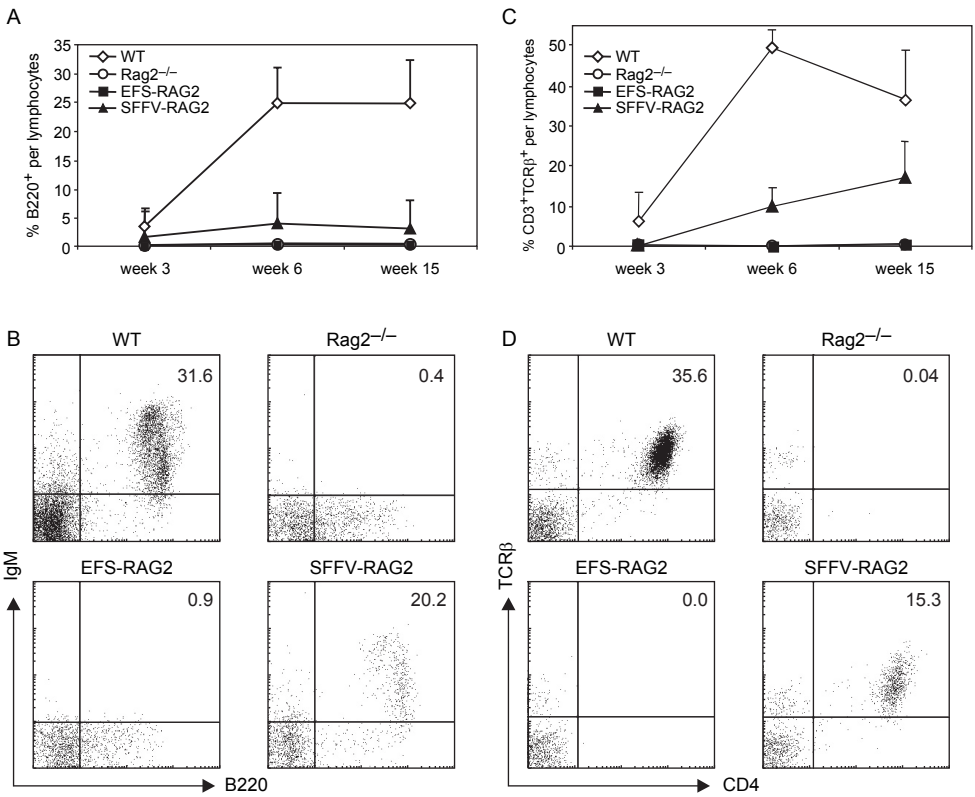

Figure 2. B and T lymphocyte reconstitution in peripheral blood.
Over time, the presence of B and T lymphocytes in peripheral blood was monitored by flow cytometry. A. Percentage of B220^{+} cells per total lymphocytes in peripheral blood. Means of 4 mice (WT) or 5 mice (other groups) are shown. Bars represent one standard deviation. B. Flow cytometric analysis of peripheral blood cells for IgM and B220 staining within the lineage negative lymphocyte gate. The lineage cocktail consisted of Ter-119, NK1.1, CD11b, GR-1 and CD43 antibodies. Numbers in the dot plots represent percentages within the quadrants. C. Percentage of CD3^{+}TCRβ^{+} cells of total lymphocytes in peripheral blood. D. Flow cytometric analysis of peripheral blood cells for TCRβ and CD4 staining within the lineage negative lymphocyte gate. The lineage cocktail consisted of B220, Ter-119, NK1.1, CD11b, GR-1 and CD43 antibodies. Numbers in the dot plots represent percentages within the quadrants.

after transplantation, $B220^+$ B cells could be detected in animals that received WT cells. At this time, B cells were also detectable in animals that received SFFV-RAG2-transduced cells, although at lower levels (Figure 2A). All $B220^+$ cells in WT and SFFV-RAG2 animals were also positive for surface membrane (sm)IgM, a product of RAG-mediated Ig gene rearrangements (Figure 2B). This confirmed that the $B220^+$ cells were indeed B cells and that the *RAG2* gene was functional in terms of inducing V(D)J recombination. After six weeks, $CD3^+TCR\beta^+$ T cells could be discerned in the WT group as well as the SFFV-RAG2 group. Analogous to what was seen for the B cells, the percentage of $CD3^+TCR\beta^+$ T cells in the SFFV-RAG2 was lower than the WT-group (Figure 2C). Both $CD4^+TCR\beta^+$ cells (Figure 2D) and $CD8^+TCR\beta^+$ were detectable in all WT and SFFV-RAG2 animals.

After 15 weeks, all animals were sacrificed and lymphoid organs were taken out for analysis. Cellularity of bone marrow, thymus, spleen and peripheral blood was determined (Table I). Compared to the WT-group, mice in the *Rag2*$^{-/-}$ and EFS-RAG2 groups displayed a low white blood cell (WBC)-count. However, the SFFV-RAG2 mice showed a recovery of white blood cell numbers in the periphery of about 50% compared to WT animals and had clearly higher (2-3 fold) mean counts than mice in the negative control group. No significant difference between groups was found in the number of mononuclear cells in spleen or the number of white blood cells in the bone marrow. In the spleen, no $CD3^+TCR\beta^+$ T cells, $B220^+IgM^+$ or $B220^+IgD^+$ B cells could be detected in the *Rag2*$^{-/-}$ or the EFS-RAG2 group (Figure 3). IgM^+ and IgD^+ $B220^+$ cells were present in animals that received SFFV-RAG2 transduced cells, be it at lower percentages than found in the WT group. Among spleen cells of WT animals and SFFV-RAG2 animals, T cells were also detectable. Although the percentage of T cells was lower in the SFFV-RAG2 mice, the ratio of $CD4^+$ to $CD8^+$ cells was comparable in both groups (Figure 3). Summarizing these results, in the SFFV-RAG2 transduced mice, but not the EFS-RAG2 transduced mice, B and T cells could be detected in the periphery.

T-cell development and TCR repertoire

The WT group was the only group in which the cellularity of the thymus reached normal levels. Even though a reasonable percentage of T cells was found in the peripheral blood of SFFV-RAG2 mice, their thymi contained only about 10% of the normal number

Table I. Cell counts of lymphoid organs 15 weeks after transplantation.

	Peripheral blood WBC ($x10^9$/L)	Spleen MNC ($x10^8$)	Thymus MNC ($x10^6$)	Bone marrow WBC ($x10^6$)
WT (n=4)	24.3 ± 7.6	1.6 ± 0.5	41.3 ± 14.0	40.8 ± 3.8
Rag1$^{-/-}$ (n=5)	5.2 ± 1.2	2.2 ± 0.5	5.6 ± 1.5	38.1 ± 6.9
EFS-RAG1 (n=5)	3.8 ± 1.2	1.9 ± 0.5	5.0 ± 1.3	37.4 ± 10.5
SFFV-RAG1 (n=5)	13.4 ± 3.2	1.2 ± 1.0	4.4 ± 1.7	41.1 ± 20.2

Data are expressed as mean ± SD.

of thymocytes. Immunophenotypic analysis of thymocytes demonstrated that all of the mice in the WT group showed a normal percentage of CD4/CD8 double-positive cells in the thymus, while the percentage found in SFFV-RAG2 mice was somewhat lower (Figure 4). Mature thymocytes of WT mice as well as SFFV-RAG2 mice had TCRβ

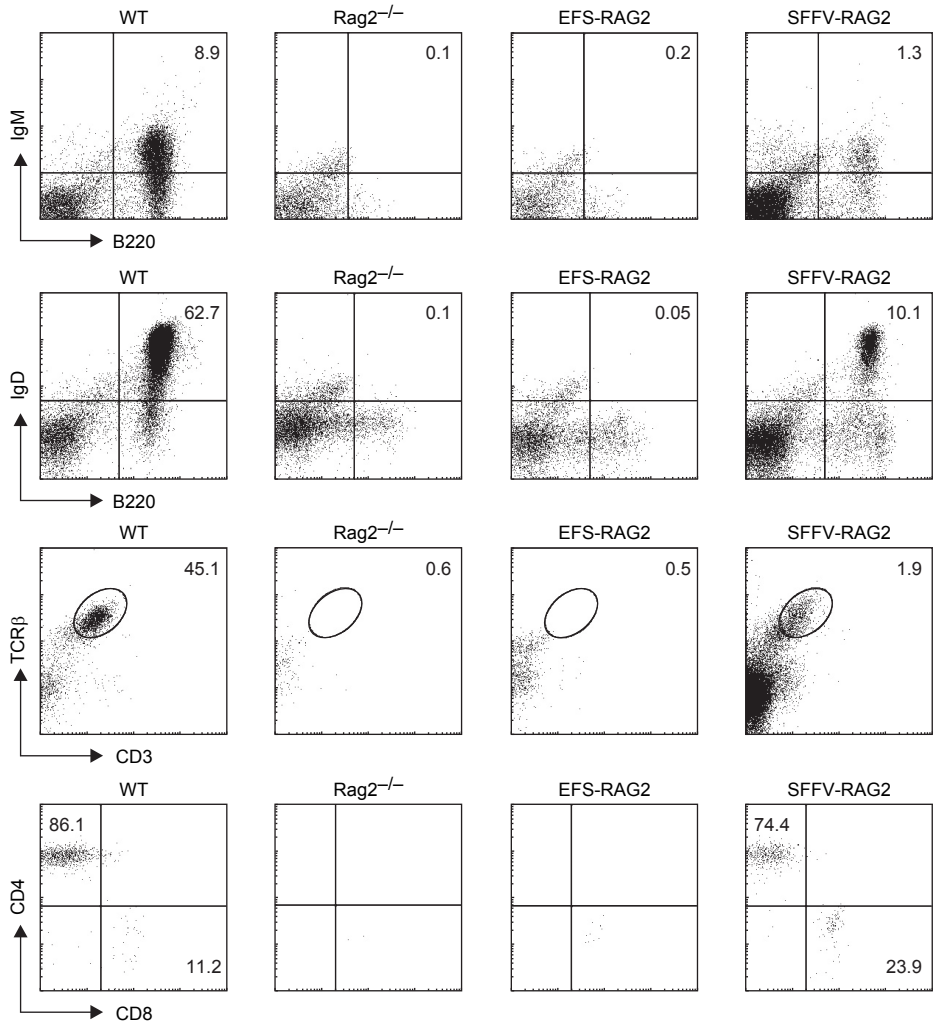

Figure 3. B and T lymphocyte reconstitution in the spleen.
Flow cytometric analysis of spleen cells 15 weeks after transplantation. Upper panels show flow cytometric analysis for IgM, IgD and B220 staining within the lineage negative lymphocyte gate. The lineage cocktail consisted of Ter-119, NK1.1, CD11b, GR-1 and CD43 antibodies. Lower panels show flow cytometric analysis for TCRβ, CD3, CD4 and CD8 staining within the lineage negative lymphocyte gate. The lineage cocktail consisted of B220, Ter-119, NK1.1, CD11b, GR-1 and CD43 antibodies. Numbers in the dot plots represent percentages within the quadrants or gates.

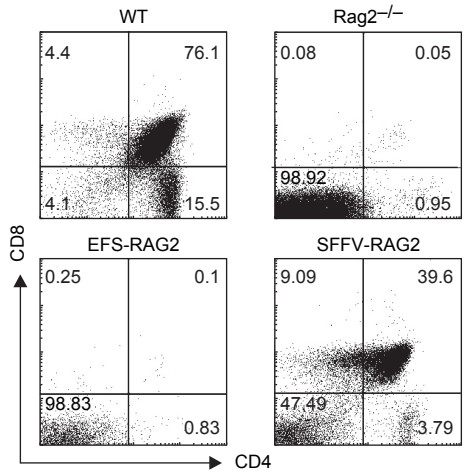

Figure 4. T-lymphocyte development in the thymus.
Flow cytometric analysis of thymocytes 15 weeks after transplantation. Dot plots of flow cytometric analysis for CD4 and CD8 staining within the lineage negative gate. The lineage cocktail consisted of B220, Ter-119, NK1.1, CD11b, GR-1 and CD43 antibodies. Numbers in the dot plots represent percentages within the quadrants.

expressed on their cell surface. Some of the *Rag2*$^{-/-}$ and EFS-RAG2 mice showed presence of a low percentage of CD4/CD8 double-positive cells, but none of these cells expressed TCRβ or TCRγδ on the surface. These peculiar cells are most likely host-derived as up-regulation of CD4 and CD8 after γ-irradiation has been reported for *Rag2*$^{-/-}$ mice.[37, 38]

Total RNA extracted from spleen cells was used to analyze TCR Vβ diversity in peripheral T cells. TCR Vβ repertoire was determined using cDNA material from 2 WT animals, one *Rag2*$^{-/-}$ animal, and 4 SFFV-RAG2 animals by PCR using a fluorochrome-labeled constant β (Cβ)-gene segment-specific primer and 24 Vβ-specific primers.[36] GeneScan analysis was performed on the labeled PCR products. WT animals demonstrated a diverse TCR repertoire as the whole spectrum of Vβ-gene segments tested showed a full range of in-frame rearrangements (Figure 5). As expected, no rearranged Vβ-gene segments could be detected in *Rag2*$^{-/-}$ samples. SFFV-RAG2 animals displayed usage of the whole spectrum of Vβ gene-segments analyzed, but the peak distribution within some Vβ-gene segment was not completely Gaussian, in contrast to the results in the WT samples. In conclusion, mice that received SFFV-RAG2 transduced cells developed TCR-bearing lymphocytes that use the full spectrum of Vβ gene families analyzed.

B-cell development and immunoglobulins

Flow cytometry was done on bone marrow cells to determine whether B-cell development was restored in animals that underwent gene therapy. Early precursor

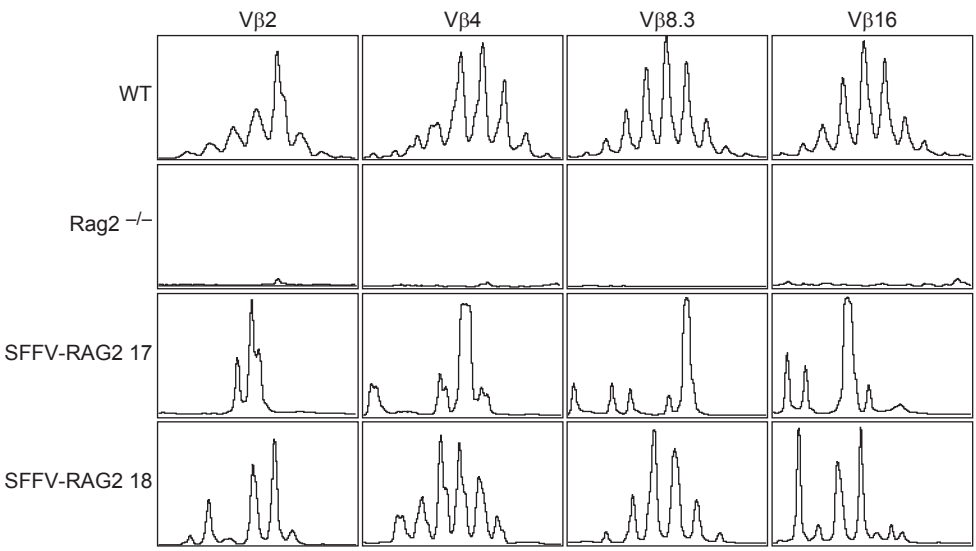

Figure 5. TCR Vβ repertoire analysis.
GeneScan analysis of PCR products obtained from 4 Vβ-specific PCR amplifications. A total of 24 Vβ-specific amplifications was done on material extracted from 2 WT mice, 1 Rag2$^{-/-}$ mouse and 5 SFFV-RAG2 mice. Representative samples of GeneScan plots are shown. The x-axis indicates CDR3 length, the y-axis shows arbitrary fluorescence intensity of the runoff products.

B cells are B220^{+}CD43^{+}, whereas B220^{+}CD43^{-} cells appear later in B-cell development. During the final stages in the bone marrow, developing B cells express IgM and IgD on their cell surface. In WT animals, both the B220^{+}CD43^{+} and the B220^{+}CD43^{-} population could be distinguished and IgM^{+}IgD^{+} cells could also be identified (Figure 6). A complete lack of B220^{+}CD43^{-} cells was measured in *Rag2*$^{-/-}$ animals as well as EFS-RAG2 animals. Consequently, no IgM^{+} or IgM^{+}IgD^{+} could be detected in the bone marrow. SFFV-RAG2 mice, however, did display all stages of B-cell development including IgM and IgD expression on the cell surface (Figure 6). To determine whether a polyclonal B-cell population had developed, Genescan analysis was done on total RNA extracted from spleen cells from 4 WT animals, 2 *Rag2*$^{-/-}$ animal, and 5 SFFV-RAG2 animals. A consensus V_H primer and a fluorochrome-labeled constant $(C)_\mu$ or C_γ gene-specific primer were used for amplification of cDNA and labeled PCR-products were used for Genescan analysis. In WT animals, a Gaussian distribution of PCR products was observed when using the C_μ-as well as the C_γ-specific primer, indicating a polyclonal B-cell population (Figure 7). No IgH rearrangements were detectable in *Rag2*$^{-/-}$ mice. In SFFV-RAG2 mice, a polyclonal rearrangement pattern was measured, be it that the peak distribution was not entirely Gaussian. Serum immunoglobulin levels (IgM and IgG1) were assessed by ELISA (Figure 8). Sera of animals lacking mature B cells in the bone marrow and the periphery

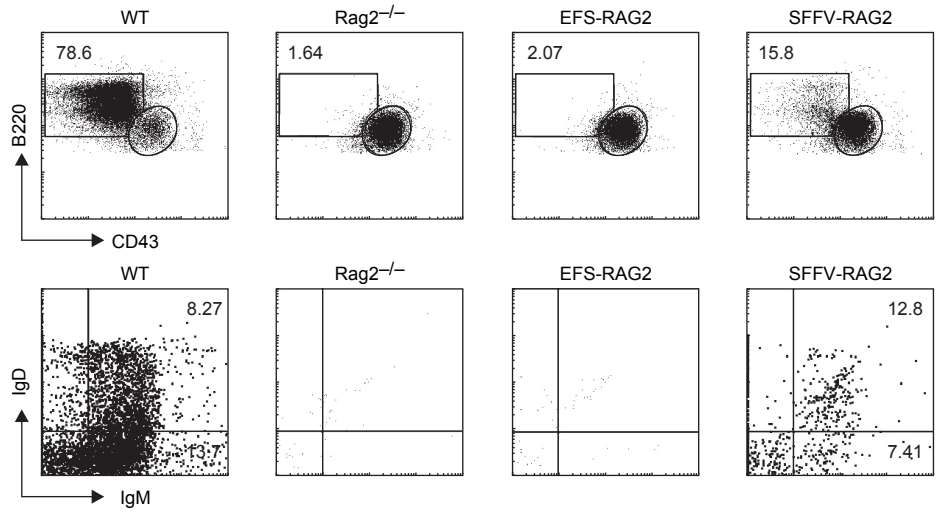

Figure 6. B-lymphocyte development in the bone marrow.
Flow cytometric analysis of bone marrow cells 15 weeks after transplantation. Dot plots of flow cytometric analysis for B220, CD43, IgD and IgM staining within the B220 positive gate. Numbers in the dot plots represent percentages within the quadrants or gates.

(*Rag2*$^{-/-}$ and EFS-RAG2) did not have any detectable levels of IgM or IgG1. In WT as well as SFFV-RAG2 transduced animals normal quantities of IgM and IgG1 were detected. Taken together, these data demonstrate that the *Rag2*$^{-/-}$ cells that underwent gene therapy with the SFFV-RAG2 construct are capable of developing into phenotypically and functionally normal B cells with polyclonal V_H-segment usage. Despite the lower frequency of B cells in the periphery, these B cells were capable of undergoing class switch recombination. In addition, serum Ig concentrations were restored to normal levels in the SFFV-RAG2 mice.

Provirus integration and transgene expression

The presence of proviral copies in genomic DNA was determined 15 weeks after transplantation. DNA was extracted from bone marrow cells, thymocytes and spleen cells derived from EFS-RAG2 as well as SFFV-RAG2 animals (Figure 9, upper panel). Compared to the starting material, in which we could detect 0.29 EFS-RAG2 and 8.70 SFFV-RAG2 proviral copies per cell, the mean number of integrations we could detect at week 15 had decreased. In all three organs that were assayed, about 0.01 EFS-RAG2 and 2.5 SFFV-RAG2 proviral copies per cell could be found. The difference found between *ex vivo* and *in vivo* gene marking might be the consequence of a high transduction efficiency of non-repopulating cells. The level of *RAG2* expression was measured in bone marrow cell and spleen cells (Figure 9, lower panel). Since precursor B-cells were not purified from bone marrow, we did not detect any *RAG2* expression in animals transplanted with

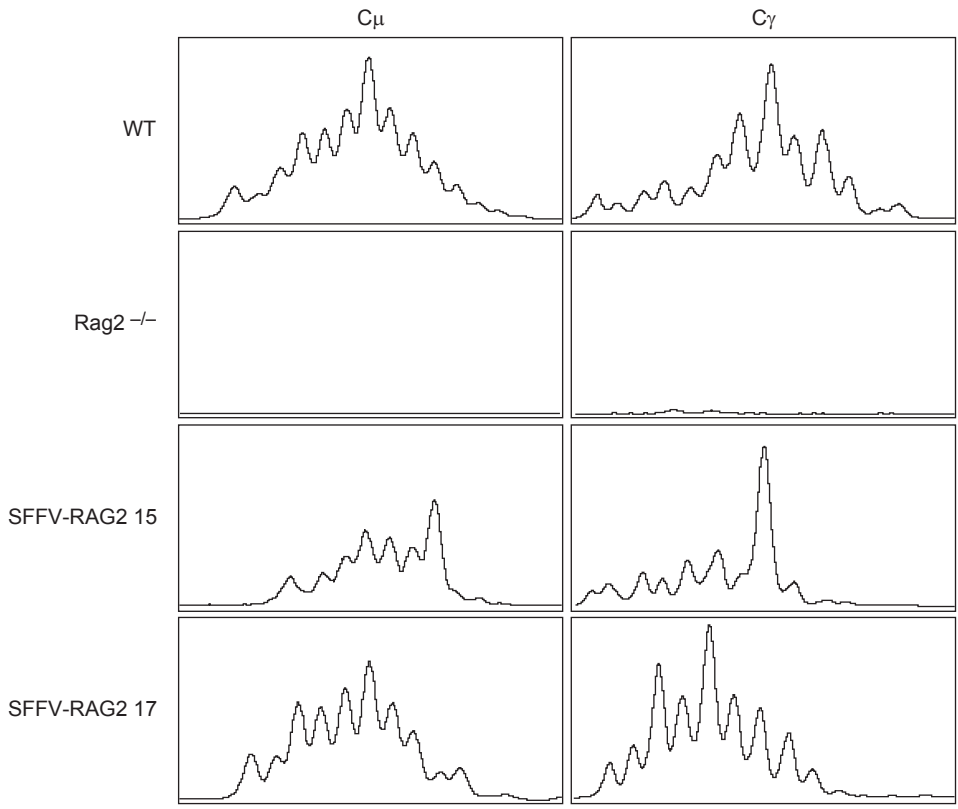

Figure 7. Ig repertoire analysis.
GeneScan analysis of PCR products obtained from Cμ and Cγ-specific PCR amplifications. Genescan analysis was done on material extracted from 4 WT mice, 2 Rag2$^{-/-}$ mice and 5 SFFV-RAG2 mice. Representative samples of GeneScan plots are shown. The x-axis indicates PCR product length, the y-axis shows arbitrary fluorescence intensity of the runoff products.

WT cells. As expected, no *RAG2* expression was detected in either organ taken from *Rag2*$^{-/-}$ mice or WT spleen cells. Marginal to undetectable *RAG2* expression was found in bone marrow and spleen samples from EFS-RAG2 mice. This in contrast to SFFV-RAG2 bone marrow and spleen samples, in which high levels of *RAG2* expression were detected. The level of *RAG2* expression detected in EFS-RAG2 transduced cells at the onset of the experiment as well as 15 weeks after transplantation was not sufficient to restore T- and B-cell development. On the other hand, the level of *RAG2* expression in SFFV-RAG2 transduced cells was effective in at least partial restoration of T- and B-cell development.

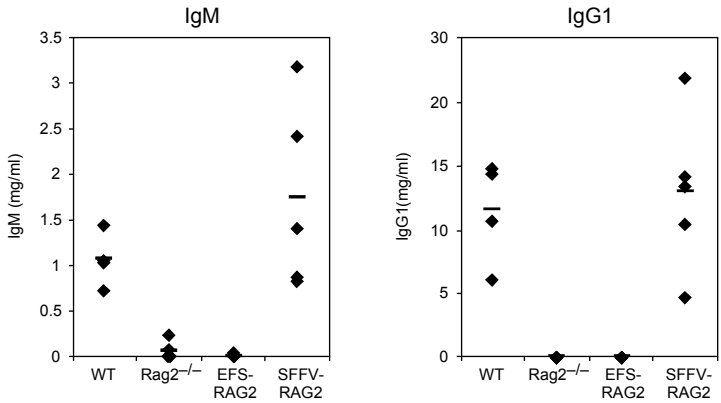

Figure 8. Serum Ig levels.
IgM and IgG1 serum levels of all mice were quantified by ELISA. Each diamond represents a value obtained in one mouse. Mean values are represented by horizontal bars.

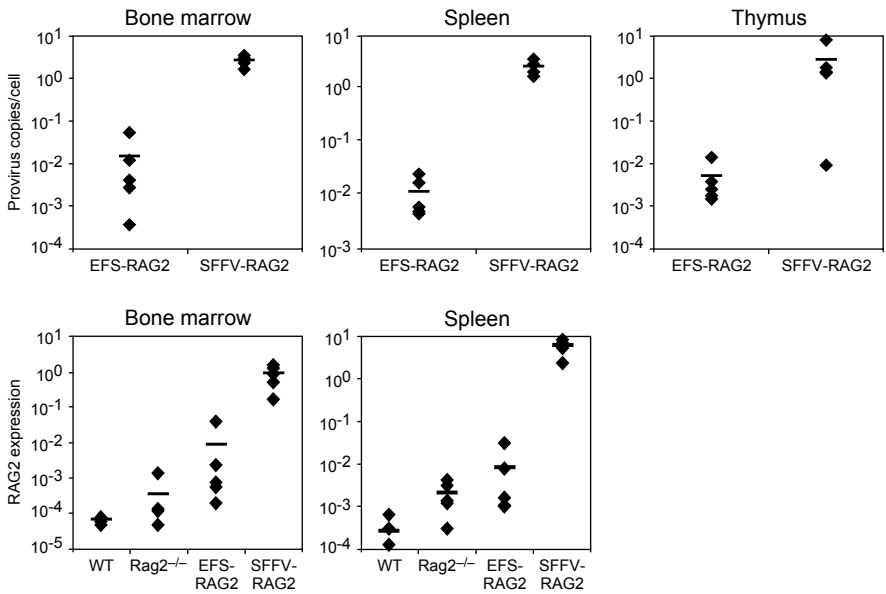

Figure 9. Provirus integration and transgene expression.
The number of proviral copies and the transgene expression was detemined by RQ-PCR. The upper panel shows the provirus copy number per cell as determined on DNA extracted from bone marrow cells, spleen cells and thymocytes taken from EFS-RAG2 and SFFV-RAG2 mice. The lower panel shows RAG2 expression in bone marrow and spleen samples from WT, Rag2$^{-/-}$, EFS-RAG2 and SFFV-RAG2. Diamonds represent values obtained from individual mice. Means are represented by horizontal bars.

DISCUSSION

In this report we describe at least partial correction of the RAG2-deficient phenotype using a lentivirus-based SIN vector, demonstrating the feasibility of lentivirus based correction of RAG-SCID in a preclinical model. We found that one of the constructs that was tested, EFS-RAG2, did not succeed in restoring lymphoid development. However, cells transduced with SFFV-RAG2 virus were capable of overcoming the RAG2-deficient phenotype. Most likely, the success or failure of the *ex vivo RAG2*-gene transfer depended on the transduction efficiency and resulting *RAG2* expression levels. At the start of the experiment a low proviral copy number per cell was detected in the EFS-RAG2. This resulted in low *RAG2* expression, which was not enough to correct the T-negative, B-negative phenotype. The average provirus copy number found in SFFV-RAG2 transduced cells at the onset and the end of the experiment was fairly high, 8.7 and 2.5, respectively. This proved to give sufficient *RAG2* expression for lymphoid reconstitution to occur but the copy numbers are relatively high. This creates a situation that is undesirable in view of safety since the consensus in the field is that copy numbers in a therapeutic setting should be as low as possible, ideally around 1 copy per cell. At higher transduction efficiencies, the EFS-RAG2 construct might also be capable of correcting the RAG2-deficient phenotype. In an effort to optimize *RAG2* expression we will start testing constructs that contain *RAG2* sequences that are optimized for their codon-usage and GC-content. This is expected to result in higher expression per integrated provirus thus reducing the number of integrations needed on a per cell basis, as we have observed for codon-optimized *RAG1* lentiviral vectors (Chapter 5.2 of this thesis).

RAG-SCID patients that receive stem cell transplantations are either preconditioned with cytotoxic drugs or do not receive any pretransplant treatment.[39, 40] ADA-SCID patients undergoing gene therapy have been receiving non-myoablative conditioning[9] and X-linked SCID patients were left untreated prior to gene therapy treatment.[10, 41] In this initial study on the efficacy of lentiviral SIN vectors for *RAG2* gene transfer we chose to give a lethal dose of γ-irradiation but future studies will have to be adapted to mimic the clinical situation more closely.

In the current group of SFFV-RAG2 mice, we were able to detect transitional B cells, naïve follicular B cells as well as marginal zone B cells in the spleen (data not shown). With serum Ig levels as well as Ig-repertoire being comparable between WT and SFFV-RAG2 animals, B cells that developed after *RAG2* gene therapy seem to be functionally normal. In addition, it has been shown that *ex vivo RAG2*-gene transfer, using a Moloney leukemia virus (MLV)–based vector, can result in fully immuno-competent mice.[14] In this study we observed the mice for 15 weeks, after which organs were taken for analysis. Future experiments will be designed with a longer follow-up period, as well as secondary transplantations, to assess long-term efficacy and safety of the lentiviral vectors used. Long-term studies will have to include immunizations that assess T-cell dependent and T-cell independent immune responses.

It is has been show repeatedly that introduction of therapeutic transgenes in hematopoietic stem cells of immunodeficient patients or animals confers a selective advantage.[9, 10, 14, 15, 41, 42] This was also observed in MLV-based *RAG2*-gene transfer experiments.[14] We expect that *Rag2*$^{-/-}$ cells transduced with a *RAG2* therapeutic lentivirus-based vector would result in similar developmental advantages in the T- and B-cell lineage. Due to limitations of the material, we were not able to confirm whether *RAG2*-transduced cells had gained a selective advantage over non-transduced cells or cells from other blood lineages. Interestingly, the lower percentage of B cells found in peripheral blood and spleen of SFFV-RAG2 mice did not result in a lower serum immunoglobulin levels, as compared to WT animals. Apparently, a relatively low number of B lymphocytes is sufficient to reconstitute immunoglobulin production. In contrast, T cells seem to undergo peripheral expansion more readily since the percentage of T cells in peripheral blood was much higher than the number of mature T cells found in the thymus. It would be interesting to verify these observations in future gene therapy experiments.

Combining measurements of transgene expression and therapeutic vector copy number with a thorough analysis of the B- and T-cell system will tell us more about the dynamics of immune reconstitution in a gene therapy setting. Proper functioning of the immune system should be evaluated through vaccination of the animals, which could be followed by analyzing T-cell receptor excision circles (TRECs)[43, 44] and kappa-deleting recombination excision circles (KRECs)[45] in the periphery. In conjunction with TCR and Ig repertoire analysis, the information about proliferation of lymphocytes after immunization will give us more insight into the reconstitution and function of the adaptive immune system after *RAG*-gene therapy.

The reconstitution kinetics of peripheral blood T and B cells in mice that received SFFV-RAG2 transduced cells resembles that of mice transplanted with WT cells, although the fraction of B and T lymphocytes found was lower. The spleen also displayed lymphoid reconstitution upon SFFV-RAG2 gene therapy and all major subsets of B-and T-cell development were detected in primary lymphoid organs. Even though one of the constructs that we tested did not result in B- or T-cell reconstitution, our experiments establish that *RAG2* gene-transfer using a lentiviral vector is possible.

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CHAPTER 5

RAG1 GENE THERAPY

CHAPTER 5.1

PRE-CLINICAL STUDY OF LENTIVIRAL GENE THERAPY FOR RAG1 DEFICIENCY

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ABSTRACT

SCID patients with an inactivating mutation in recombination activating gene 1 or 2 (*RAG1* or *RAG2*) lack peripheral B and T cells due to the inability to rearrange immunoglobulin (Ig) and T-cell receptor (TCR) genes. Hematopoietic stem cell transplantation can cure many types of SCID, but an HLA-matched donor is in most cases not available. Therefore, efforts have been made to develop gene therapy as an additional treatment option for SCID. We investigated the use of lentiviral self-inactivating (SIN) vectors, which have a favorable safety profile relative to LTR-driven gammaretroviral vectors, for human *RAG1* gene transfer in a *Rag1*$^{-/-}$ mouse model. Lentiviral SIN vectors containing human *RAG1* cDNA under control of either the human elongation factor 1α (EFS) promoter or the MLV-derived enhancer–promoter from the spleen-focus-forming virus (SFFV) were used to transduce lineage depleted hematopoietic progenitor cells from *Rag1*$^{-/-}$ bone marrow that were subsequently transplanted into lethally irradiated *Rag1*$^{-/-}$ mice. Reconstitution of B and T lymphocytes in peripheral blood was monitored and primary and secondary lymphoid organs were phenotypically analyzed. We did not detect B or T lymphocytes in mice treated with EFS-RAG1 in any of the immunological compartments analyzed. In mice that received SFFV-RAG1 treated cells only B-cell development was successfully restored. The B-cell fraction was smaller than that found in recipients of wild-type cells and as a result serum immunoglobulin (Ig) levels were not fully normalized. Neither in the periphery nor in the thymus of SFFV-RAG1-treated mice T cells were detected. With this study we show that *RAG1*-gene transfer using lentiviral SIN vectors is feasible, since recombinase activity was restored and resulted in successful generation of B cells. However, *RAG1* transgene expression levels were insufficient for reconstitution of T-cell development and therefore needs to be improved.

INTRODUCTION

Severe combined immunodeficiency (SCID) is a rare disorder in which cells of the specific immune system do not properly develop. All SCID patients lack T cells in their peripheral blood. Depending on the underlying defect, NK cells and/or B cells may also be absent.[1-4] Genetic aberrations in recombination activating gene 1 and 2 (*RAG1* or *RAG2*) can lead to B- and T-cell deficiency, a so called T^{-}B^{-}SCID.[5-8] RAG proteins are critical for the rearrangement of immunoglobulin (Ig) and T-cell receptor (TCR) genes. In the absence of rearrangements, functional Ig and TCR proteins will not be formed. This causes a developmental arrest in the B- as well as the T-cell lineage.

Until recently, the only curative therapy for SCID was a hematopoietic stem cell transplantation. Because an HLA-matched donor is not always available, effort has been put into developing gene therapy as an alternative treatment option for SCID. Three clinical trials have been successful in the treatment of ADA-SCID and X-linked

SCID patients.[9-11] In addition to these gene therapy trials for SCID, therapeutic transfer vectors have been successfully developed and tested for other SCID-causing genetic aberrations, including gammaretroviral transfer vectors for *RAG1* and *RAG2*.[12-14] Sadly, adverse effects have emerged from the French X-linked SCID gene therapy trial[15-17] in which clonal T-cell proliferations were observed in 4 patients; the fourth case was reported at the 33rd Annual Meeting of the European Group for Blood and Marrow Transplantation (Lyon, France, March 25-28, 2007). In at least 2 patients, the therapeutic vector was found integrated near the *LMO2* oncogene.[18] Ectopic expression of this oncogene impedes T-cell development in both murine and human test systems.[19-21] After the occurrence of the clonal T-cell proliferations, ongoing efforts to improve gene transfer methods have been given high priority. One of the options for improving safety is the use of a different vector system. *In vitro* studies have shown that integrations of lentiviral vectors are more spread out over the transcriptional segment of genes. This is a more favorable pattern than seen when using gammaretroviral vectors, which generally tend to integrate toward the transcription start site of genes,[22] a region that typically contains regulatory elements. In the past decade, lentiviral transfer systems have been adapted to enhance their safety.[23-26] An additional alteration to the viral long terminal repeat (LTR) resulted in lentiviral self-inactivating (SIN) vector system. Viral promoter-enhancer sequences were deleted from the U3 region of the 3' LTR, rendering the LTR transcriptionally inactive when integrated in the host genome.[24, 27] This design allows for incorporation of more physiological promoters (e.g. elongation factor 1α (EFS)).

In addition to the safety concerns with respect to proviral integration, continuous expression of RAG proteins could pose an extra safety issue. When both RAG1 and RAG2 proteins are ubiquitously expressed, mice rapidly develop lymphadenopathy, splenomegaly, and lymphocytic perivascular infiltrates.[28] However, observations in *Rag1* and *Rag2* single-transgenic mice as well as *in vivo* gene therapy studies using gammaretroviral transfer vectors expressing *Rag* imply that constitutive overexpression of one of the two RAG proteins does not cause malignancies or other pathological changes.[28] This is in agreement with the fact that both RAG proteins are required for recombination and that mutations in one of the *RAG* genes can lead to total lack of recombinase activity.[7, 29, 30] Considering these observations, it is not likely that lentiviral (over-)expression of a single *RAG1* or *RAG2* gene would be harmful.

A mouse model that parallels the RAG-SCID patients is available to study the efficacy of *RAG1* gene transfer vectors.[29] In these *Rag1*$^{-/-}$ mice development of B cells is blocked at the pro-B-cell stage (B220^{lo}CD43^{+}IgM^{-}) and T-cell development does not advance beyond the double negative 3 stage (DN3; CD4^{-}CD8^{-} and CD25^{+}CD44^{-}). Here we report the results of a study in which we explored the use of lentiviral SIN vectors for *RAG1* gene transfer. Two lentiviral SIN transfer vectors were generated in which expression of human *RAG1* cDNA is driven by the human EFS promoter[31] or the MLV-derived enhancer-promoter from the spleen-focus-forming virus (SFFV). The transfer vectors also contain a woodchuck hepatitis virus-derived post-transcriptional regulatory element (WPRE)

which augments both viral titer and transgene expression.[32, 33] The WPRE present in our vectors does not have the X protein open reading frame and mutations were introduced in 4 possible ATGs.[34] Lineage-depleted hematopoietic progenitor cells from *Rag1*$^{-/-}$ bone marrow underwent one round of *ex vivo* transduction with EFS-RAG1 or SFFV-RAG1 lentiviral SIN vectors and were injected into lethally irradiated *Rag1*$^{-/-}$ recipient mice. Reconstitution of the peripheral as well as central lymphoid compartment was analyzed.

MATERIAL AND METHODS

Mice

C57BL/6 *Rag1*$^{-/-}$ mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6 wild-type mice were purchased from Charles River Nederland (Maastricht, The Netherlands). Mice were maintained in the specified pathogen-free (SPF) breeding section of the Experimental Animal Center of the Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands. This study was approved by the institutional Animal Ethical Committee of the Erasmus MC, Rotterdam.

Lentiviral vectors and vector production

Lentiviral gene transfer constructs were derived from pRRL.PPT.PGK.GFPpre.[24] The pRRL.PPT.EFS.RAG1.pre (hereafter: EFS-RAG1) and pRRL.PPT.SFFV.RAG1.pre (hereafter: SFFV-RAG1) transfer vectors were constructed by replacing the PGK promoter by a human EFS promoter or a MLV-derived enhancer–promoter from the SFFV. The *GFP* sequence was replaced by human *RAG1* cDNA. The *WPRE* used in these constructs is lacking the X protein ORF and 4 possible ATGs were mutated.[34] Gene transfer constructs were validated by full DNA sequencing of the transgene. Helper plasmids pMDLg/pRRE, pRSV-Rev and pMD2.VSVG[24] for lentivirus production were kindly provided by L. Naldini (San Raffaele Telethon Institute for Gene Therapy Milano, Italy). Large-scale helper-plasmid preparations were produced by PlasmidFactory (Bielefeld, Germany). 293T cells were transiently transfected with transfer and helper plasmids using the calcium-phosphate method. Cells received fresh media 16h after transfection. Lentivirus was harvested 24h, 38h and 48h after transfection. Cleared supernatant was filtered through 0.22 µm pore cellulose acetate filters and stored at -80°C. Pooled lentiviral supernatant was concentrated by ultracentrifugation for 16 hours at 18,000 x g and 4°C. Pellets were resuspended in StemSpan serum-free expansion medium (StemSpan-SFEM, Stemcell Technologies Inc, Vancouver, BC, Canada). Because suitable anti-RAG1 antibodies were not available, we determined the viral titer using RQ-PCR as described below.

Transduction and transplantation of bone marrow cells

To obtain bone marrow cells, femurs and tibias of C57BL/6 wild-type mice and C57BL/6 *Rag1*$^{-/-}$ mice were flushed, passed through a 0.7 µm pore nylon mesh and washed. Cells were viably frozen. After thawing, lineage negative cells were isolated using a mouse lineage depletion kit and a magnetic cell sorter (Miltenyi Biotech, Germany). Cells were grown in StemSpan-SFEM supplemented with 100 ng/ml recombinant mouse stem cell factor (rmSCF), 10 ng/ml recombinant mouse thrombopoietin (rmTPO) and 50 ng/ml recombinant human FMS-related tyrosine kinase 3 ligand (rhFLT 3-L) for 1 day. *Rag1*$^{-/-}$ cells were subsequently transduced by way of spin-occulation, in the presence of 4 µg/ml protamine sulphate (Sigma-Aldrich, Saint Louis, MO, USA) at 800 x g and 32°C for 1 hour. Using a lentiviral RAG2-IRES-EGFP construct, we found that viral titers as determined by RQ-PCR were approximately 65 times higher than transduction-based viral titers (data not shown), allowing us to make a rough estimation of the multiplicity of infection (MOI). Cells transduced with the EFS-RAG1 vector received a viral dose of 5,200 WPRE copies per cell (approximate MOI of 80). Cells transduced with the SFFV-RAG1 vector received a viral dose of 4,850 WPRE copies per cell (approximate MOI of 75). The difference in virus concentration was based on results obtained from preliminary experiments that tested the transduction efficiency of

lentiviral batches. After transduction, cells were grown for another 24h in the presence of cytokines. Transduced cells (5 x 10^5/mouse) were mixed with supportive *Rag1*$^{-/-}$ spleen cells (3 x 10^5/mouse) and injected into the tail vein of female *Rag1*$^{-/-}$ recipient mice (aged 8 to 15 weeks) that were exposed to a single dose of 850 cGy total-body γ-irradiation. Two control groups were included in this study; *Rag1*$^{-/-}$ mice that received wild-type C57BL/6 lineage depleted bone marrow cells (referred to as WT in the text) and *Rag1*$^{-/-}$ mice that received mock-transduced *Rag1*$^{-/-}$ lineage depleted bone marrow cells (referred to as *Rag1*$^{-/-}$ in the text). Mice were given ciproxin-containing water and their welfare was monitored daily.

Real-time quantitative-polymerase chain reactions

Real-time quantitative polymerase chain reactions (RQ-PCR) were used for the quantitative analysis of genomic lentiviral RNA, proviral DNA copies, and transgene mRNA expression using *WPRE*, *RAG1* and *Abelson* (*ABL*) as targets. Total RNA from viral supernatant and cells was extracted using the RNeasy Total RNA isolation kit (Qiagen, Hilden, Germany). RNA was reverse transcribed into cDNA. Genomic DNA was extracted using the GeneElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich, Saint Louis, MO, USA). RQ-PCR reactions were performed on the ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The following primers and probes were used. *WPRE*: forward primer, 5'-CCGTTGTCCGTCAACGTG-3'; reverse primer, 5'-AGTTGACAGGTGGTGGCAAT-3'; probe, 5'-FAM-TGCTGACGCAACCCCCACTGGC-TAMRA-3'. *RAG1*: forward primer, 5'- GCCAAAC CTAACTCTGAACTGTGTT-3'; reverse primer, 5'- GCGTCTCGTGGTCAGACTCA-3'; probe, 5'-FAM-CCATTGTGCCTTATGCT-TAMRA-3'. For copy number quantification of unknown samples, standard curves were created by preparing ten-fold serial dilutions of plasmid constructs of known concentration that contained the WPRE sequence. For samples derived from cellular RNA, the expression levels were normalized to the expression of the *ABL* gene: forward primer, 5'-TGGAGATAACACTCTAAGCATAACTA AAGGT-3'; reverse primer, 5'-GATGTAGTTGCTTGGGACCCA-3'; probe, 5'-FAM-CCATTTTTGGTTTGG GCTTCACACCATT-TAMRA-3'.

Flow cytometric studies and cell sorting

Peripheral blood, spleen, thymus and bone marrow were obtained from CO_2-euthanized mice and cell suspensions were prepared. Cells were counted and stained with the following anti-mouse antibodies: fluorescein isothiocyanate (FITC)-conjugated IgD (11-26c.2a) and T-cell receptor β (TCRβ) (H57-597); R-phycoerythrin (PE)-conjugated CD19 (ID3), IgM (R6-60.2) and TCR γδ (GL3); peridinin chlorophyll protein (PerCP)-conjugated CD8α (53-6.7); allophycocyanin (APC)-conjugated CD3ε (145-2C11) and CD19 (ID3); PE-Cy7-conjugated CD4 (L3T4) and CD45R/B220 (RA-6B2); biotin-conjugated CD11b (M1/70), CD43 (S7), CD45R/B220 (RA-6B2), GR-1 (RB6-8C5), NK1.1 (PK136) and TER-119 (Ly-76) (all antibodies BD Biosciences, Santa Clara, CA, USA). When necessary, streptavidin-conjugated APC-Cy7 (BD Biosciences) was added as a second step. Data were acquired on a FACSAria (BD Biosciences). Analyses were performed using FlowJo software (Treestar, Ashland, OR, USA). $CD19^+$ B cells and $CD11b^+$ cells were sorted from pooled SFFV-RAG1 spleen cells using the FACSDiva (BD Biosciences).

Ig and TCRβ repertoire analysis

Total RNA was extracted from spleen cells using the RNeasy Total RNA isolation kit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA. For Ig repertoire analysis, cDNA was amplified using a FAM-labeled constant $(C)_\mu$ or C_γ gene segment-specific primer (C_μ: 5'-FAM-TCTCTGCGACAGCTGGAATGG-3' and C_γ: 5'-FAM-GGA CAG GGA TCC AGA GTT CCA-3') and a consensus V_H primer (5'- AGG TCA AAC TGC AGC AGT CTG G-3'). Labeled PCR-products were size-separated on an ABI Prism Genetic Analyzer (Applied Biosystems, Foster City, CA). The fluorescent intensity of each band was recorded and analyzed using GeneScan Analysis Software, version 3.7.1 (Applied Biosystems)

Serum immunoglobulin quantification

A sandwich enzyme-linked immunosorbent assay (ELISA) was done to determine IgM and IgG1 serum levels using unlabeled and peroxidase-labeled anti-mouse IgM (1B4B1) and IgG1 (H143.225.8) antibodies (SouthernBiotech, Birmingham, AL, USA) for capture and detection, respectively. Serially diluted sera were incubated at room temperature for 3h, and azino-bis-ethylbenzthiazoline sulfonic acid (ABTS) was

used as a substrate. Antibody concentrations were calculated by using purified IgM and IgG1 proteins as standards.

Immunohistochemistry

For immunohistochemical studies of intraepithelial lymphocytes (IEL), pieces of small intestine were fixed using formalin and sections were stained by immunohistochemistry using CD4 (clone 4B12, Sanbio, Uden, The Netherlands) and CD8 (clone C8/144B, Dako, Glostrup, Denmark) antibodies.

RESULTS

Lentiviral *RAG1* gene transfer

To examine whether it would be possible to use lentiviral SIN vectors for *RAG1* gene transfer, two transfer constructs were generated. The pRRL.PPT.PGK.GFPpre construct[24] was altered to include a modified WPRE.[34] In addition, the GFP sequence was replaced by *huRAG1* cDNA and either the human EFS promoter or the SFFV promoter was used to control expression of *RAG1* (Figure 1). One round of transduction was done on lineage depleted precursor cells from *Rag1*$^{-/-}$ bone marrow, which were subsequently transplanted into lethally irradiated *Rag1*$^{-/-}$ mice. A small part of the transduced cells was cultured for an additional 6 days, allowing for full expression of the lentiviral construct. These cells were used to determine transgene expression and proviral copy number by RQ-PCR. We detected, on average, 6.2 integrations per cell and *RAG1* expression of 0.43 relative to *ABL* in EFS-RAG1 transduced cells. SFFV-RAG1-transduced cells had 2.2 integrations per cell and a *RAG1* expression-level of 0.69 relative to *ABL*. Transgene expression levels could not be determined at the protein level, because suitable anti-RAG1 antibodies are currently not available.

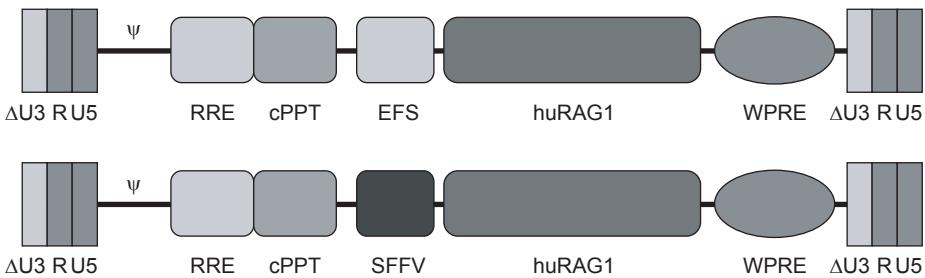

Figure 1. Schematic design of lentiviral vector constructs.
The figure shows the relevant elements that are present on the transfer vectors used in this study. Vectors are depicted as proviruses and are not to scale. ΔU3, unique 3' regulatory sequences with viral enhancer and promoter sequences deleted; R, repeat sequence; U5, unique 5′ regulatory sequences; Ψ, packaging signal; RRE, Rev-responsive element; cPPT, central polypurine tract sequence; EFS, elongation factor 1α promoter; SFFV, spleen-focus-forming virus promoter- enhancer; *huRAG1*, human recombination activating gene 1; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

Peripheral lymphoid reconstitution in *RAG1*-transduced mice

We analyzed peripheral blood for the presence of B and T lymphocytes 3, 6 and 14 weeks after transplantation of the animals. At none of the time points B or T lymphocytes were detected in peripheral blood of recipients of *Rag1*$^{-/-}$ cells (*Rag1*$^{-/-}$ group) or EFS-RAG1 transduced *Rag1*$^{-/-}$ cells (EFS-RAG1 group) (Figure 2A and C). Animals that received wild type cells showed a steady increase in the percentage of B220^{+} B cells over time. At the

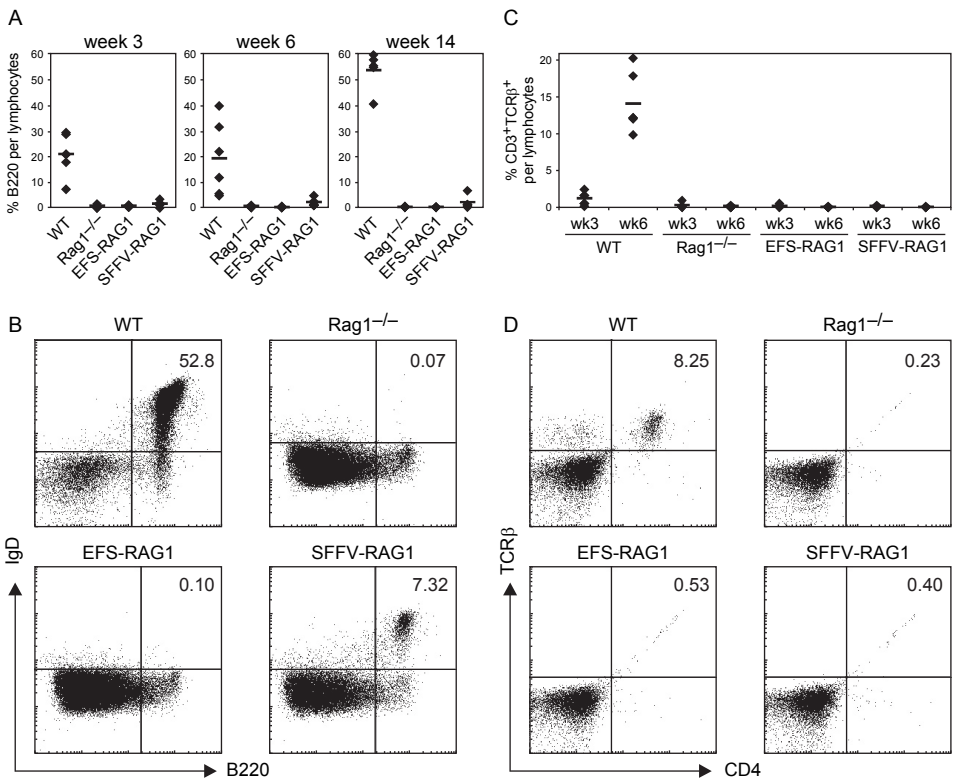

Figure 2. B and T lymphocyte reconstitution in peripheral blood.
Over time, the presence of B and T lymphocytes in peripheral blood was monitored by flow cytometry. A. Percentage of B220^{+} cells of total lymphocytes in peripheral blood at 3, 6, and 14 weeks after transplantation. Means of 5 mice (WT) or 6 mice (other groups) are shown. Bars represent one standard deviation. B. Flow cytometric analysis of peripheral blood cells for IgD and B220 staining within the lineage negative lymphocyte gate at week 14 after transplantation; the lineage cocktail consisted of Ter-119, NK1.1, CD11b, GR-1 and CD43 antibodies. Numbers in the dot plots represent percentages within the quadrants. C. Percentage of CD3^{+}TCRβ^{+} cells of total lymphocytes in peripheral blood at 3 and 6 weeks after transplantation. D. Flow cytometric analysis of peripheral blood cells for TCRβ and CD4 staining within the lineage negative lymphocyte gate at week 6 after transplantation; the lineage cocktail consisted of B220, Ter-119, NK1.1, CD11b, GR-1 and CD43 antibodies. Numbers in the dot plots represent percentages within the quadrants.

first bleeding we were also able to detect $B220^+$ B cells in most SFFV-RAG1 animals. However, the percentage of B cells was much lower than that found in animals from the WT group (Figure 2A). Most $B220^+$ cells from WT and SFFV-RAG1 animals were also positive for surface membrane (sm)IgD, which can only be expressed if RAG-mediated Ig gene rearrangement has successfully been completed (Figure 2B). In peripheral blood drawn 6 and 14 weeks post-transplantation $CD3^+TCR\beta^+$ T cells could be found in the WT group. Animals from all other groups did not have any T cells (Figure 2C and D). Due to technical problems with the red cell-lysis procedure in the T-cell staining at week 14, we were unable to perform a gating strategy comparable to the one done at weeks 3 and 6. Therefore, these data are not shown in Figure 2C.

Fourteen weeks after transplantation, all animals were sacrificed and lymphoid organs were dissected for further analysis. Cells derived from bone marrow, thymus, spleen and peripheral blood were counted (Table I). In comparison to the WT group, mice from all other groups had low white blood cell (WBC) counts. The cellularity of spleens taken from *Rag1*$^{-/-}$, EFS-RAG1 and SFFV-RAG1 animals was also lower (2- to 3-fold) than that of spleens taken from animals in the WT group. A significant difference was not found in white blood cell numbers from the bone marrow, although animals in the WT group seemed to have a slightly higher reconstitution. The number of thymocytes in the EFS-RAG1 and SFFV-RAG1 animals was comparable to the number found in *Rag1*$^{-/-}$ animals. Phenotypic analysis was done on spleen cells by flow cytometry (Figure 3). At week 14 after transplantation, all WT animals had $B220^+IgM^+$ and $B220^+IgD^+$ spleen cells, whereas *Rag1*$^{-/-}$ and EFS-RAG1 animals were negative for these populations. In SFFV-RAG1 mice $B220^+IgM^+$ and $B220^+IgD^+$ spleen cells were clearly present, albeit at lower percentages than seen in WT animals. $CD3^+TCR\beta^+$ T cells were detected in mice from the WT group. In line with the data obtained from peripheral blood, $CD3^+TCR\beta^+$ T cells were not found in the spleens of *Rag1*$^{-/-}$, EFS-RAG1 or SFFV-RAG1 mice. Taken together, mice that received cells treated with ESF-RAG1 lacked peripheral lymphoid reconstitution and mice receiving SFFV-RAG1 transduced cells showed B-cell reconstitution, but no T-cell reconstitution.

Table I. Cell counts of lymphoid organs 14 weeks after transplantation.

	Peripheral blood WBC ($x10^9$/L)	Spleen MNC ($x10^8$)	Thymus MNC ($x10^6$)	Bone marrow WBC ($x10^6$)
WT (n=5)	17.4 ± 7.4	0.61 ± 0.23	76.2 ± 38.8	96.7 ± 32.9
Rag1$^{-/-}$ (n=6)	4.5 ± 3.1	0.23 ± 0.16	5.7 ± 0.7	59.6 ± 7.0
EFS-RAG1 (n=6)	2.5 ± 1.0	0.19 ± 0.07	6.3 ± 1.2	72.0 ± 13.4
SFFV-RAG1 (n=6)	2.8 ± 0.4	0.19 ± 0.08	11.7 ± 11.8	58.2 ± 6.8

Data are expressed as mean ± SD.

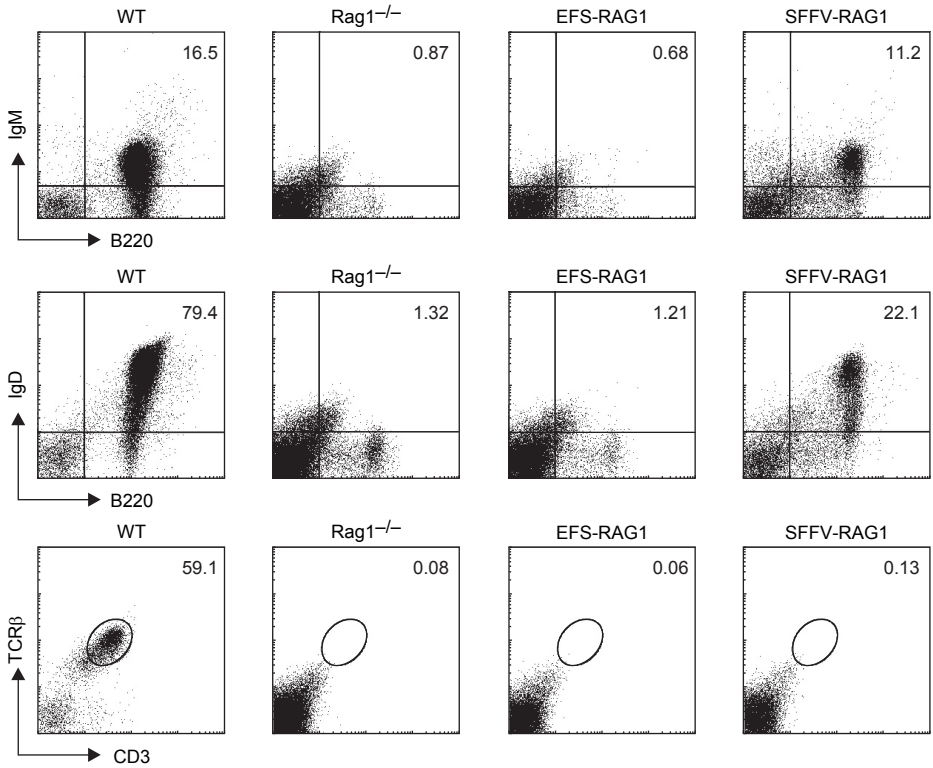

Figure 3. B and T lymphocyte reconstitution in the spleen.
Flow cytometric analysis of spleen cells 14 weeks after transplantation. Upper panels show flow cytometric analysis for IgM, IgD and B220 staining within the lineage negative lymphocyte gate; the lineage cocktail consisted of Ter-119, NK1.1, CD11b, GR-1 and CD43 antibodies. The lower panel shows flow cytometric analysis for TCRβ and CD3 staining within the lineage negative lymphocyte gate; the lineage cocktail consisted of B220, Ter-119, NK1.1, CD11b, GR-1 and CD43 antibodies. Numbers in the dot plots represent percentages within the quadrants or gates.

T-cell development

As shown in Table I, WT animals were the only animals in which the thymus was reconstituted on a cellular level. Immunophenotypic analysis revealed a normal staining pattern for CD4 and CD8 in these mice (Figure 4, upper panel). Corresponding to the lack of T cells in peripheral blood and spleen, recipients of *Rag1*$^{-/-}$, EFS-RAG1 and SFFV-RAG1 transduced cells mostly had CD4^{-}CD8^{-} (double negative, DN) thymocytes. Some mice had a small CD4/CD8 double positive (DP) population, but none of these cells expressed TCRβ or TCRγδ on the surface. It has been described before that CD4 and CD8 can be upregulated in *Rag1*$^{-/-}$ mice after irradiation.[35] This atypical population could have resulted from DN cells that were not eliminated by irradiation. One mouse

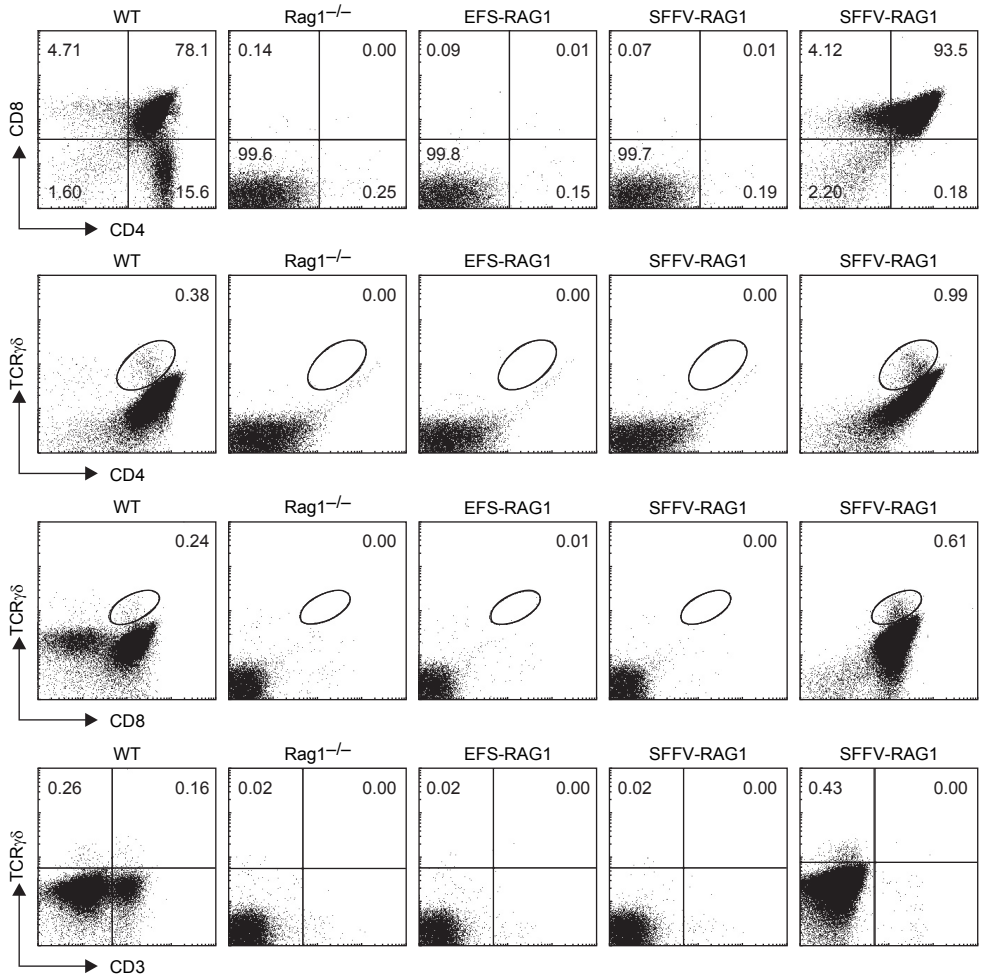

Figure 4. T-lymphocyte development in the thymus.
Flow cytometric analysis of thymocytes 14 weeks after transplantation. The upper panel shows flow cytometry analysis for CD4 and CD8 staining within the lineage negative gate. The lower panels show flow cytometry analysis for TCRγδ, CD4, CD8 and CD3 staining within the lineage negative lymphocyte gate. The lineage cocktail consisted of B220, Ter-119, NK1.1, CD11b, GR-1 and CD43 antibodies. Numbers in the dot plots represent percentages within the quadrants or gates.

in the SFFV-RAG1 group had a sizable DP population (Figure 4, plots on the far right). When examined for the expression of the TCRβ chain, this DP population turned out to be negative, while DP cells from WT mice did express TCRβ (data not shown). Cells were also checked for TCRγδ expression in combination with CD3, CD4 and CD8 (Figure 4,

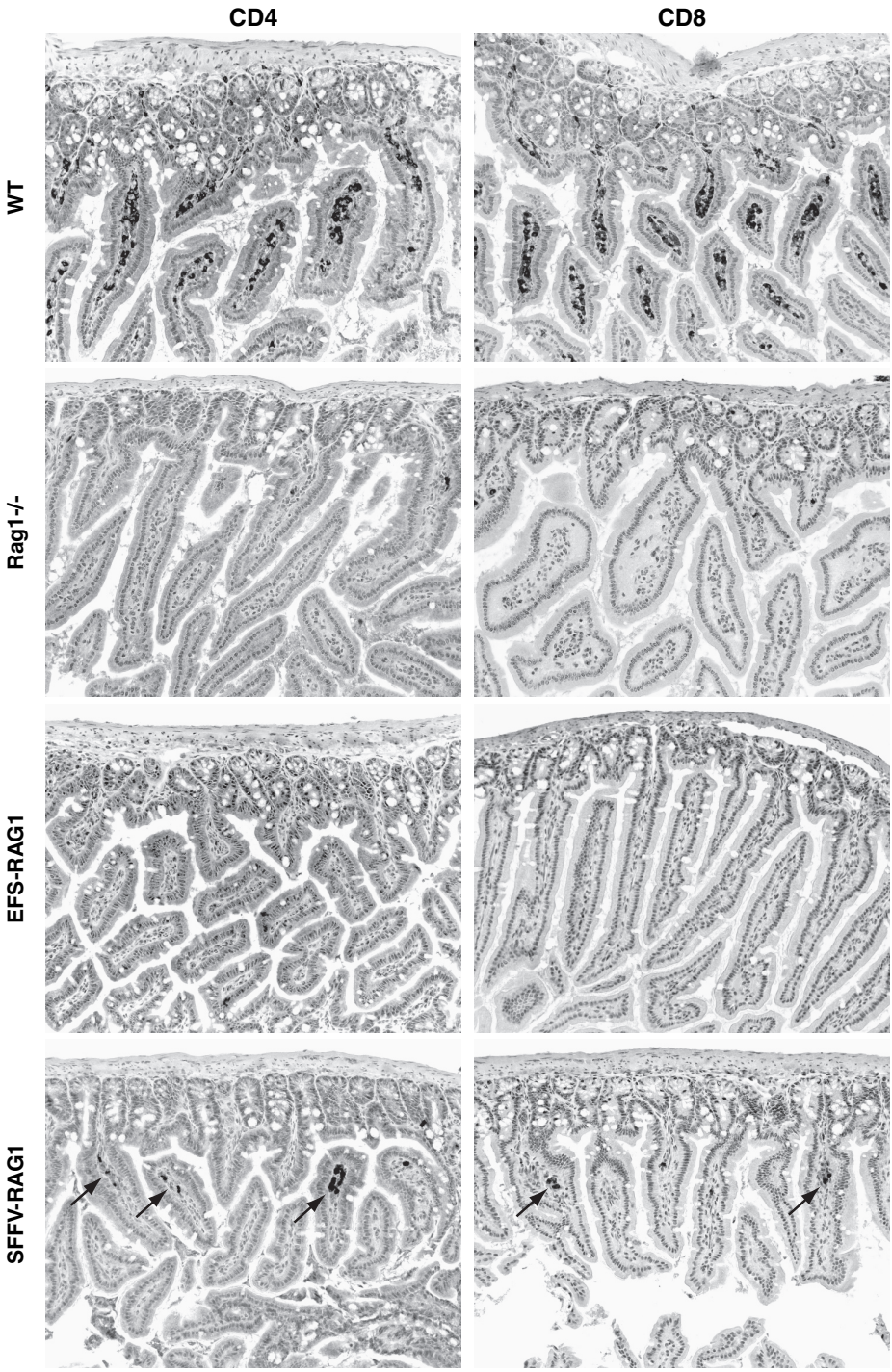
CD4
CD8
WT
Rag1-/-
EFS-RAG1
SFFV-RAG1

Figure 5 (facing page). Development of intraepithelial lymphocytes after gene transfer.
Frozen small intestine sections of mice transplanted with WT, *Rag2*$^{-/-}$, EFS-RAG2 transduced and SFFV-RAG2 transduced cells were made 14 weeks after transplantation. To identify intraepithelial lymphocytes (IEL), sections were stained by immunohistochemistry with CD4 and CD8 antibodies. Images were captured using a Zeiss Axioskop 2 plus microscope (original magnification 20x) and an Axiocam camera.

lower panels). TCRγδ^{+} cells are infrequently found in the thymus. In WT mice as well as one of the SFFV-RAG1 mice TCRγδ^{+} thymocytes could be detected. This can be taken as evidence that some RAG activity took place, since this is a requirement for the formation of all types of TCR chains. Of note, the TCRγδ^{+} cells in the SFFV-RAG1 mouse were negative for CD3, an observation we cannot explain at this moment. To test whether IEL were present in the small intestine, immunohistochemistry was done (Figure 5). In WT animals, both CD4^{+} and CD8^{+} cells were detectable. Both *Rag1*$^{-/-}$ and EFS-RAG1 animals were negative for CD4 and CD8. In the single mouse with TCRγδ^{+} cells in the thymus, CD4^{+} and CD8^{+} positive cells were detectable, though very low in frequency. Taken together, a significant development of T cells was not found in animals after gene therapy, although a small fraction of TCRγδ^{+} T cells was detected in the thymus of a single SFFV-RAG1 treated animal.

B-cell development and immunoglobulins in serum

Immunophenotypic analysis was also done on bone marrow cells to assess whether all compartments of B-cell development were present after gene therapy (Figure 6). Early precursor B cells are defined as B220^{+}CD43^{+}, while B220^{+}CD43^{-} cells appear later in B-cell development. In this particular experiment B220^{+} as well as B220^{hi} cells could be identified (Figure 6, upper panel). During the final developmental stages in the bone marrow, B cells start to express IgM and IgD on the cell surface. In animals from the WT group, all stages of B-cell development were represented. The CD43^{-}B220^{hi} population also expressed IgM and IgD (Figure 6, lower panel), whereas cells in the CD43^{-}B220^{+} gate did not (data not shown). Curiously, the phenotype of recipients of *Rag1*$^{-/-}$ cells was not fully consistent with 'normal' *Rag1*$^{-/-}$ mice, which exhibit a block at the CD43^{+} stage. Apparently, regenerating bone marrow is phenotypically different from steady-state bone marrow. Importantly, *Rag1*$^{-/-}$ animals did not have an IgM/IgD positive population. EFS-RAG1 mice were phenotypically comparable to the *Rag1*$^{-/-}$ mice in this experiment. However, all stages of B-cell development were also present in mice from the SFFV-RAG1 group, including cells with surface expression of IgM and IgD. Genescan analysis was done on total RNA extracted from splenocytes from 5 WT animals, 2 *Rag1*$^{-/-}$ animal, and 6 SFFV-RAG1 animals, to establish whether a polyclonal B-cell population had developed. A fluorochrome-labeled constant $(C)_{\mu}$ or C_{γ} gene segment-specific primer and a consensus V_H primer were used to amplify cDNA. Labeled PCR-products were used for Genescan analysis. In WT animals, PCR products showed a Gaussian distribution when using the C_{μ} or the C_{γ}–specific primer, indicating a polyclonal B-cell population

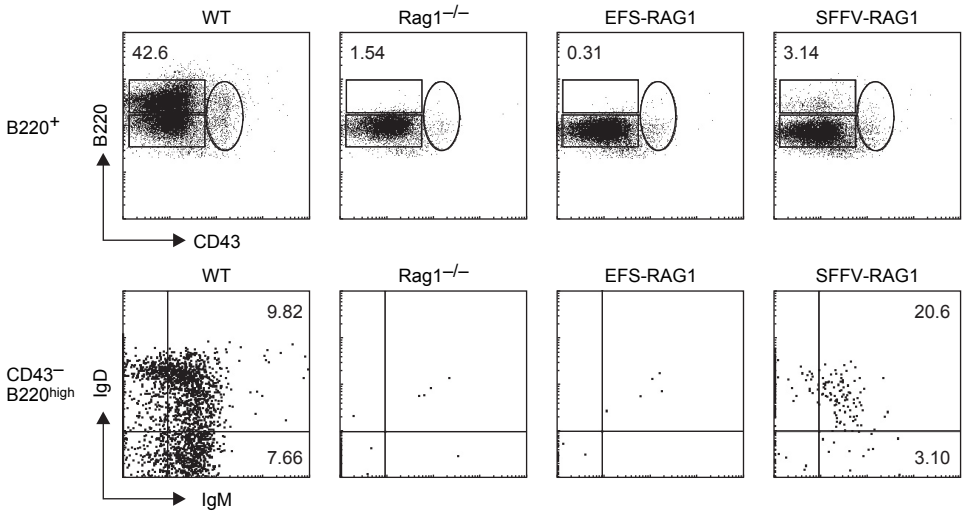

Figure 6. B-lymphocyte development in the bone marrow.
Flow cytometric analysis of bone marrow cells 14 weeks after transplantation. The upper panel shows dot plots of flow cytometric analysis for B220 and CD43 staining within the $B220^+$ gate. The lower panel shows dot plots of IgD and IgM staining within the $CD43^-B220^{hi}$ gate. Numbers in the dot plots represent percentages within the quadrants or gates.

(Figure 7). No IgH rearrangements were detected in *Rag1*$^{-/-}$ mice. In SFFV-RAG1 mice, a polyclonal rearrangement pattern was measured, but the peak distribution was not completely Gaussian.

Serum Ig levels were determined by ELISA (Figure 8). *Rag1*$^{-/-}$ and EFS-RAG1 animals, which lacked mature B cells in bone marrow, spleen and peripheral blood, did not have any detectable levels of IgM or IgG1. In serum of WT animals and SFFV-RAG1 animals IgM as well as IgG1 were detectable, though the levels of immunoglobulins present in SFFV-RAG1 were considerably lower than the levels found in WT mice. Taken together, these data show that mice receiving SFFV-RAG1 transduced cells end up with phenotypically normal B cells, albeit at reduced numbers.

Provirus integration and transgene expression

At the end of the experiment proviral copy numbers were determined on genomic DNA extracted from bone marrow cells, splenocytes and thymocytes of all EFS-RAG1 and SFFV-RAG1 mice (Figure 9A, upper panel). As stated before, we detected 6.2 proviral copies per cell in EFS-RAG1 transduced cells and 2.2 proviral copies per cell in SFFV-RAG1 transduced cells prior to transplantation. Fifteen weeks after transplantation, the mean number of proviral copies detected in the lymphoid organs was clearly lower. The discrepancy between gene marking found *ex vivo* and *in vivo* could possibly be the

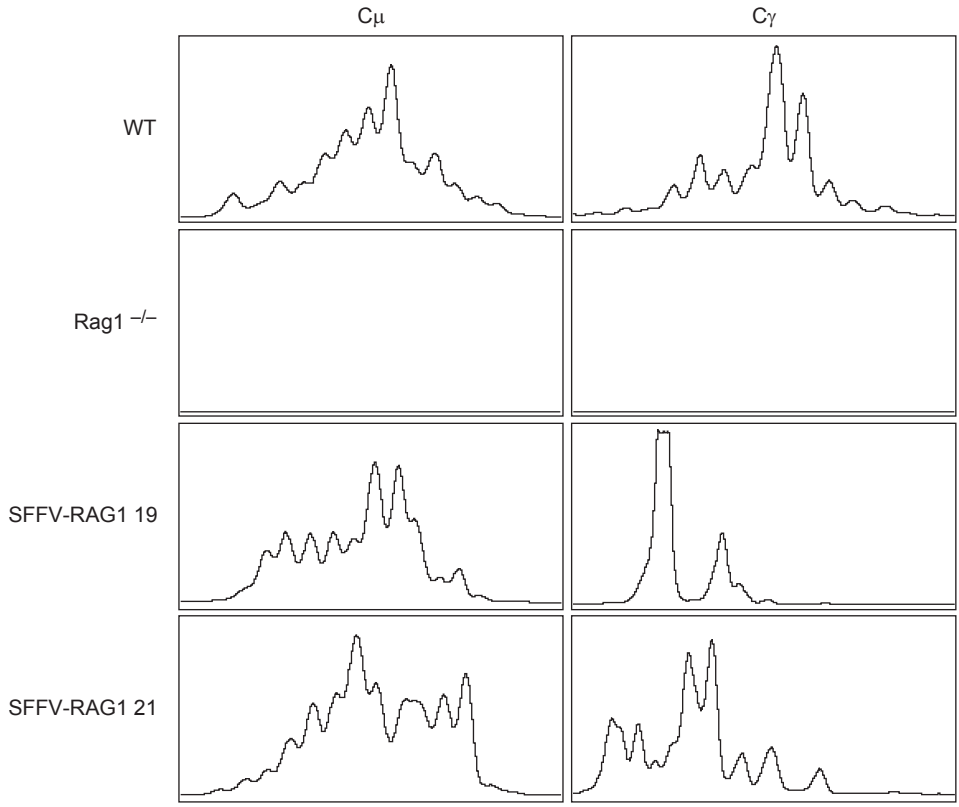

Figure 7. Ig repertoire analysis.
GeneScan analysis of PCR products obtained from Cμ and Cγ-specific PCR amplifications. Genescan analysis was done on material extracted from 5 WT mice, 2 *Rag1*$^{-/-}$ mouse and 6 SFFV-RAG1 mice. Representative samples of GeneScan plots are shown. The x-axis indicates PCR product length, the y-axis shows arbitrary fluorescence intensity of the runoff products.

result of a high transduction efficiency of non-repopulating cells. For both vectors, the highest copy number was found in bone marrow and was about 2-fold lower in spleen as well as thymus. In addition, the number of copies in post-transplant material from EFS-RAG1 mice had decreased more dramatically than in SFFV-RAG1 mice: 6.2 to 0.02-0.05 and 2.2 to 0.5-1.3, respectively. The level of human *RAG1* expression was also determined in bone marrow cells, spleen cells and thymocytes (Figure 9A, lower panel). Expression of *RAG1* was barely detectable in *Rag1*$^{-/-}$ samples and expression in WT samples, which contains mouse *Rag1*, was marginal. The majority of SFFV-RAG1 samples had a substantial transgene expression level, with expression in the thymic samples being the lowest. In specimens from EFS-RAG1 mice, *RAG1* expression is about

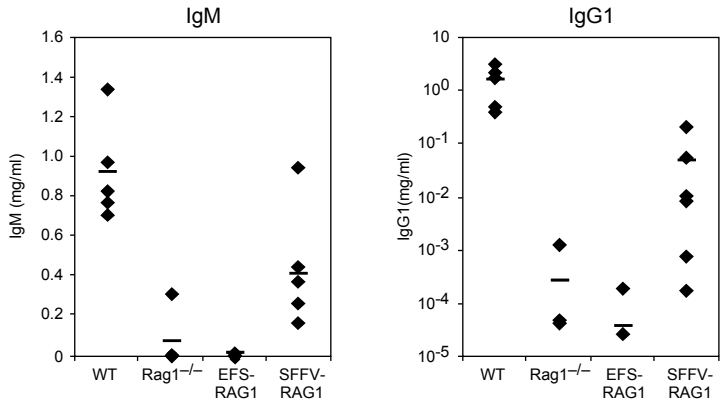

Figure 8. Serum Ig levels.
IgM and IgG1 serum levels of all mice were quantified by ELISA. Each diamond represents a value obtained in a single mouse. Mean values are represented by horizontal bars.

100-fold lower than in SFFV-RAG1 mice which can probably be attributed to the low proviral copy number found in these mice, and to a lesser extent to the lower promoter strength. The degree of *RAG1* expression found in cells transduced with the EFS-RAG1 lentivirus was insufficient to result in mature B and T cells. *RAG1* expression levels were adequate for reconstitution of the B cell compartment in SFFV-RAG1 animals but insufficient for T-cell development. Spleen cells from SFFV-RAG1 mice were pooled, myeloid and B cells were sorted to >90% purity and proviral copy numbers and transgene expression was determined (Figure 9B). We observed similar proviral copy numbers in both lineages (left graph), but levels of transgene expression dramatically differed between the two: *RAG1* expression reached much higher levels in B cells when compared to myeloid cells (middle graph). The same result was seen when expression was normalized for the number of proviral copies (right graph), suggesting that *RAG1* expression is positively selected for in B cells or selectively down regulated in myeloid cells.

DISCUSSION

The results of this initial series of preclinical experiments, aimed at clinical application of lentiviral SIN vectors for *RAG1* gene, demonstrated limited efficacy. One of the constructs used in this study, EFS-RAG1, did not result in the restoration of B- and T-cell development. At the onset of the transplantation experiment the EFS-RAG1 proviral copy number was relatively high (6.2) but *RAG1* expression levels were fairly low (0.43 relative to ABL). Most likely, this caused the failure of T- and B-cell reconstitution. When using the SFFV-RAG1 vector, the developmental block was only partially lifted,

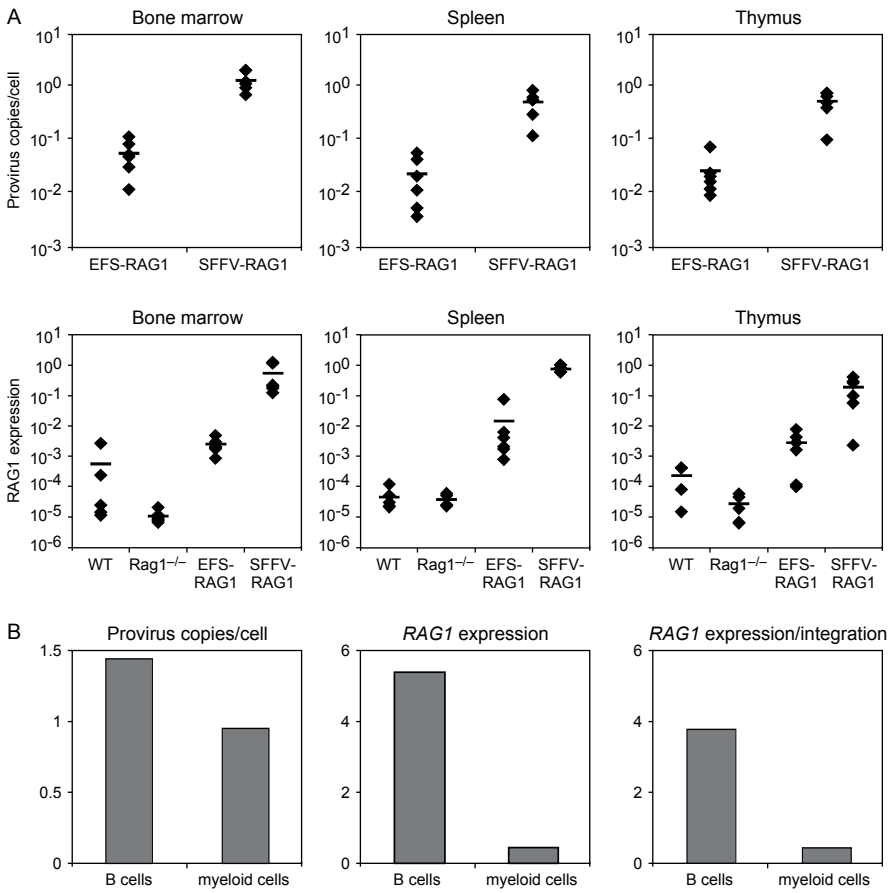

Figure 9. Provirus integration and transgene expression.
The number of proviral copies and the transgene expression was determined by RQ-PCR. A. The upper panel shows the proviral copy number per cell as determined on DNA extracted from bone marrow cells, spleen cells and thymocytes taken from EFS-RAG1 and SFFV-RAG1 mice. The lower panel shows *RAG1* expression in bone marrow, spleen and thymus samples from WT, *Rag1*$^{-/-}$, EFS-RAG1 and SFFV-RAG1. Diamonds represent values obtained from individual mice. Means are represented by horizontal bars. B. The number of proviral copies and transgene expression in total spleen cells, B cells and myeloid cells sorted from pooled SFFV-RAG1 spleen cells. The left graph shows the proviral copy number. The middle graph shows the level of *RAG1* expression relative to *ABL*. The right panel shows the *RAG1* expression level related to proviral integration.

in that B cells were able to develop but T cells were undetectable, with the exception of TCRγδ^{+} cells in the thymus and intestinal IEL of a single animal. This probably implies that B-cell development requires a lower level of *RAG1* expression than T-cell development. This finding is in line with gene expression profiling of various stages of human B- and

T-cell development that we have conducted.[36, 37] Interestingly, detection of TCRγδ$^+$ cells in the thymus of a single animal suggests that low levels of *RAG1* expression are sufficient for rearrangement of TCR gamma and delta gene segments. We did not find any TCRγδ$^+$ cells in peripheral blood or spleen but most TCRγδ$^+$ cells reside in the gut and the skin.[38] Indeed, some CD4$^+$ and CD8$^+$ cells could be detected in the epithelial layer of the small intestine. Overall, the level of *RAG1* expression was not sufficient for full lymphoid reconstitution in our experiments. However, aiming for higher expression levels would mean that more proviral copies per cell are needed, which is a highly undesirable situation in light of insertional mutagenesis. To be able to reduce the number of integrations per cell, while at the same time increasing transgene expression, we are assessing transfer vectors that contain *RAG1* sequences that are optimized for codon usage and GC-content. Tests in cell lines have shown that using a codon-optimized *RAG1* sequence results in dramatically higher transgene expression per integrated provirus thus lowering the number of integrations needed per cell. This topic is discussed in a separate section of this thesis (Chapter 5.2).

In the current investigation we analyzed primary and secondary lymphoid organs 14 weeks after gene therapy. In order to evaluate long-term efficacy as well as safety of the *RAG1* lentiviral SIN vectors, longer follow-up and secondary transplantations will have to be done. In addition, immunizations will have to be included in long-term studies to assess T-cell dependent and T-cell independent immune responses. After correcting for the inability to recombine Ig and TCR gene segments, *Rag1*$^{-/-}$ mice are not expected to have any intrinsic problems in mounting immune responses. Indeed, it has recently been shown that *ex vivo RAG1*-gene transfer using Moloney leukemia virus (MLV)–based vectors give rise to fully immuno-competent mice.[13]

In this study on the efficacy of *RAG1* gene transfer using lentiviral SIN vectors, we elected to subject mice to a lethal dose of γ-irradiation. Most RAG-SCID patients that undergo bone marrow transplantation are either pre-treated with low dose cytotoxic agents or receive no pretransplant conditioning at all.[39, 40] Prior to gene therapy treatment, X-linked SCID patients are also not subjected to conditioning[10, 41] and ADA-SCID patients treated with gene therapy were subjected to non-myeloablative conditioning.[9] Future preclinical *RAG1* gene transfer studies will have to address whether mild or no pre-treatment allows for lymphoid reconstitution after gene therapy.

In the context of SCID, the introduction of therapeutic genes in hematopoietic stem cell has repeatedly demonstrated to confer a selective growth and developmental advantage to those cells that need the involved therapeutic gene for further differentiation.[9, 10, 13, 14, 41, 42] We anticipated that *Rag1*$^{-/-}$ cells transduced with a vector that contains *RAG1* would result in similar developmental advantages for T- and B-cell precursors. Unfortunately, only B cells were detectable in our gene therapy treated animals. We were therefore limited to a comparison between B cells and myeloid cells. When comparing the average number of integrations between these populations, the B-cell fraction gave only a slightly higher value than the myeloid fraction. Similar copy numbers did not result in comparable *RAG1*

expression levels. Rather, *RAG1* expression was about 10 fold lower in the myeloid lineage. This could point towards positive selection in the B-cell lineage for precursor B-cells expressing *RAG1* at relatively high levels or down regulation of *RAG1* expression in myeloid cells, or both.

In summary, with the transfer vector that contains the SFFV promoter controlling *RAG1* expression, we reached *RAG1* expression levels that were sufficient only for B-cell development. Nevertheless, the results presented in this paper represent an encouraging first step towards *RAG1* gene therapy. Future efforts are primarily aimed at identification of the transgene expression levels needed for restoration of B- as well as T-lymphocyte development. However, reaching sufficient levels of transgene expression using lentiviral *RAG1* gene transfer vectors has proven extremely challenging. Optimizing the codon usage of the *RAG1* sequence is a promising option to increase transgene expression levels and we will test the effectiveness of this modification *in vivo*. In addition, animals in future experiments will be monitored longer and immune responses to vaccination will be tested in conjunction with extensive phenotypic analysis, Ig and TCR repertoire analysis and investigation of peripheral B- and T-cell expansion through kappa-deleting recombination excision circles (KRECs)[43] and T-cell receptor excision circles (TRECs).[44, 45] These type of experiments will allow us to fully evaluate immune reconstitution in a RAG-SCID gene therapy setting.

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CHAPTER 5.2

IMPROVING LENTIVIRAL TRANSGENE EXPRESSION USING CODON OPTIMIZED *RAG1*

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To be submitted

ABSTRACT

Gene therapy is an alternative for bone marrow transplantation as treatment of severe combine immunodeficiency (SCID). In our laboratory, we are testing lentiviral SIN vectors for recombination activation gene 1 and 2 (*RAG1* and *RAG2*) gene transfer. Reaching therapeutic levels of transgene expression is a major hurdle to overcome for these transgenes. In an effort to increase *RAG1* expression levels we optimized the *RAG1* sequence. Codon usage was optimized for *Homo sapiens*: certain cis-acting motifs and cryptic splice sites were removed and the GC-content was increased. These modifications to the *RAG1* sequence led to 2- to 3-fold higher viral titers and 10- to 100-fold increased transgene expression levels. We anticipate that the use of lentiviral SIN vectors containing an optimized *RAG1* sequence might result in sufficient expression of the therapeutic gene and will lead to a lower number of viral integrations per cell required for correction of the RAG-SCID phenotype, thereby improving safety.

INTRODUCTION

In our laboratories, we intend to develop lentivirus-based gene therapy for human recombination activation gene 1 and 2 (*RAG1* and *RAG2*) deficiency. Individuals suffering from severe combine immunodeficiency (SCID) caused by mutations in *RAG1* or *RAG2* show a total lack of T and B lymphocytes, the cells that make up the adaptive immune system. To develop a fully competent adaptive immune system, most RAG-SCID patients receive a bone marrow transplantation, until recently the only curative treatment option. In the past decade great progress has been made in the development of gene therapy for several causes of SCID. In this type of therapy an unmutated copy of the affected gene is transferred into the hematopoietic stem cells and/or progenitor cells of the patient by a modified, replication defective retrovirus. In the absence of an HLA-matched donor, this is the only treatment that compares favorably to the results of HLA-mismatched or matched unrelated donor transplantation. Clinical gene therapy trials on ADA-SCID and X-linked SCID have demonstrated that this kind of approach provides an effective alternative for the treatment of SCID patients.[1-3]

A major problem encountered during testing of gene therapy vectors for RAG1-SCID is the production of gammaretroviral and lentiviral vector batches with a titer sufficient for efficient transduction of hematopoietic stem cells. In addition, *RAG1* gene expression levels turned out to be too low for full correction of the effects of RAG1 deficiency *in vivo*, since they were insufficient for reconstitution of T-cell development in the thymus (see Chapter 5.1). To achieve correction of RAG1 deficiency, a higher level of transgene expression would be necessary. To realize this, we would have to use either more virus particles during the transduction procedure, or optimize the expression levels. The use of a higher concentration of viral particles is undesirable from a safety point of view, since

more provirus integration events would occur, thus increasing the chance of oncogene activation or disruption of tumor suppressor genes.

Adjusting the DNA sequence to optimize codon usage is a frequently used technique to enhance expression of genes in a heterologous host. For instance, the genetic sequence of one of the most widely used reporter proteins, eGFP, was also codon optimized to enhance protein expression.[4] The differential frequency of tRNAs between the host species and the original species is the main cause of reduced translation efficiency.[5] RAG proteins mediate DNA nicking and hairpin formation using chemistry that is identical to the DDE class of transposases, strongly suggesting these genes originated outside the mammalian genome. Therefore, the codon-usage is likely to be suboptimal for translation in mammalian cells. Analysis of the human *RAG1* cDNA sequence by GeneArt (Regensburg, Germany) indeed revealed several opportunities to improve the DNA sequence without affecting the amino acid sequence, since quite a few rare codons are present in the native *RAG1* gene. Most of these codons were replaced by codons that are more frequently used by *Homo sapiens* genes. In addition, the GC-content was increased to augment mRNA stability. Finally, further analysis identified the presence of cis-acting motifs in the wildtype gene, i.e., prokaryotic inhibitory motifs (14), splice donor sites (4), polyA sites (1), and RNA instability motifs (2). These motifs could negatively influence expression and were therefore removed. Because no alterations were made to the amino acid sequence, we anticipate that regulatory mechanisms occurring at the protein level would function normally. The newly designed *RAG1* gene was synthesized and cloned into our lentiviral SIN vectors. In this manuscript we evaluate the effect of the optimized *RAG1* sequence on lentivirus titers, transduction efficiencies, and transgene expression levels.

MATERIAL AND METHODS

Lentiviral vectors

The gene transfer constructs we used were originally derived from pRRL.PPT.PGK.GFPpre.[6] The pRRL.PPT.EFS.RAG1.pre (hereafter: EFS-RAG1) and pRRL.PPT.SFFV.RAG1.pre (hereafter: SFFV-RAG1) transfer vectors were constructed by replacing the PGK promoter by a human elongation factor 1α (EFS) promoter or a MLV-derived enhancer–promoter from the spleen-focus-forming virus (SFFV). The GFP sequence was replaced by human RAG1 cDNA. The WPRE used in these constructs is lacking the X protein ORF and 4 possible ATGs were mutated.[7] The *RAG1* gene sequence was optimized by modifying 573 of its 1044 codons, resulting 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 (hereafter: c.o.RAG1) was cloned into pRRL.PPT.EFS.pre and pRRL.PPT.SFFV.pre resulting in pRRL.PPT.EFS.c.o.RAG1.pre (hereafter: EFS-c.o.RAG1) and pRRL.PPT.SFFV.c.o.RAG1.pre (hereafter: SFFV-c.o.RAG1). Gene transfer constructs were validated by full DNA sequencing of the transgene. Helper plasmids pMDLg/pRRE, pRSV-Rev and pMD2.VSVG[6] for lentiviral production were kindly provided by L. Naldini (San Raffaele Telethon Institute for Gene Therapy Milano, Italy). Large-scale helper-plasmid preparations were obtained through PlasmidFactory (Bielefeld, Germany).

Lentiviral vector production

293T cells were transiently transfected with transfer and helper plasmids using the calcium-phosphate method. Cells received fresh media 16h after transfection. Lentivirus was harvested 24h, 38h and 48h after transfection. Cleared supernatant was filtered through 0.22 µm pore cellulose acetate filters and stored at -80°C. Total RNA was extracted from viral supernatant using the RNeasy Total RNA isolation kit (Qiagen, Hilden, Germany). Viral titers were determined by RQ-PCR as described below.

Transduction

HeLa cells (human cervical cancer cell line) were transduced by way of spin-occulation, in the presence of 4 µg/ml protamine sulphate (Sigma-Aldrich, Saint Louis, MO, USA) at 800 x g and 32°C for 1 hour. Transduced cells were cultured for 5 days after which genomic DNA and total RNA was isolated. Genomic DNA was extracted using the GeneElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich, Saint Louis, MO, USA). Total RNA from was extracted using the RNeasy Total RNA isolation kit (Qiagen, Hilden, Germany).

Real-time quantitative-polymerase chain reactions

Real-time quantitative polymerase chain reactions (RQ-PCR) were used for the quantitative analysis of genomic lentiviral RNA, proviral DNA copies, and transgene mRNA expression using *WPRE* and the Abelson (*ABL*) gene as targets. RNA was reverse transcribed into cDNA. RQ-PCR reactions were performed on the ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The following primers and probes were used. *WPRE*: forward primer, 5'-CCGTTGTCCGTCAACGTG-3'; reverse primer, 5'-AGTTGACAGGTGGTGGCAAT-3'; probe, 5'-FAM-TGCTGACGCAACCCCCACTGGC-TAMRA-3'. For copy number quantification of unknown samples, standard curves were created by preparing ten-fold serial dilutions of plasmid constructs of known concentration that contained the WPRE sequence. For samples derived from cellular RNA, the expression levels were normalized to the expression of *ABL*: forward primer, 5'-TGGAGATAACACTCTAAGCATAACTAAAGGT-3'; reverse primer, 5'-GATGTAGTTGCTTGGGACCCA-3'; probe, 5'-FAM-CCATTTTTGGTTTGGGCTTCACACCATT-TAMRA-3'.

RESULTS

We tested the effect of optimization of the *RAG1* sequence on the production of lentiviral viral vectors in 7 individual transfection experiments in which constructs expressing *RAG1* and *c.o.RAG1* were used side-by-side. Viral titers were determined by RQ-PCR using WPRE, an element present in all constructs, as a target (Figure 1). When comparing the two EFS transfer constructs, higher titers are indeed obtained when the EFS-c.o.RAG1 construct is used to produce virus. On average, about 3-fold higher titers were obtained when using the EFS-c.o.RAG1 transfer construct (Figure 1, left panel). Similar results were obtained when titers obtained with SFFV-RAG1 and SFFV-c.o.RAG1 transfer constructs were compared. Using SFFV-c.o.RAG1 resulted in a more than 2-fold increase in titer (Figure 1, right panel). In conclusion, the optimized *RAG1* sequence improved viral titers obtained after transfection of the plasmids in 293T cells.

To test the quality of the viral batches, we determined transduction efficiency on the HeLa cell line. In all cases, 250 viral particles per cell were used in the transduction procedure. Five days after transduction, DNA was isolated from the transduced cells. We used RQ-PCR to determine the average number of provirus copies per cell (Figure 2). Even though the variation between samples was large, the average provirus copy number

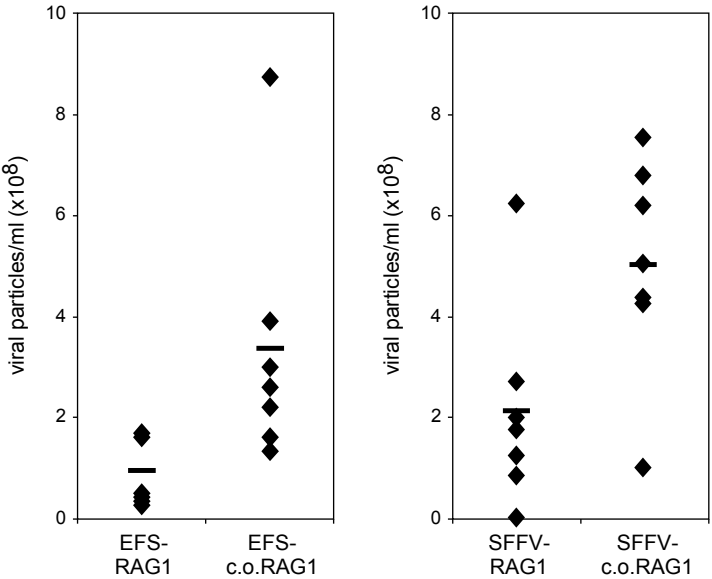

Figure 1. Lentivirus titers.
Viral titers were determined by RQ-PCR. The left panel shows titers obtained with lentiviral SIN vectors using an EFS promoter. The right panel shows titers obtained with lentiviral SIN vectors using a SFFV promoter. Titers are expressed as viral particles per milliliter. Diamonds represent values obtained from single transfections. Means are indicated by horizontal bars.

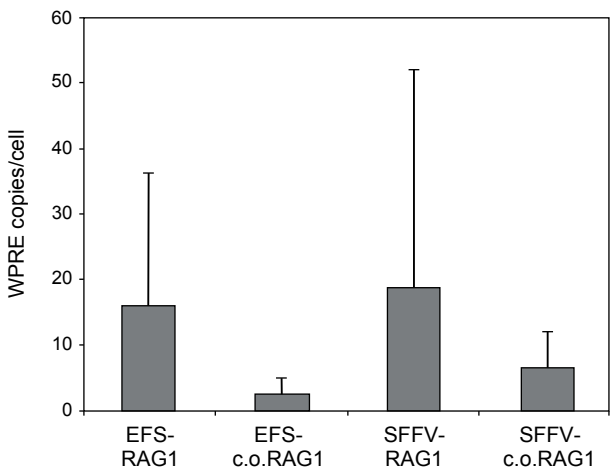

Figure 2. Transduction efficiency.
Proviral copies were determined by RQ-PCR. Transduction efficiencies are expressed as WPRE copies per cell. Means are shown for 7 individual experiments. The error bar represents one standard deviation.

was 3- to 6-fold higher in the cells transduced with constructs carrying the wildtype *RAG1* sequence. Therefore, optimizing the *RAG1* sequence did not result in a more efficient transduction procedure.

The main reason for the use an optimized *RAG1* sequence in our transfer constructs, was to improve on the low transgene expression levels obtained with wildtype *RAG1* cDNA. From the cells described in Figure 2 we also extracted mRNA to determine the level of transgene expression. RQ-PCR on cDNA revealed that transgene expression (normalized for the number of provirus copies) was much higher when viral vectors containing c.o.*RAG1* were used to transduce the cell line (Figure 3). The most striking difference was seen between the EFS-RAG1 and the EFS-c.o.RAG1 vector, in that the optimized sequence resulted in a more than 100-fold increase in transgene expression. Less dramatic differences were found when comparing transgene expression levels in SFFV-RAG1 and SFFV-c.o.RAG1 transduced cells, but a 10-fold increase was consistently found using the codon-optimized version of the virus. These observations demonstrate that a high transgene expression level can be reached with a relatively low number of viral copies per cell.

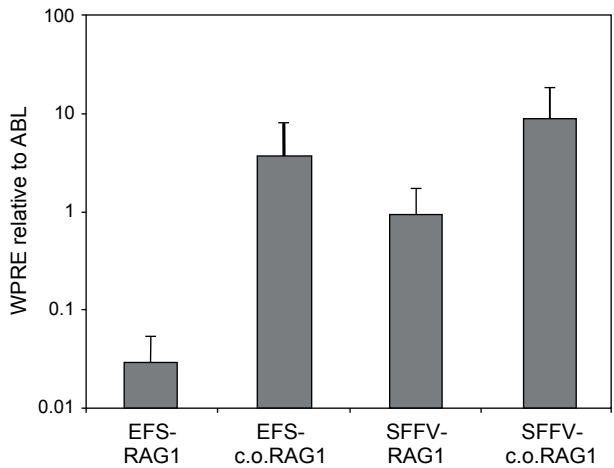

Figure 3. Transgene expression.
Transgene expression was determined by RQ-PCR and is expressed as *WPRE* expression relative to *ABL* expression. Means are shown for 5 or 6 individual experiments. The error bar represents one standard deviation.

DISCUSSION

In this report we assessed the effect of a codon-optimized *RAG1* sequence on lentivirus titers, transduction efficiencies, and transgene expression levels. We found that the use of an optimized *RAG1* sequence had a positive influence on the viral titers obtained, although the effect was moderate (2- to 3-fold). Transduction efficiencies

turned out to be slightly lower when using the optimized *RAG1* sequence. In contrast, the effect on transgene expression levels was highly significant, especially for the EFS-c.o.RAG1 construct, which demonstrated a more then 100-fold increase in expression as compared to the levels reached using the wildtype EFS-RAG1 construct. When placed in the context of gene therapy, the high transgene expression level per proviral integration should provide a great advantage, since gene transfer protocols should preferably aim at a minimum number of integrated proviruses to achieve efficacy in view of minimizing the risk of insertional mutagenesis.

These experiments need to be validated by testing the optimized *RAG1* sequence in primary human and murine hematopoietic stem cells. Furthermore, we will use lentiviral vectors carrying the optimized *RAG1* sequence in a preclinical murine *Rag1*$^{-/-}$ model to determine the minimum proviral copy number that will give a sufficiently high expression level to reverse the RAG1 deficient phenotype.

In summary, the higher transgene expression levels reached using an optimized *RAG1* sequence will allow us to attempt *RAG1* gene transfer using the weakest possible promoter, which would provide an additional layer of safety to the gene therapy procedure.

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We would like to thank Christopher Baum and Axel Schambach for advising us to optimize codon-usage of the *RAG1* sequence. The authors are grateful to Niek van Til for providing the transduced HeLa cell line used as a positive control sample in RQ-PCR experiments. This work was supported by Grants of the European Union (INHERINET, QLK3-CT-2001-0427 and CONSERT, LSHB-CT-2004-005242) and the Netherlands Organization for Health Research and Development (Translational Gene Therapy Research Programme of ZonMw, 43100016).

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CHAPTER 6

GENERAL DISCUSSION

A feature common to all forms of severe combined immunodeficiency (SCID), is the occurrence of a block in T-cell development. The studies in this thesis aimed at gaining more detailed knowledge of normal human T-cell development and applying this knowledge to the development of hematopoietic stem cell (HSC) gene therapy for SCID patients, including the understanding of adverse events that occurred in a recent gene therapy trial. This chapter discusses the findings from our studies and their implications in relation to current issues in the field of gene therapy.

In **Chapter 2**, subsets corresponding to consecutive stages of human T-cell development were isolated and their gene expression profile was correlated to the T-cell receptor (TCR) gene rearrangement status. This analysis resulted in the identification of candidate factors involved in regulation of TCR gene rearrangements.

In **Chapter 3**, it was shown that over-expression of the T-cell acute lymphoblastic leukemia (T-ALL) oncogene *LMO2* during human T-cell development in a gamma-retroviral setting resulted in a developmental block. In the same setting, the therapeutic *IL2RG* gene was shown not to have any adverse effect on the development of T cells. These studies partially dissect events that induced T-lymphoproliferations in X-linked SCID patients that underwent gene therapy.

Pre-clinical studies to investigate the feasibility of gene therapy for recombination activating gene (RAG)-SCID using lentivirus-based transfer vectors were described in **Chapters 4 and 5**. Full correction of both T- and B-cell development was established when using a *RAG2* transfer vector in *Rag2*$^{-/-}$ mice. Due to low transgene expression levels, initial studies using the *RAG1* transfer vector in Rag1$^{-/-}$ mice were only partially successful, resulting in reconstitution of B-cell, but not T-cell development. In an effort to increase *RAG1* expression, it was shown that a *RAG1* transfer vector containing a codon optimized *RAG1* sequence indeed results in a higher *RAG1* expression level per integrated vector.

New insights into human T-cell development

A developing T cell has to undergo several rounds of genomic DNA recombination, followed by selection processes. Rearrangement at the TCR loci happens in a specific order, namely, *TCRD>TCRG>TCRB>TCRA*. This specific sequence of TCR gene rearrangements, found in murine as well as human T-cell development, implies a high level of regulation. For rearrangements to occur, a TCR locus needs to become accessible for the recombination machinery. This is achieved through alterations in the chromatin structure. Histone proteins are the main building blocks of chromatin and can be modified by various post-translational modifications resulting in a change in DNA packing. Histone acetylation results in loosening of the chromatin structure, allowing for processes such as replication and transcription. On the other hand, histone methylation promotes a tight chromatin structure, restricting access to genomic DNA. Histone modification processes are mediated by histone modifying enzymes. These modifying enzymes are in turn recruited to areas of transcriptional regulation by sequence-specific DNA-binding

proteins: transcription factors, through protein–protein interactions. Only the transcription factors E2A and HEB have proven capable of inducing TCR gene rearrangements.[1] To date, other transcription factors involved in control of the sequential opening and rearrangement of the TCR loci are not yet identified. In an effort to increase our knowledge of human T-cell development and concomitant processes, we purified all known subsets of T-cell development using detailed immunophenotyping and high-speed cell sorting (**Chapter 2**). Gene expression profiling at the RNA level as well as quantification of TCR gene rearrangements at the DNA level were done on all subsets. One of the findings in this study was the complete correspondence of the occurrence of human TCR gene rearrangements with those in murine T-cell developmental subsets. The pattern of TCR gene rearrangement that we observed is summarized in Figure 1. In addition, correlation of the onset of TCR rearrangements at the different loci with expression profiles of genes involved in transcriptional regulation and DNA binding yielded a substantial, but testable, list of factors potentially involved in the regulation and/or initiation of TCR gene rearrangements (see **Chapter 2**).

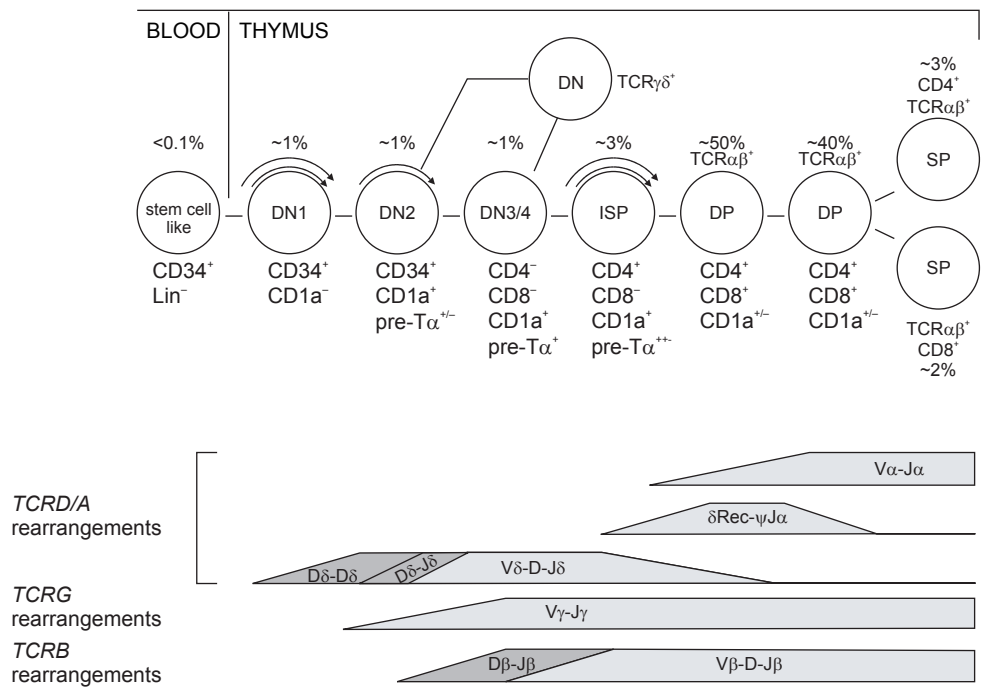

Figure 1. T-cell development.
Subsequent stages of human T-cell development are described according to the surface markers that are expressed. For each subset, the percentage within the total thymocyte population is given. In the lower part of the figure, rearrangement activity of the T-cell receptor loci is depicted.

Experimental approaches studying regulation of TCR gene rearrangements

Correlation of transcription factor expression with rearrangements is merely indirect evidence of their involvement in the rearrangement process. To establish the direct involvement of the identified factors in regulation or initiation of TCR gene rearrangements more firmly, functional experiments have to be done. To test individual factors for their direct contribution to the TCR gene rearrangement process, we propose several experimental approaches. Firstly, the *in vitro* transfection system used to study the effects of HEB and E2A on TCR gene rearrangement[1] can be employed to study the contribution of the newly identified candidate factors to the different types of TCR gene rearrangements. Secondly, *in vivo* over-expression studies could provide important information. Murine models that sustain the development of a complete human immune system could be utilized.[2] Human hematopoietic stem cells (HSC) in which candidate factors are retrovirally over-expressed, could be transplanted into, for instance, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. In this way, the effects of our candidate factors could be studied in developing human T-cells in a gain-of-function setting. Thirdly, loss-of-functions studies could be done in a similar 'humanized' setting, using siRNA to knock down expression of a specific factor. As a fourth experimental approach, the analysis of genomic DNA sequences bound by our candidate regulatory proteins could be studied using the recently developed ChIP-on-chip technique.[3-5] This technique integrates chromatin immunoprecipitation (ChIP) with microarray technology (chip). For this technique to succeed, a specific antibody that recognizes the factor under investigation is a prerequisite. Importantly, ChIP-on-chip requires antibodies that recognize its epitope in free solution as well as under fixed conditions. Another limitation of this technique lies in the relatively high number of cells needed for immunoprecipitation. Given these restrictions and the fact that only one target can be studied at a time, it is important to carefully select candidate factors using an *in vitro* functional assay before doing a ChIP-on-chip assay.

Challenges of gene therapy

In the early days, challenges of developing gene therapy revolved around the ability to transfer genes into mammalian cells and reaching sufficient transgene expression. The Moloney murine leukemia virus (Mo-MLV) was the most widely used retrovirus-based transfer vector. In Mo-MLV, the viral promoter resides in the long terminal repeat (LTR) of the vector and is strong enough to achieve high levels of transgene expression. Once methods for viral transduction of HSC were optimized, MLV vectors were successful in a clinical setting.[6-11] More recently, additional challenges and questions have surfaced for gene therapy development. In the French gene therapy trial for X-linked SCID, 4 patients have suffered from lymphoproliferation in the T-cell lineage, to date. The first two cases were reported in 2003.[12] The third case was described at the 10th annual AGST Meeting, 2007, Seattle, WA, USA. The fourth case was reported at the 33rd Annual Meeting of the European Group for Blood and Marrow Transplantation in Lyon 2007, France. In all four cases, the leukemic clones contained insertions of the therapeutic

vector near known oncogenes. Several factors could have contributed to the induction of these severe adverse effects (SAE). The age of the patients at the moment of treatment was initially named as one of the risk factors as the first two patients that presented with SAE were treated in the first trimester after birth. However, the third patient with SAE was older at the time of treatment. In addition, the first two patients with SEA received a fairly high dose of transduced cells compared to other patients in the same trial.[12] Conversely, in the British gene therapy trial for X-linked SCID, patients received comparable or higher doses of transduced cells but did not present with SAE to date.[9] Other factors, such as HSC culture conditions and the use of different viral envelopes, could also have influenced the differences in the outcome of the two trials. However, no evidence has been obtained to support these hypotheses as of yet. In addition, it has been debated whether the *IL2RG* transgene itself could have contributed to the lymphoproliferations, since in a successful ADA-SCID gene therapy trial[6] without SAE, a transfer vector with the same backbone was used as the one used in the French X-linked SCID trial. A more detailed understanding of the mechanism and contribution of different factors to the outcome of gene therapy trials should lead to advances from which future trials can benefit. Several aspects of developing safe and effective gene therapy for SCID are discussed in the remaining part of this chapter.

Insertional mutagenesis

One of the main advantages of using transfer vectors based on retroviruses is also one of the main problems: their stable integration into the genome of the target cell. Traditionally, this integration was thought to be fairly random. However, both HIV and MLV integrases favor integration in bent DNA, at the outer face of the helix.[13] Previously, retroviral integration sites had already been mapped to DNase I hypersensitivity sites.[14] In addition, it was shown that both MLV and HIV-derived vectors preferentially integrate in actively transcribed genes.[15, 16] Until clonal lymphoproliferation was observed in the French gene therapy trial for X-linked SCID, the frequency of adverse effects due to vector integration was thought to be low. The first two patients that presented with SAE in the gene therapy trial both suffered from a clonal T-lymphoproliferation due to an insertion of the therapeutic gene near the known T-ALL oncogene *LMO2.*[12] Immediately, the question was asked whether insertion of the transfer vector near *LMO2* occurring in two patients was coincidental, or whether this locus was especially susceptible to viral insertion. With the retroviral preference for insertion in actively transcribed regions in mind, we queried the gene expression data obtained in **Chapter 2**. $CD34^+$ umbilical cord blood (UCB) cells are highly similar to $CD34^+$ cells from bone marrow, the target cells for gene therapy. We found *LMO2* to be expressed at high levels in $CD34^+$ UCB cells, followed by a rapid decrease in expression in the early phases of T-cell development. $CD34^+$ UCB cells are highly similar to $CD34^+$ cells from bone marrow, the target cells for gene therapy. The high expression of *LMO2* in $CD34^+$ UCB cells makes the *LMO2* gene is more vulnerable to viral integration. Taking into account that SCID is primarily a

disease affecting the T-cell lineage, we took a closer look at the expression of other known T-ALL oncogenes in **Chapter 3**. After $CD34^+$ UCB cells underwent cytokine-stimulation *in vitro*, similar to stimulations done during viral transduction for gene therapy, we found that T-ALL oncogenes *LMO2*, *LYL1*, *TAL1*, *TAN1* were actively transcribed. The gene recently found to be involved in T-ALL, *CCND2*,[17] was also expressed at high levels. Of all T-ALL oncogenes, *LMO2* and *CCND2* showed the highest level of expression. The expression profiles found in $CD34^+$ UCB cells, were also seen in peripheral blood stem cells (PBSC) that underwent stimulation exactly following the clinical protocols used for X-linked SCID gene therapy. It is fair to assume that the above mentioned oncogenes are more susceptible to viral integration then other oncogenes. Despite its high transcriptional activity, to date, no viral integrations were found in the *LMO2* locus in the British trial for X-linked SCID gene therapy.[18] In the gene therapy trial for ADA-SCID, the therapeutic vector was found to be integrated in or near the *LMO2* locus, but the integration did not result in aberrant expression of *LMO2*, and consequently, SEA have not been observed in this trial.[19] Apart from *LMO2*, several vector integrations were identified in or near other genes involved in T-ALL in the clonal T-cell expansions found in the French X-linked SCID gene therapy trial. Among them were *BMI1*, *LYL1*, *c-Jun*, and *CCND2*. We identified some of these genes as susceptible to viral integration because of their high transcription levels in cytokine-stimulated $CD34^+$ UCB and PBSC cells.

When we tested for the functional effects of retrovirus-driven expression of *LMO2*, a block in T-cell development was observed. An effect that was not seen for the B, NK, or myeloid development. Leukemogenesis is a multi-step process.[20] Therefore, viral integration near a T-ALL oncogene in itself is most likely not sufficient to establish full-blown leukemia. However, based on data obtained in **Chapter 3**, we propose a model for the initial steps of leukemogenesis in a SCID gene therapy setting, depicted in Figure 2. Most genetic defects in SCID cause a block early in T-cell development. This block can be overcome through genetic correction via HSC gene therapy. High transcriptional activity in HSC renders certain genes vulnerable for viral insertion. Gene-corrected cells are capable of going through the initial steps T-cell development. After the initial stages of T-cell development, the expression of many genes is down-regulated, unless viral insertion causes sustained expression. In the early developmental stages, thymocytes undergo tremendous proliferation. Ectopic expression of most genes does not cause a problem, but almost by definition, T-ALL oncogene activation deregulates T-cell development. Therefore, thymocytes could accumulate in proliferative stages, making them more susceptible to additional oncogenic hits. If this model is correct, ectopic expression of T-ALL oncogenes that are highly expressed in HSC and need to be down-regulated in their transcription during early T-cell development, constitute a high risk factor in outcome of viral SCID gene therapy. This model does not apply to all forms of SCID. Adenosine deaminase (ADA) is mainly involved in metabolic processes. ADA-deficiency causes toxicity, especially to more mature proliferating lymphocytes. Cells that carry the correct copy of the *ADA* gene can potentially support the development of neighboring non-corrected cells through

extracellular ADA expression. This could explain why insertions near the *LMO2* gene in the ADA-SCID gene therapy trial have not resulted in lymphoproliferation,[19] since the advantage of gene therapy is not limited to gene-corrected cells but is conferred to a population of neighboring cells. This would reduce the selective advantage of individual, gene-corrected cells. High levels of *LMO2* mRNA were not detected in ADA-SCID patient samples with an insertion near *LMO2*.[19] In addition, the exact position of the viral integration could have influenced the level of upregulation. Recent characterization of T-ALL samples showed that levels of *LMO2* expression strongly depend on whether or not the negative regulatory element upstream of the transcription start site (TSS) is uncoupled from the coding sequences.[21]

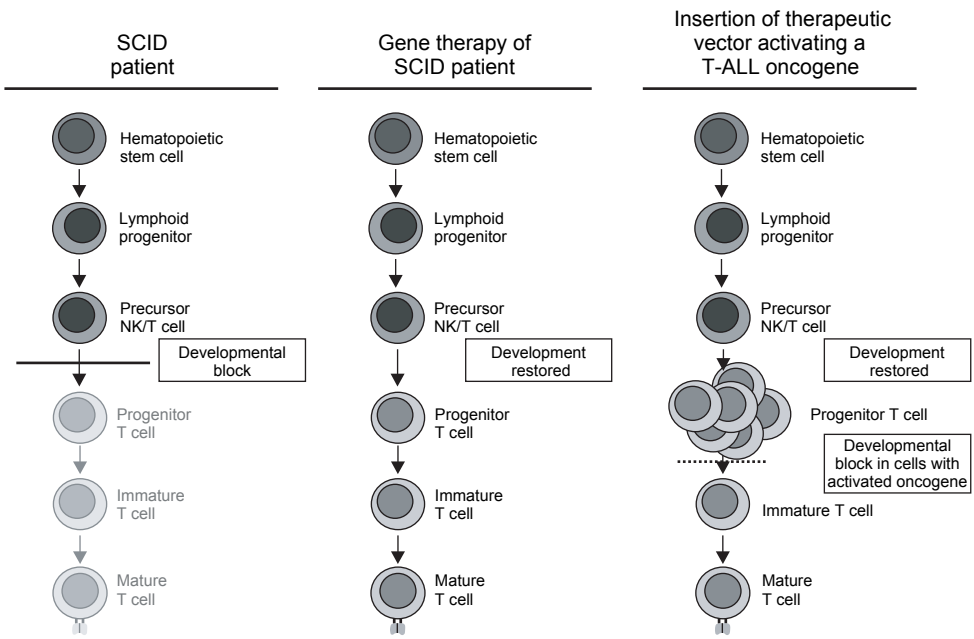

Figure 2. Model for leukemogenesis in SCID-XI gene therapy.
Left panel: Mature T cells are absent due to a mutation in one of the genes important for T-cell development. Middle panel: T-cell development is restored after retroviral gene therapy. Right panel: Overexpression of an oncogene causes deregulation of T-cell development.

Improving vector safety

The strong promoter/enhancer activity of the LTR of MLV-based transfer vectors is most likely the largest contributing factor to oncogenic activation after viral integration. For both HIV and MLV based vectors, a large part of the U3 region of the LTR have been deleted.[22, 23] As part of the lifecycle of retroviruses their RNA is reverse transcribed.

During this process, the deletion in the U3 region is transferred to the 5' LTR, resulting in the transcriptional inactivation of the provirus. Vectors with a part of their U3 region deleted are called self-inactivating (SIN) vectors. In these SIN vectors, the transgene expression is driven by an internal promoter. Although the SIN design reduces promoter/enhancer activity of the viral LTR, this type of transfer vector can still result in oncogenic activation and subsequent transformation of cells when a strong internal promoter is used, i.e., the strong internal promoter can transactivate cellular genes beyond the boundaries of the LTR.[24]

Different types of viral vectors interact with their host in different ways. Proviral integration patterns in the host genome differ from strain to strain. As mentioned before, both MLV and HIV preferentially integrate in loci that undergo active transcription. However, the two viruses display different preferences with respect to the integration site within a gene. MLV vectors show a strong bias for integration near the TSS of genes, while HIV based vectors preferentially integrate throughout transcriptional units.[16, 25] The preferential integration near the TSS of MLV-based vectors increases the chance of deregulated transcription, as confirmed by promoter trapping studies,[26] in which a reporter is expressed only when integrated downstream of an active promoter. The less favorable integration pattern of MLV, combined with the capacity of lentiviral vectors to transduce quiescent cells, such as HSC, led us to choose a lentivirus-based SIN vector for the development of gene therapy for RAG-SCID (**Chapters 4 and 5**).

To further limit the interaction between viral vector elements and the host genome, chromatin insulators could be incorporated in the transfer vector. Initially, insulators were used to prevent vector silencing brought about by the surrounding genome, resulting in low transgene expression levels.[27] The enhancer-blocking function of chromatin insulators could potentially lower oncogenic activation by viral vectors. Indeed, one study showed that an insulator placed in the LTR can reduce clonal dominance.[28] Of note, the viral vectors that contained insulators had a weak tendency to integrate near TSSs.[28] Another feature of SIN transfer vectors that has been investigated is their effectiveness of transcriptional termination. SIN transfer vectors display leakiness in their transcriptional termination, which could lead to oncogenic activation in the host genome. Incorporation of upstream polyadenylation enhancer elements in the 3' U3 region can improve polyadenylation and subsequent transcriptional termination.[29] The presumed positive effects of chromatin insulators and upstream polyadenylation enhancer elements in HSC should be evaluated more extensively using *in vitro* and (disease specific) *in vivo* assays.[24, 28, 30-32]
Modifications introduced to gene transfer vectors may profoundly reduce the chance of oncogenic activation, but abberant activation can not be avoided completely, since the rationale for using retroviral vectors is based on integration in the host genome.

Transgene safety

The discussion about transgene safety started after the observation of clonal lymphoproliferations in patients enrolled in an X-linked SCID gene therapy trial[12]. A similar event was also detected in a murine gene marking study.[33] Cooperation between the

transgene, *IL2RG*, and the oncogene *LMO2* was proposed as a possible mechanism for the occurrence of clonal proliferation.[34] Using replication-competent MLV, a murine retroviral tagged cancer gene database (RTCGD) was built. A murine T-cell leukemia clone and a tumor clone of the B lineage present in this database were found to have insertions near *Lmo2* as well as *Il2rg*, suggesting their cooperation in leukemogenesis.[34] However, retroviral insertions near several other known oncogenes, such as *Bmi1*, *Rap1gds1*, *Laptm5* and *Ccnd1*, were found in the same clones as well (RTCGD query date: August 6, 2007), making it difficult to determine which hits cooperated in leukemogenesis and which hits were "innocent bystanders". Another report has also suggested a role for the IL2Rγ in leukemogenesis.[35] A murine model system was used in which *Il2rg*$^{-/-}$ HSC were transduced using a lentiviral SIN vector carrying the *IL2RG* gene. Mice transplanted with the *IL2RG*-transduced HSC developed tumors with a higher incidence than control mice or mice receiving *LMO2*-transduced HSC. High levels of *IL2RG* expression were achieved, exceeding endogenous *IL2RG* expression levels, as well as the *IL2RG* expression levels found in the X-linked SCID gene therapy trials. The report did not specify whether the tumors were host or donor derived and whether the proliferations were clonal. Both Jak3 and Stat5 were found to be activated in the tumors (as reported at the 9th annual meeting of the ASGT in Baltimore, 2006), unlike what was found in leukemic samples from patients enrolled in the X-linked SCID gene therapy trial. High transgene expression levels as well as the insertion of multiple copies of the transfer vector per cell might have contributed to tumor formation, but the direct involvement of IL2Rγ in oncogenesis remains to be established.

Experiments described in **Chapter 3** set out to investigate the effect of retroviral expression of *IL2RG* on human T-cell development. CD34^{+} cells were isolated from human UCB and transduced with a IL2Rγ-encoding MLV-based virus. Transduced cells were subsequently grown in a human-mouse hybrid fetal thymic organ culture (FTOC) to induce T-cell development. Immunophenotypic analysis showed that retroviral expression of *IL2RG* did not affect human T-cell development, unlike the developmental block we observed upon retroviral *LMO2* expression. This is in line with results obtained from murine experiments.[36] Transgenic *IL2RG* mice did not develop T-cell proliferations over a period of at least 12 months, and from mice injected with gammaretroviral or lentiviral *IL2RG*-transduced HSC, only 3 out of 68 mice developed a lymphoma. In addition, leukemic samples from patients that underwent IL2RG gene therapy did not show constitutive JAK3 activation.[12] Combined, these data argue against an oncogenic or toxic nature of *IL2RG* transgenes.

RAG1 and *RAG2* as transgenes

In **Chapters 4 and 5**, we investigated the feasibility of lentivirus-mediated gene therapy for RAG-SCID. Inappropriate RAG activity has been implicated in human as well as mouse lymphomas.[37] Therefore, RAG expression in a gene therapy setting could also pose problems. Expression of both human RAG proteins under control of the *lck*

promoter in normal mice results in incomplete thymopoiesis and a severe reduction in cellular immunity.[38] Expression of both murine Rag proteins under the control of the ubiquitous MHC class I H2K promoter or the lymphocyte-specific mb-1 promoter was also associated with a severe block in both B- and T-cell development.[39] Such genotoxicity is not seen when only one of the RAG proteins was overexpressed.[38] This suggests that viral expression of one RAG protein, as would be the case in gene therapy for RAG-SCID, should not have unfavorable effects due to expression of the corrective *RAG1* or *RAG2* transgene. In a gene therapy setting, only patients with debilitating mutations in one of the two *RAG* genes would be treated. Expression of the unmutated RAG gene most likely undergoes normal regulation processes. Both RAG proteins have to be present for DNA cleavage to occur, therefore downregulation of one of the two RAG proteins will result in complete abolishment of recombinase activity.

A problem that many researchers working with lentiviral vectors encounter, is the low viral titers that are often obtained. This has also been the major hurdle to be taken when attempting lentivirus-mediated RAG gene transfer. In particular, the relatively large size of full-length RAG1 (1040 amino acids) appeared to be a problem in obtaining viral stocks with sufficiently high titers. Many studies of the mechanism of V(D)J recombination have been done using shortened versions of the RAG proteins, mainly because of their enhanced solubility. The core regions of RAG proteins are defined as the minimal regions required to catalyze DNA cleavage. Although tempting, we did not use the less sizeable core-RAGs in our pre-clinical studies for several reasons. Truncation of part of the N-terminal non-core region of RAG1 can result in inefficient V(DJ) recombination as well as immune deficiency.[40, 41] The N-terminal non-core region of RAG1 also contains a zinc-binding RING domain that is capable of autoubiquitylation,[42] thus implying regulation of RAG1 protein levels through degradation in the proteasome. The C-terminal non-core region of RAG2 enhances V to DJ recombination[43], and represses transposition events.[44, 45] In addition, RAG2 stability is controlled in a cell-cycle specific way through phosphorylation of a threonine residue in the non-core region. Upon phosphorylation, RAG2 is sequestered in the cytoplasm where it is poly-ubiquitylated and degredated by the proteasome.[46] Since both functional and regulatory domains can be found in non-core regions of both RAG proteins, we conclude that full-length RAGs should be used for gene transfer in a SCID gene therapy setting.

Efficacy of lentivirus-based RAG vectors

Before therapeutic vectors can be used in patients, extensive pre-clinical testing has to be done to determine both efficacy and safety. Safety studies should be done in disease specific models, in murine models using human cells, and non-human primates, such as rhesus macaques. In **Chapters 4 and 5**, the efficacy of self-inactivating lentiviral RAG vectors was tested in murine Rag-deficient models. For both *RAG1* and *RAG2*, lentiviral SIN vectors were constructed with two different promoters controlling transgene expression; the human elongation factor 1α (EFS) promoter or the MLV-derived enhancer-promoter

from the spleen-focus-forming virus (SFFV). Vectors that contained the EFS promoter driving *RAG1* or *RAG2* expression did not result in immune reconstitution. Low vector copy numbers resulted in low expression levels in both cases, precluding therapeutic effectiveness, in contrast to the vectors that contained the SFFV promoter. In the pre-clinical *RAG2*–gene transfer study (**Chapter 4**), sufficient transgene expression levels were reached to reconstitute B- as well as T-cell development. We were able to detect B and T lymphocytes in the peripheral immune compartments, be it at lower frequencies than wild-type control animals, which suggest that higher *RAG2* expression levels are needed for full reconstitution. The mice in which the immune system was successfully restored, received cells with a relatively high vector copy number (about 9 per cell). A high provirus copy number per cell will increase the chance of oncogenic activation, and should therefore be avoided. In the *RAG1*-gene transfer study described in **Chapter 5**, mice receiving cells transduced with the SFFV-vector showed reconstitution of B-cell development and B-cell function, but T cells did not develop. At the onset of the experiment, transduced HSC contained about 2 proviral copies per cell, while 3 months after transplantation an average of 1 copy per cell was detectable. The provirus copy number found in these mice falls within a safer range than seen in the *RAG2* study, but immune reconstitution was by no means complete. To achieve full immunological reconstitution, higher transgene expression per cell is needed. With the vectors tested, this would mean more proviral insertions per cell, increasing the risk of insertional mutagenesis. Therefore, both studies could benefit from higher expression levels per integrated provirus.

Optimizing RAG expression

The coding sequence of the *RAG1* gene was analyzed for its codon usage, GC-content and the presence of cis-acting motifs that could negatively influence expression. The native *RAG1* gene has a high GC-content and contains a lot of codons that are rarely used in human genes. In addition, 20 cis-acting motifs were identified that could negatively influence gene expression. Alterations were made to the coding sequence, removing all cis-acting motifs, increasing the GC-content and optimizing codon-usage. Because the amino acid sequence was not changed, regulation occurring at the protein level is still expected to take place. The effects of optimized codon usage in the *RAG1* gene on viral titer and transgene expression were studied in **Chapter 5**. We found that constructs containing codon optimized *RAG1* resulted in higher viral titers. But the most striking effect was found on transgene expression. Especially in constructs with an EFS promoter, the difference between expression of *RAG1* from a wild-type sequence or the codon optimized was substantial (more than 100-fold). This result is very encouraging in light of safety of gene therapy. The high *RAG* gene expression that is needed for immune reconstitution can now be achieved with fewer viral copies per cell, reducing the chance of oncogene activation. These *in vitro* findings need to be confirmed in an *in vivo* setting. The *RAG2* coding sequence will also be codon optimized and tested for its *in vitro* and *in vivo* expression capacity.

Future directions for gene therapy for SCID

Retrovirus-mediated gene therapy for SCID has proven to be very successful in the clinical setting. The major residual problem is mutagenesis and the consequential risk of oncogenesis due to the relatively random insertion of the therapeutic virus into the host genome. Ideally, insertion of the therapeutic gene is targeted to precise loci in the genome. New technologies are being employed to make site-specific integration suitable for a gene therapy setting. One of the tools explored for targeted gene delivery is the adeno-associated virus (AAV), which integrates its genome in a 3-kb site on human chromosome 19.[47] Several modifications have yielded a state-of-the-art hybrid vector design, capable of inserting an AAV expression cassette in 20% of target cells (cell line) with a site specificity of approximately 70%.[48] However, the system is far from being ready for clinical applications, mainly due to low efficiency of gene transfer into primary cells (1-5%). Non-specific integration also occurs, and to date, the non-specific integration pattern has been insufficiently studied to make proper risk-assessments.

Another avenue for site-directed gene transfer utilizes the properties of meganucleases. Meganucleases are sequence specific endonucleases recognizing large targets of more then 14 bases,[49] and can be found in eukaryotes, bacteria and archae. Engineered forms of these endonucleases, such as engineered zinc finger nucleases are capable of stimulating integration of long stretches of DNA after the introduction of a double-strand break at a specific location,[50] Many of these targeted approaches have to be tested for specificity. In addition, gene transfer into primary cells needs to be improved before site-directed gene transfer techniques can move to a clinical setting.

Towards clinical application of RAG-SCID gene therapy

In the absence of a suitable bone marrow donor, retroviral gene therapy is the best possibility for treatment of SCID at this moment. Risks that come with more or less randomly integrating viruses, can be minimized by aiming for low vector copy number per cell as well as minimal infusion of transduced hematopoietic stem cells. In addition, much effort is put into improving the safety features of transfer vector backbones. Effects of modifications to vectors need to be evaluated systematically using *in vitro* assays as well as relevant *in vivo* animal models. Our pre-clinical studies show the feasibility of using SIN lentiviral vectors for *RAG*-gene transfer. Efficacy needs to be reached at lower vector copy numbers, expressing codon-optimized *RAG*-sequences. Safety of *RAG* expression as well as the use of the lentiviral SIN backbone in combination with the chosen promoters need to be tested in long-term follow-up studies. Using the *Rag2*$^{-/-}$/*IL2rg*$^{-/-}$ mouse models, efficacy of the transfer vectors could be tested using RAG-SCID patient material.

For clinical application of HSC gene therapy, large batches of lentivirus-based vectors need to be produced. This is one of the bigger problems of lentiviral transfer systems. With this in mind, retroviral SIN vectors might be a better option. On the other hand, the incidence of RAG-SCID is fairly low, and only patients without an HLA-matched HSC donor would be considered for gene therapy, reducing the need for big viral batches.

Once gene therapy for RAG-SCID is applied in a clinical setting, patients will have to be monitored closely. Reconstitution of T and B lymphocytes can easily be determined by immunophenotyping of peripheral blood cells. If the therapy is successful, B and T cells should be detectable within a couple of months after re-infusion of the treated cells. In addition, serum immunoglobulin levels should be restored. To monitor the safety of the therapeutic procedure, keeping a close eye on white blood cell counts will be essential in detecting clonal lymphoproliferations. One of the means to reduce the risk of lymphoproliferations, is to limit the number of cells that is given back to the patient. However, this number should still be high enough to result in permanent B- and T-cell reconstitution. In other words, a sufficient number of transduced cells with both repopulation and self-renewal capacities should be transplanted. A clinical trial for *RAG*-gene therapy will provide an answer to the question whether this therapy will result in a life-long cure for SCID or whether several rounds of gene therapy need to be carried out.

RAG deficiency results in a block in B- and T-cell development due to loss of the capacity to recombine antigen receptor gene segments. The nature of this developmental block calls for a careful analysis of the repertoire of the B- and T-cell receptors that arise after gene therapy. Primarily, it is essential for the specific defense of the patient that a broad immunological repertoire is built up. In addition, monitoring the dynamics of the immunological repertoire of a regenerating immune system is of great scientific interest. Data obtained from a clinical trial for RAG-SCID gene therapy can be used to optimize future gene therapeutic treatment of RAG-SCID patients.

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ABBREVIATIONS

AAV	adeno-associated virus
ABL	Abelson
ADA	adenosine deaminase
ALL	acute lymphoblastic leukemia
ANOVA	analysis of variance
CD	cluster of differentiation
CMV	cytomegalovirus
c.o.	codon optimized
DN	double negative
DNAPKcs	DNA-dependent protein kinase catalytic subunit
DP	double positive
EFS	elongation factor 1α
EGFP	enhanced green fluorescent protein
FACS	fluorescence activated cell sorting
FLT3L	FMS-related tyrosine kinase 3 ligand
FTOC	fetal thymic organ culture
GALV	gibbon ape leukemia virus
G-CSF	granulocyte-colony stimulating factor
GVHD	graft-versus-host disease
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HSC	hematopoietic stem cell
hu	human
IEL	intraepithelial lymphocytes
Ig	immunoglobulin
IL	interleukin
ISP	immature single positive
ITAM	immunoreceptor tyrosine-based activation motif
IVIG	intravenous immunoglobulins
JAK3	Janus kinase 3
KREC	kappa-deleting recombination excision circle
LMO2	LIM-only protein 2
LTR	long terminal repeat
MHC	major histocompatibility complex
Mo-MLV	Moloney murine leukemia virus
MSV	murine sarcoma virus
mu	murine
NK	natural killer

PBSC	peripheral blood stem cells
PEG	polyethylene glycol
PID	primary immunodeficiency
PNP	purine nucleoside phosphorylase
PPT	polypurine tract
RAG	recombination activating gene
RMA	robust multichip assay
RQ-PCR	real-time quantitative polymerase chain reaction
RSS	recombination signal sequences
RSV	Rous sarcoma virus
SAE	severe adverse effect
SCF	stem cell factor
SCID	severe combined immunodeficiency
SFFV	spleen-focus-forming virus
SIN	self-inactivating
sm	surface membrane
SP	single positive
TdT	terminal deoxynucleotidyl transferase
TEA	T-early alpha
TCR	T-cell receptor
TPO	thrombopoetin
TREC	T-cell receptor excision circle
TSS	transcription start site
UCB	umbilical cord blood
VSVG	vesicular stomatitis virus glycoprotein
WBC	white blood cell
WPRE	woodchuck hepatitis virus post-transcriptional regulatory element
WT	wild-type

SUMMARY

Within the human immune system, several types of cells can be distinguished that all have their own designated function in host defense. This includes the B and T lymphocytes, which are highly specialized cells that make up the cellular compartment of the adaptive immune system. Both cell types arise from hematopoietic stem cells (HSC) that undergo a series of developmental steps, intended to equip the B and T cells with receptors that are capable of recognizing antigens in a highly specific manner. T-cell receptors (TCR) are bound to the membrane, whereas B-cell receptors, immunoglobulins (Ig), exist in a membrane-bound as well as a soluble form. The soluble antigen receptors produced by B cells are known as antibodies, which make up the humoral part of the adaptive immune system.

A large number of different antigen receptors need to be generated in order to recognize the wide range of antigens that is encountered by the host. To achieve this broad receptor repertoire, developing B and T cells undergo a series of rearrangements at the genome level. The antigen receptor segments involved in gene rearrangement are subdivided in variable(V), diversity(D), and joining(J) gene segments. More or less random rearrangement of these segments, also called V(D)J recombination, results in a large variety of *de novo* exons that code for the antigen recognition portion of the antigen receptor. This process of V(D)J recombination occurs exclusively during B- and T-cell development. The initial stages of B-cell development occur in the bone marrow, whereas T-cell development takes place in the thymus. B or T cells only migrate to the periphery when they have obtained a functional set of rearrangements that produce a fully functional antigen receptor.

In **Chapter 2** of this thesis we studied the TCR gene rearrangement patterns during eight consecutive stages of human T-cell development by quantitative PCR analysis. The same developmental stages were used for global gene expression profiling using DNA microarrays. By combining the *TCR* gene rearrangement data with gene expression data, we identified candidate factors for the initiation/regulation of TCR gene recombination. We also confirmed that TCR loci rearrange in a highly ordered fashion; *TCRD-TCRG-TCRB-TCRA*. Our data demonstrated that a number of key events, such as initiation of *TCRD* and *TCRB* gene rearrangements, occur earlier than reported previously. Therefore, human T-cell development is much more similar to murine T-cell development than reported before.

Severe combined immunodeficiency (SCID) is a rare inherited disease that can be caused by several genetic defects. Clinical symptoms usually present within the first few months of live and often include opportunistic infections, persistent diarrhea, and a failure to thrive. If SCID is not treated, the infant will die. The hallmark of SCID is the absence of functional T lymphocytes, but in certain types of SCID, the function and number of B cells and NK cells can also be affected. SCID can be cured through HSC transplantation. Because a suitable donor is not always available, gene therapy has been developed

as an alternative treatment. During gene therapy, the correct version of the affected gene is delivered to the HSC that are taken from the patients' own bone marrow. Retroviral vectors are used as a delivery system because of their ability to integrate into the genome, ensuring the presence of the therapeutic gene in all progeny of the HSC, which include T and B lymphocytes.

For two types of SCID, ADA-SCID and X-linked SCID, gammaretrovirus-based gene therapy has proven a successful treatment. However, the occurrence of leukemia in a gene therapy trial for X-linked SCID has highlighted insertional mutagenesis as an adverse effect. Although retroviral integration near the T-cell acute lymphoblastic leukemia (T-ALL) oncogene LIM-only protein 2 (*LMO2*) appears to be a common event, it is unclear why *LMO2* was preferentially targeted. In **Chapter 3** of this thesis, we showed that in $CD34^+$ progenitor cells *LMO2* has the highest transcription level of classical T-ALL oncogenes. Upon stimulation with growth factors typically used in gene therapy protocols, transcription of *LMO2*, *LYL1*, *TAL1* and *TAN1* is most prominent. Therefore, we conclude that these oncogenes may be susceptible to viral integration. Experiments in which we used a retroviral vector to overexpress the *LMO2* gene in $CD34^+$ cells, resulted in severe abnormalities in T-cell development, whereas B-cell and myeloid development remained unaffected. The interleukin-2 receptor gamma chain (IL2Rγ), which is deficient in X-linked SCID, has been proposed as a cooperating oncogene to *LMO2*. However, we found that overexpression of the *IL2RG* gene had no effect on T-cell development. We conclude that retrovirus-mediated expression of *IL2RG* during T-cell development is unlikely to be directly oncogenic.

The use of a different vector system has been proposed to improve the safety of gene transfer. Lentivirus-based vectors have been shown to have a more favorable integration pattern then gammaretroviruses, and self-inactivating (SIN) versions of this transfer system have been developed. SIN vectors contain a 3' long terminal repeat (LTR) from which viral promoter-enhancer sequences have been deleted, leaving the LTR transcriptionally inactive when integrated in the host genome. In this system, internal promoters are used to drive transgene expression.

Some forms of SCID are caused by a deficiency in proteins that are essential for V(D)J recombination, such as recombination activating gene 1 or 2 (RAG1 or RAG2). Inactivating mutations in *RAG1* or *RAG2* lead to SCID with a T^-B^- phenotype. In **Chapters 4 and 5.1**, the use of lentiviral SIN vectors for human *RAG* gene transfer was tested using RAG-deficient murine models. Lineage-depleted RAG-deficient bone marrow cells were treated with lentiviral SIN vectors that contained human *RAG1* or *RAG2* cDNA under control of either the human elongation factor 1α (EFS) promoter or the enhancer-promoter from the spleen-focus-forming virus (SFFV) and transplanted into lethally irradiated RAG-deficient mice. Over time, B- and T-cell reconstitution was monitored in peripheral blood.
After 3 months, immunophenotypic analysis of central and peripheral lymphoid organs was done, serum Ig levels were measured, and the presence of antigen receptor rearrangements was tested.

So far, we did not see a therapeutic effect of the vectors that contained the EFS promoter in both *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ mice. *Rag1*$^{-/-}$ mice that received SFFV-RAG1-treated bone marrow cells showed reconstitution of B-cell development, but not of T-cell development. These initial findings in peripheral blood were confirmed by immunophenotypic analysis of primary and secondary lymphoid compartments. Ig gene rearrangements as well as serum immunoglobulins were found in the SFFV-RAG1 treated mice. In *Rag2*$^{-/-}$ mice treated with the SFFV-RAG2 gene transfer vector, we were able to detect IgM^{+}IgD^{+} B cells as well as TCRβ^{+} T cells in primary as well as secondary lymphoid organs. In addition, serum immunoglobulin levels had normalized and rearranged Ig and TCR gene products were detectable. Overall, the presence of antigen receptor gene rearrangements and their corresponding proteins confirmed complementation of the recombinase deficiency in these models.

The inability to restore rearrangement function in developing T cells of animals that received *RAG1* gene therapy most likely reflects the requirement for higher transgene expression levels. Increasing transgene expression with the vectors described above, would mean that a higher viral dose should be given to the cells. This would lead to the undesirable situation of an increased number of integrated vectors per cell. To improve transgene expression levels, the sequence coding for *RAG1* was optimized for its codon usage (**Chapter 5.2**). When testing the codon-optimized *RAG1* construct *in vitro*, we observed that viral titers as well as *RAG1*-expression per integrated virus increased. These encouraging findings will be tested *in vivo*.

The research described in this thesis demonstrates that acquiring more detailed knowledge of normal human T-cell development is important for understanding the adverse events that occurred in a recent gene therapy trial for X-linked SCID. Furthermore, the pre-clinical gene therapy experiments in RAG-deficient mice showed proof-of-principle for the use of lentiviral SIN vectors for *RAG* gene transfer. Finally, the experiments with the codon-optimized *RAG1* construct shows that it is probably possible to use low numbers of viral integrations per cell, while the transgene expression is high. Future studies will focus on pre-clinical *in vivo* gene therapy experiments and the development and implementation of solid methods to demonstrate fully functional B-lineage and T-lineage reconstitution.

SAMENVATTING

Het humane immuunsysteem kent een aantal verschillende celtypen die ieder hun eigen functie hebben binnen het afweersysteem, waaronder ook de B en T lymfocyten. Dit zijn zeer gespecialiseerde witte bloedcellen die de specifieke afweer op cellulair niveau vertegenwoordigen. Beide celtypen ontstaan uit bloedstamcellen in het beenmerg. Deze stamcellen ondergaan een aantal ontwikkelingsstappen die bedoeld zijn om de cellen receptoren te geven die op zeer specifieke wijze antigenen kunnen herkennen. T-celreceptoren (TCR) zijn membraangebonden moleculen. Daarentegen komen B-celreceptoren, de zogenaamde immuunglobulines (Ig), voor in zowel membraangebonden als uitgescheiden vorm. De door B cellen uitgescheiden immuunglobulines zijn beter bekend als specifieke antistoffen, die voorkomen in het serum en de weefsels.

Voor het herkennen van het grote aantal antigenen dat we dagelijks tegenkomen, moeten veel verschillende antigeenreceptoren worden gemaakt. Om tot deze verscheidenheid aan receptoren te komen, ondergaan voorloper B en T cellen een aantal herschikkingen in de genen die coderen voor de Ig en TCR moleculen. Deze genen zijn onderverdeeld in meerdere 'variabele' (V), 'diversity' (D) en 'joining' (J) gensegmenten. Door op min of meer willekeurige wijze de gensegmenten aan elkaar te koppelen (V(D)J recombinatie), ontstaan er veel verschillende nieuwgevormde exonen die coderen voor het variabele deel van de receptor, dat een antigeen op specifieke wijze herkent. V(D)J recombinatie komt uitsluitend voor gedurende B- en T-celontwikkeling. Het eerste deel van de B-celontwikkeling vindt plaats in het beenmerg, terwijl T cellen zich in de thymus ontwikkelen. B en T cellen kunnen pas naar het bloed en de lymfeklieren migreren, wanneer de genherschikkingen een goed functionerende antigeenreceptor hebben opgeleverd.

In **Hoofdstuk 2** van dit proefschrift hebben we door middel van kwantitatieve PCR analyse de TCR genherschikkingspatronen bestudeerd in 8 opeenvolgende stadia van de humane T-celontwikkeling. Van diezelfde ontwikkelingsstadia zijn ook de genexpressieprofielen bepaald met behulp van DNA microarrays. Door de resultaten van deze twee analyses te combineren hebben we factoren geïdentificeerd die mogelijk betrokken zijn bij de initiatie of regulatie van TCR genherschikkingen. Onze studie bevestigde ook dat de verschillende TCR loci in een specifieke volgorde herschikken: *TCRD-TCRG-TCRB-TCRA*. Onze data toonden aan dat een aantal belangrijke processen, zoals initiatie van *TCRD* en *TCRB* genherschikkingen, eerder plaatsvinden dan tot nog toe werd aangenomen. Hieruit volgt dat T-celontwikkeling in de mens veel meer lijkt op die in de muis dan eerder gerapporteerd.

Ernstige gecombineerde immuundeficiëntie, ook wel 'severe combined immunodeficiency' (SCID) genoemd, is een zeldzame, erfelijke ziekte die veroorzaakt kan worden door verschillende genetische defecten. De klinische symptomen van SCID manifesteren zich meestal in de eerste maanden na de geboorte waarbij herhaaldelijke, vaak opportunistische, infecties en chronische diaree vaak voorkomen. SCID is een dodelijke aandoening als er geen behandeling plaatsvindt. Het kenmerk van SCID is de

afwezigheid van functionele T cellen in het bloed, maar ook B cellen en/of NK cellen kunnen ontbreken bij dit ziektebeeld. Beenmergtransplantie is een goede behandeling voor SCID. Aangezien niet voor alle SCID patiënten een passende donor beschikbaar is, wordt gentherapie ontwikkeld als alternatieve behandelmethode. Bij gentherapie wordt de correcte versie van het aangedane gen ingebracht in bloedstamcellen afkomstig uit het beenmerg van de patiënt. Voor de genoverdracht wordt gebruik gemaakt van retrovirale vectoren, omdat deze virussen van nature integreren in het gastheer DNA. Dit zorgt ervoor dat het therapeutische gen bij celdeling wordt overgedragen op iedere dochtercel, waaronder ook de zich ontwikkelende B en T cellen.

Voor twee vormen van SCID, ADA-SCID en geslachtsgebonden SCID, zijn al patiënten succesvol behandeld met gentherapie op basis van een gammaretrovirus. Het ontstaan van leukemie na gentherapie voor geslachtsgebonden SCID heeft echter de gevaren van foutieve insertie van de therapeutische vector benadrukt. Bij een aantal patiënten met leukemie werd een insertie van de therapeutische vector teruggevonden in de buurt van *LMO2*, een bekend T-cel oncogen. Het was aanvankelijk niet duidelijk waarom deze insertie met een dergelijk hoge frequentie voorkomt. In **Hoofdstuk 3** van dit proefschrift laten we zien dat in voorloper bloedcellen *LMO2* van alle bekende T-cel oncogenen het hoogst tot expressie komt. Na stimulatie van voorloper bloed cellen met groeifactoren, een standaard behandeling bij gentherapie, worden de T-cel oncogenen *LMO2*, *LYL1*, *TAL1* en *TAN1* tot expressie gebracht. Wij concluderen hieruit dat met name deze oncogenen open staan voor virale insertie. In experimenten waarbij we het *LMO2* gen met behulp van een retrovirus tot overexpressie brachten in $CD34^+$ voorlopercellen, zagen we dat B cellen en myeloïde cellen zich hieruit normaal konden ontwikkelen, maar dat T cellen in hun ontwikkeling werden geremd. Men heeft voorgesteld dat de interleukine 2 receptor gamma (IL2Rγ), het eiwit dat niet functioneert bij geslachtsgebonden SCID, als oncogen zou kunnen samenwerken met LMO2. Echter, bij retrovirale overexpressie van het *IL2RG* gen in $CD34^+$ voorlopercellen zagen wij geen effecten op de T-celontwikkeling. We concludeerden hieruit dat retrovirale overexpressie van *IL2RG* gedurende T-celontwikkeling niet direct oncogeen is.

Men heeft voorgesteld een ander vector systeem te gebruiken om genoverdracht veiliger te maken. Het is aangetoond dat lentivirale vectoren een minder nadelig integratiepatroon hebben dan gammaretrovirussen. Daarbij zijn er 'zelf-inactiverende' (SIN) versies van dit systeem ontwikkeld. Bij SIN vectoren is de sterke virale 'promoter-enhancer' verwijderd, zodat de vector na integratie transcriptioneel inactief is. In dit systeem wordt voor het tot expressie brengen van het therapeutische gen een zwakkere interne promoter gebruikt.

Sommige vormen van SCID worden veroorzaakt door het ontbreken van functionele eiwitten die cruciaal zijn voor V(D)J recombinatie, zoals 'recombination activating gene' (RAG) 1 en 2. Inactiverende mutaties in *RAG1* en *RAG2* lijden tot SCID met een T^-B^- fenotype. In **Hoofdstuk 4 en 5.1** worden de resultaten beschreven van experimenten waarbij het gebruik van lentivirale SIN vectoren voor RAG-genoverdracht getest worden in RAG-deficiënte muizenmodellen. Voorloper bloedcellen werden behandeld met

lentivirale SIN vectoren die het humane *RAG1* of *RAG2* gen tot expressie brachten onder invloed van 2 verschillende promotoren: 'human elongation factor 1α' (EFS) of 'spleen-focus-forming virus' (SFFV). Na genoverdracht werden de cellen ingespoten in RAG-deficiënte muizen. Het verschijnen van B en T cellen in het bloed werd in de tijd gevolgd.

Drie maanden na de behandeling werd het immunofenotype van centrale en perifere lymfoïde organen bepaald, de Ig concentratie in het serum gemeten en bepaald of er antigen receptor herschikkingen hadden plaatsgevonden.

In zowel *Rag1*$^{-/-}$ als *Rag2*$^{-/-}$ muizen die behandeld waren met vectoren met een EFS promoter zagen we geen therapeutisch effect. In *Rag1*$^{-/-}$ muizen die SFFV-RAG1-behandeld beenmerg ontvingen werden wel B cellen gevonden, maar geen T cellen. Deze bevindingen uit perifeer bloed werden bevestigd door immunofentypische analyse van centrale en perifere lymfoïde organen. Ig genherschikkingen alsmede serum Igs waren detecteerbaar in SFFV-RAG1-behandelde muizen. In *Rag2*$^{-/-}$ muizen die met de SFFV-RAG2 vector behandeld waren, waren zowel IgM^{+}IgD^{+} B cellen als TCRβ^{+} T cellen detecteerbaar in centrale en perifere lymfoïde organen. In deze muizen waren serum Ig aanwezig in normale hoeveelheden en herschikte Ig en TCR genen waren detecteerbaar. De aanwezigheid van antigenreceptor genherschikkingen en hun eiwitproducten bevestigt dat er in deze modellen correctie plaats heeft gevonden van de recombinase activiteit.

Het ontbreken van T-cel ontwikkeling in de dieren die *RAG1* gentherapie hebben ondergaan, wijst waarschijnlijk op de noodzaak voor hogere expressie van het therapeutische gen. Om de expressie van het transgen te verhogen met de vectoren die hierboven zijn beschreven, zou er een hogere dosis virus aan de cellen gegeven moeten worden. Dit zou lijden tot de onwenselijke situatie van een hoger aantal virale inserties per cel. Om de expressie van het therapeutische gen op een andere manier te verhogen, hebben we besloten de gensequentie van *RAG1* te optimalizeren met betrekking tot het codon gebruik (**Hoofdstuk 5.2**). Uit *in vitro* experimenten blijkt dat we met deze verbeterde sequentie hogere virus titers bereiken en bovendien hogere expressie van *RAG1* per geïntegreerd virus. Deze bemoedigende resultaten zullen in toekomstige experimenten in het RAG-deficiënte muizenmodel getest worden.

Het onderzoek dat in dit proefschrift is beschreven, laat zien dat het verkrijgen van een meer gedetailleerde kennis van de normale humane T-celontwikkeling essentieel is voor het verklaren van de bijwerkingen die ontstonden in recentelijk uitgevoerde gentherapie voor geslachtsgebonden SCID. Verder lieten de preklinische experimenten in RAG-deficiënte muizen zien dat het gebruik van lentivirale SIN vectoren voor *RAG* genoverdracht succesvol is. Tot slot hebben we laten zien dat bij een laag aantal virale integraties van het *RAG1* construct met een geoptimaliseerde gensequentie, toch een hoge transgen expressie bereikt kan worden. De nadruk in de vervolgstudies zal liggen op de preklinische *in vivo* gentherapie experimenten en het ontwikkelen en implementeren van solide methodes die de volledige reconstitutie van B en T cellen aantonen.

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Nee labgenoten, ik vergeet jullie niet! Floor, je hebt me goed op weg geholpen met de in het lab lopende technieken en je was ook nog eens een supergezellige kamergenoot. Ik ben blij voor je dat je een nieuwe uitdaging hebt gevonden, maar ik zal je erg missen. Edwin, we hebben samen heel wat cellen van elkaar gescheiden. Zonder jouw vaardigheid had ik waarschijnlijk maar een half boekje gehad. De Affy-apparatuur wordt altijd goed verzorgd door Tom, waardoor ik menig mooie array heb kunnen draaien. De data van deze arrays zijn vervolgens op onnavolgbare wijze doorgelicht door Dick; bedankt dat je de getallen een stuk logischer maakte dan ze in eerste instantie leken. Het gentherapie team heeft al een tijdje een BTK-tak. Peter, het is nu al gezellig bij jou op de kamer en ik denk dat we het nog ver gaan schoppen met de gentherapie. Tiago and Gita, your experience with sizable animal experiments and FACSAria measurements have helped me out a lot, thanks. Tiago, it was a pleasure being roommates with you for almost 2 years (!).

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Miranda, jij hebt het gentherapieteam de afgelopen tijd versterkt. Jouw werk aan het BTK-project heeft ook bijgedragen aan de vooruitgang van het RAG-project. Buiten dat ben jij ook nog eens de persoon die er al jaren voor zorgt dat het lab op een goede

manier blijft draaien. Sinds we op dezelfde treinroute zitten, heb ik bovendien al heel wat kilometers tegen je aangepraat. Dat je mijn paranimf wilt zijn, vind ik een hele eer.

De laatste tijd nemen de proeven in de groep van Frank dusdanig monsterlijke proporties aan, dat het hele lab gemobiliseerd moet worden om ze binnen 24 uur tot een goed einde te brengen ☺. Dit geldt ook voor de laatste 2 'proefjes' die ik voor mijn boekje had bedacht. Ik wil graag de Dynomics dames en alle mensen van 838 bedanken voor hun uitstekende teamwerk. De eervolle vermeldingen gaan hier naar de nachtwerkers, Edwin, Mark en Sjanneke, bedankt!

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In het laatste deel van hun promotieonderzoek komen promovendi vaak met de wildste ideeën op de proppen. Meestal zijn dit ook nog eens ideeën waarvoor technieken nodig zijn die ze zelf niet beheersen. Waardering voor de hulp van buitenaf is dan ook wel op z'n plaats. Berlinda, bedankt voor de spoedcursus 'darmpjes invriezen'. De invroren darmen zijn vervolgens door Marcel Vermeij van de afdeling Pathologie op maat gesneden en gekleurd. Hartelijk bedankt Marcel.

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Promotieonderzoek is niet bepaald bevorderlijk voor je sociale leven. Ik hoop dan ook dat ik de komende tijd weer wat meer tijd zal hebben voor de vrienden die ik al in tijden niet gezien of gesproken heb. Misschien moet ik mezelf even opnieuw voorstellen. Kirsten en Sandra (en Floor?), het is binnenkort in ieder geval weer eens tijd voor een 'dagje turnhal'!

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Karin

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