In vivo modelling of normal and pathological human T-cell development

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Chapter 7

General Discussion

The hematopoietic stem cell (HSC) maintains all the different cells present in our blood during our life by a delicate and balanced process of both self-renewal and differentiation. These processes are controlled by intricate networks of many signaling pathways regulating the different lineage choices. Most of the cells from the different cell lineages develop within the bone marrow (BM) except for T cells that need another specialized environment, which is provided by the thymus[1]. In mice, T-cell development has been studied extensively and in depth due to availability of transgenic mice, which enables studying the effects of genes on T-cell development. In addition, different *ex vivo* and *in vitro* assays exist to study development of T cells, such as gene expression studies of subsets, fetal thymic organ cultures (FTOC) and cocultures on different OP9 stromal cell lines to study kinetics and differentiation potential of precursor populations. For studies on human T-cell development researchers were only able to use these last assays as transplantation assays that supported human T-cell development were not available. With the development of humanized mouse models these assays have become available as now it is possible to transplant human precursor populations in severely immunodeficient mice that do allow for development of human lymphoid cells[2-4].

In this thesis, one of these immunodeficient mouse strains has been used to study different aspects of human T-cell development in an *in vivo* setting. Using these mice with an optimized transplantation protocol provided new insights in human T-cell development, thereby demonstrating the localization of several developmental checkpoints. Furthermore, this approach was used together with whole exome sequencing to determine the gene causative for a form of atypical severe combined immunodeficiency (SCID) and the precise developmental arrest. By overexpression of *LMO2* in HSPCs, it was demonstrated how *LMO2* can cause aberrant human T-cell development.

Humanized mouse models

Transgenic mouse models have been proven very useful in studies on hematopoiesis, as the effects of overexpression and absence of genes can be studied and more insight into underlying mechanisms can be obtained. Many of the obtained data can be extrapolated to humans, however, for T-cell development there are also many differences, as observed for mouse models of SCID compared to patient phenotypes. This disease, which is characterized by a deficiency of functional T cells, can be caused by mutations in several genes as described in **chapter 1**. The mouse model for RAG1-SCID mirrors the human situation in peripheral blood as both patients and mice show a deficiency in both T cells in B cells[5]. On the other hand, *Il7ra* knockout mice suffer from a deficiency in B cells besides their T-cell deficiency[6], while this is not observed in IL7RA-SCID patients[7]. This illustrates that some genes have comparable function in both humans and mice while other genes might have additional functions in one of the species.

The development of humanized mouse models[2-4] has allowed for studies on human hematopoiesis[8]. In **chapter 2** it was demonstrated that with a short culture a robust engraftment was obtained using one of these mouse models, the NOD/Scid-Il2rg$^{-/-}$ (NSG), with

outgrowth of different cell lineages. Both T cells and B cells that did develop were functional and secondary transplantations were performed, which demonstrated that the hematopoietic stem cells (HSCs) were not exhausted. Most importantly, the optimized protocol did allow for transplantation of HSPCs obtained from cryopreserved human bone marrow (BM). The level of engraftment of BM-derived HSPCs was lower as compared to mice transplanted with cord blood-derived HSPCs but both lineage development and relative abundance of different cell types was highly comparable. This made it possible to perform studies on arrests in development for different types of SCID as described in **chapter 3** and **chapter 4**.

One drawback of humanized mouse models is the xenograft setting; what effect does this have on selection of T-cell progenitors and to what extent are cytokines cross-reactive? T-cell progenitors undergo both positive and negative selection in the thymus whereby binding to MHC is crucial. Most probable, the progenitors will be selected on murine MHC as expressed by the thymic epithelial cells but also on human MHC, which is expressed on dendritic cells and B cells that did develop from transplanted HSPCs and have migrated to the thymus[9]. This is illustrated by the finding that both murine MHC restricted[4] and human MHC restricted responses have been detected in humanized mice[2, 4, 10]. In addition, the same populations were found both in thymi from engrafted NSG mice as in *ex vivo* human thymi as described in **chapter 2**. By studying the migrational patterns of thymocytes on slices of murine and human thymus it was demonstrated that these patterns were not altered when human thymocytes were put on murine thymic stroma and that migration was induced by the same chemokines in both species[11]. Collectively, these results demonstrate interspecies crosstalk and suggest no large abnormalities in thymocyte selection in this xenograft setting. In addition, it was demonstrated in **chapter 3** that the relative frequencies of different stages of B-cell precursor populations in the BM of transplanted NSG mice is highly comparable to the corresponding *ex vivo* BM aspirate of the donor. This further validates the use of this model for studies on human lymphopoiesis.

Different adaptations have been made to the NSG mouse model that, depending on the research question, might lower interspecies burdens. Human leukocyte antigen (HLA)-A2 transgenic NSG mice have been generated[12] and they have been compared side by side with NSG mice[9]. No differences in conventional human T-cell development were observed between the two strains and this was not different from fetal and postnatal human thymi[9]. In addition, we have detected a normal polyclonal TCR repertoire in humanized NSG mice (see **chapter 3**), thereby demonstrating that MHC differences did not lead to development of a skewed repertoire of the TCR. However, it was found that in both normal NSG mice and HLA-A2^{+} NSG mice transplanted with human hematopoietic stem and progenitor cells (HSPCs) there were differences in FOXP3^{+} thymocytes when compared to human thymi, probably caused by lack of cytokine crosstalk[9].

It has also been suggested that there is restricted crosstalk in cytokines needed for myeloid development, such as M-CSF. To overcome this, the MITRG and MISTRG mouse strains have been developed[13]; both are *Rag2$^{-/-}$Il2rg$^{-/-}$* mice that are knock-ins for the human alleles of M-CSF, IL-3/GM-CSF and TPO and the MISTRG mice are also transgenic for the human SIRPα gene.

SIRPα recognizes CD47, which is expressed on hematopoietic cells, and this interaction reduces phagocytosis of the transplanted human cells[14]. Both mouse strains better support the development of human myeloid cell subsets and NK cells and functionality[13]. Although NSG mice are severely immunodeficient, a sublethal dose of irradiation is needed for efficient engraftment of human HSPCs. Irradiation causes the release of free radicals and cytokines, which might have effect on the fitness of the HSPCs that will be transplanted thereafter. To circumvent this problem, NSG mice carrying a mutation in their Kit receptor have been created[15]. The ligand of this receptor is stem cell factor (SCF) a cytokine needed for stem cell self-renewal. The murine stem cells in the NSG-Kit$^{-/-}$ mouse are unfit as their receptor cannot respond to SCF and therefore they will be outcompeted upon transplantation of other HSPCs. Therefore, irradiation prior to transplantation is not needed in these mice, which allows for better studies on HSC capacities and clonality as demonstrated by an increased SCID repopulating cell (SRC) frequency in these mice (also called NSGW41) when compared to normal NSG that were irradiated prior to transplantation[15]. Strikingly, these NSGW41 had higher engraftment of human cells in their peripheral blood when transplanting limiting cell numbers and demonstrated better outgrowth of myeloid cells compared to NSG mice. It would be interesting to study the addition of human genes for cytokines, as in the MITRG and MISTRG mouse strains, in the NSGW41 strain on development of different cell lineages, their subsets and functionality.

Checkpoints in human T-cell development

Much of the knowledge on T-cell development stems from murine studies as previously human T-cell development could only be studied *ex vivo* or *in vitro*. This has changed with the development of the different humanized mouse models. However, murine T-cell development remains better characterized with the description of markers for many subpopulations, especially of the early double negative (DN) compartment in the thymus[16]. Human and murine T-cell development are comparable with respect to the following; the most immature populations are in de CD4$^-$CD8$^-$ DN compartment, immature single positive (ISP) cells develop into CD4$^+$CD8$^+$ double positive (DP), which after selection become either CD4$^+$ or CD8$^+$ single positive (SP)[17]. However, ISP are CD8$^+$CD3$^-$ in mice while CD4$^+$CD3$^-$ in humans and the markers described to characterize the murine DN compartment are not applicable to human T-cell progenitors[18]. This, combined with the fact that the phenotype in transgenic mice is not always comparable to the phenotype observed in patients, illustrates that there are also many interspecies differences and not all murine data can be extrapolated to human T-cell development.

The data described in **chapter 3** demonstrates that mutations in genes causative for SCID, such as IL7RA and IL2RG, show a phenotype different from their corresponding mouse mutants. When HSPCs from either IL7RA- or IL2RG-SCID were transplanted in NSG mice a very early block in T-cell development was observed, probably caused by an immediate need of signaling through these receptors after thymic entry. Also in normal thymopoiesis a restriction in the

number of hematopoietic clones was observed between HSCs sorted from BM and DN1/2 cells sorted from thymi of NSG mice transplanted with barcoded HSCs (see **chapter 5**). This might be correlated with cytokine driven expansion of a limited number of clones directly after seeding of the thymus. It has indeed been suggested that IL-7 and SCF have both a proliferative and survival effect on early thymocytes[19, 20]. Both in Il7r$^{-/-}$ and Il2rg$^{-/-}$ mice the defect in survival of early thymocytes could be rescued by expression of the anti-apoptosis Bcl2 protein[21, 22]. It is unknown whether in human T-cell development IL-2 and IL-7 play the same role, however, in **chapter 3**, we show an early block in T-cell development for both IL2RG- and IL7RA-SCID. Using TREC analysis it was demonstrated that DN cells have a high proliferative history[23]. Combined with the clonal restriction in these same stages as described in **chapter 5** this might suggest that also in human T-cell development there is expansion of a limited number of hematopoietic clones, which could be driven by IL-2 and IL-7.

In human T-cell development the point of β-selection has been ascribed to different stages; the immature single positive (ISP)[24-26] or the DN3 stage[17]. Transplantation of Artemis-SCID HSPCs in the NSG mouse model demonstrated that the initiation of *TRB* rearrangement might be earlier than previously described (**chapter 3**), namely at the CD7$^+$CD5$^+$CD1a$^-$ DN stage. The number of hematopoietic clones was also decreased between the DN1/2 and DN3 stage, in which DN3 was characterized by expression of CD1a (**chapter 5**). It remains difficult to determine the precise stage of β-selection as the DN compartment of human T-cell progenitors is not characterized in detail and different markers are being used. Based on above described data, a model of human T-cell developmental stages is herewith proposed (Fig. 1) that indicates the checkpoints that we described.

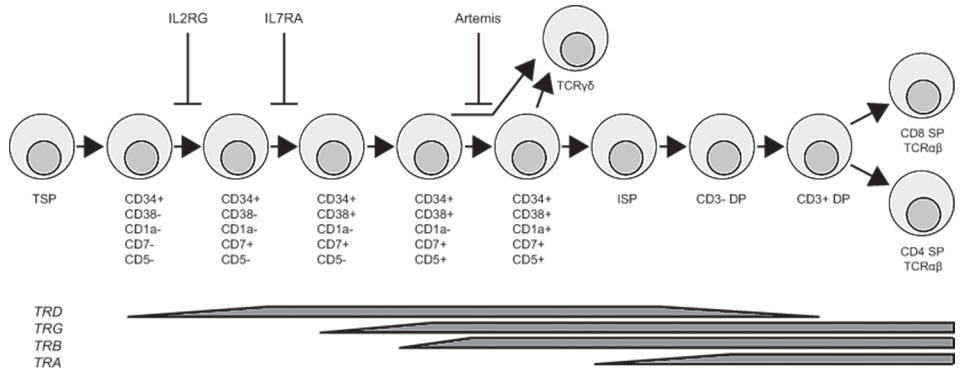

Figure 1: Proposed model of human T-cell development. Model based on data described in chapter 3 and literature indicating the arrests in human T-cell development for different types of SCID. Indicated are the stages of rearrangement of T cell receptor (TR) loci and markers to identify different stages in the CD4$^-$CD8$^-$ double negative (DN) compartment. TSP; thymus seeding progenitor, ISP; immature single positive, DP; double positive (CD4$^+$CD8$^+$), SP; single positive.

In this thesis, we demonstrated the arrests in T-cell development for SCID caused by mutations in 3 different genes, for which patients were selected with a null mutation (i.e. no residual activity of the mutated protein). Many patients exist that do have residual activity of the protein

depending on the type of mutation. These patients often present with an atypical form of SCID, such as Omenn syndrome. Omen syndrome is most often caused by hypomorphic mutations in *RAG1*[27] or *RAG2*[28] but has, for instance, also been described for *CORO1A* mutations[29]. It was even demonstrated that similar mutations in *RAG1* can lead to a diverse clinical presentation probably caused by differences in endogenous antigenic challenge and environmental factors[30]. Furthermore, upon transplantation of HSPCs from a RAG2-SCID patient in the described NSG mouse model, it was observed that there was indeed a deficiency in both T cells and B cells in the peripheral blood as in the patient. However, in the thymus there was development until the $CD3^-CD4^+CD8^+$ double positive (DP) with rearrangement of *TRD*, *TRG* and *TRB* (unpublished data). This demonstrates that even the patients that phenotypically present as a null mutant, might still have residual activity and therefore not be a molecular null mutant. In **chapter 3** it was already demonstrated that a hypomorphic IL2RG-SCID indeed is different from the null mutant after transplantation in the NSG mouse. Therefore, it would be interesting to study patients with different mutations in the same gene in the humanized NSG model to obtain more insights into the functional effects of various mutations on T-cell development.

Identification of new types of SCID

In addition to studying hypomorphic patients, the described NSG model can also be used to study patients suspected of SCID with atypical presentation. This will enable the exclusion of niche factors and determine whether the presumed defect is intrinsic to the hematopoietic cells. Using this approach it was determined that a girl with an atypical presentation of SCID indeed suffered from $T^-B^+NK^+$-SCID and that the arrest in T-cell development was at the DP to single positive (SP) transition (see **chapter 4**). Although disease characteristics of SCID with unknown genetic cause can be studied using this murine model, unfortunately, the duration of this type of experiments is too long to aid in diagnosis in this type of patients.

Whole exome sequencing (WES) of the patient and both parents was performed to search for the underlying genetic defect after exclusion of known SCID-causing genes by Sanger sequencing. Previously, WES of a patient and her parents has led to the discovery of a mutation in CARD11, which was thereby inactivated, and led to the development of SCID[31]. Casanova *et al.*, have postulated guidelines for studies on genetic causes for primary immunodeficiencies[32]. Primary immunodeficiency is a group of inherited rare diseases and therefore studies on disease causing genetic aberrations often rely on single patients. Thereby, it is more difficult to determine the affected gene in a single patient as compared to other types of diseases where more patients are available. As the analysis is more difficult in these case studies, the candidate gene needs to be validated in different assays. This can be done by cellular assays or animal models. However, most of the genes that are causative in inborn errors of immunity have been discovered in single patients[32]. Only after publication of the study that identifies the first patient, other patients can be screened for the same gene as not often these patients would present in the same center. In **chapter 4** we have identified a heterozygous *de novo* mutation in *VPS4B* using WES in a SCID patient with atypical presentation. By transplanting patient derived $CD34^+$ cells in the NSG mouse model, an arrest in T-cell development was demonstrated at the DP stage, which led to a T-cell deficiency in peripheral blood. We hypothesize that the identified mutation translates into a dominant negative form of VPS4B protein that might affect TCR signaling. However, confirmation by functional experiments is still needed. The results described in **chapter 4** potentially link a new gene to the development of $T^-B^+NK^+$-SCID.

Newborn screening

Newborn screening for SCID is implemented in many states in the USA and more countries might follow. In the Netherlands, the "Gezondheidsraad" (Health Council) has advised the Minister of Health to implement SCID, amongst other diseases, in the newborn screening[33]. This will be performed by TREC analysis on Guthrie card dried blood spots, which will detect T cell lymphopenia[34]. If tested positive, the patient will be referred for testing of the underlying defect. There already exists a fraction of SCID patients for whom the underlying genetic defect remains unknown, ranging from 7 to 33% of SCID patients[35-39], and when included in newborn screening the incidence of SCID might increase and thereby also the number of SCID patients with unknown cause. Using WES analysis of the SCID patient and both parents new genes can be identified. Combined with a gene therapy approach, the patient cells could be transplanted in NSG mice to confirm the causative effect of the gene and determine the stage of arrest(s) in lymphoid development in mice transplanted with untransduced HSPCs. Other patients that are suspected to suffer from SCID from the newborn screening could then also be screened for these newly identified genes. Newborn screening will lower the age of diagnosis for more SCID patients and this might benefit their outcomes after transplantation as it has been demonstrated that the survival after hematopoietic stem cell transplantation (HSCT) before the age of 3.5 months is higher[40]. In France, it has been calculated that transplantation before 3.5 months of age will be cost-effective[41].

T-ALL development in gene therapy trials by insertional mutagenesis

HSCT provides a cure for SCID; however survival is worse when a matched donor is not available[42]. Gene therapy might be an alternative and for many types of SCID, there are studies either in a clinical trial[43, 44] or in a preclinical phase[45-48]. Initial clinical trials for X-linked SCID (caused by mutations in *IL2RG*) and ADA-SCID have shown to be successful, but unfortunately 5 out of 20 IL2RG-SCID patients treated with gene therapy developed T-ALL caused by insertional mutagenesis (see **chapter 1**). Development of T-ALL caused by insertional mutagenesis was also found in 6 out of 10 treated patients in a gene therapy trial for Wiskott-Aldrich Syndrome (WAS), which is also a primary immunodeficiency, using a same type of vector as the initial trials for X-linked SCID and ADA-SCID[49]. Trials initiated thereafter made use of newly developed and safer SIN-lentiviral or SIN-gammaretroviral vectors and up to now no leukemias have been observed in treated patients[50-52].

What remains striking is that ADA-SCID patients did not develop T-ALL after gene therapy while similar gammaretroviral vectors have been used as in the other trials in which expression of the transgene was driven by the long terminal repeat (LTR)[53-56]. The T-ALLs that developed in the X-linked SCID trials and the WAS trial all had an integration in or nearby *LMO2*, a gene frequently upregulated in T-ALL[49, 55, 56]. LMO2 is normally highly expressed in HSCs and is downregulated early in human T-cell development[57]. In the ADA-SCID trials these integrations were also found in cells from peripheral blood of treated patients but no leukemia development was observed[58]. It remains a question why T-ALL did not develop in the ADA-SCID trials. The most likely answer is the difference in disease background[44].

SCID disease background

In **chapter 3** the stages of arrest in development were studied for different types of SCID including X-linked SCID and ADA-SCID. For X-linked SCID, caused by a mutation in *IL2RG*, a very early arrest just after seeding of the thymus was observed. It has been reported by Kohn *et al.* that in these patients there is no aberrancy in prethymic commitment and that effects of *IL2RG* deficiency will most likely be present in the thymus and not in the thymus seeding progenitor (TSP)[59]. For ADA-SCID no phenotype could be observed after transplantation in the NSG mouse model, most likely caused by complementation of murine ADA as this enzyme is also secreted. Therefore it remains elusive where the arrest in human T-cell development is in ADA-SCID patients. Joachims *et al.* have mimicked this by inhibition of ADA in human cells in FTOC and have observed a gradual decrease of populations during development most severely affecting the more mature populations[60]. However, the data obtained from transplantation of ADA-SCID derived HSPCs in NSG mice does demonstrate that in a gene therapy setting the transduced cells might have a bystander effect of the non-transduced cells thereby decreasing the selective pressure. For X-linked SCID the block in development is very early; at a normally high proliferative stage. The cells that were transduced with the correct version of *IL2RG* will have a highly selective advantage, as the block is alleviated, and undergo rapid expansion. When LMO2 expression is not downregulated due to insertional mutagenesis, this will give additional proliferative advantage during T-cell development. It was indeed observed in **chapter 6** that overexpression of *LMO2* in HSPCs, which were transplanted in NSG mice, gives rise to accelerated human T-cell development leading to higher frequencies of T cells in peripheral lymphoid organs together with an altered CD8/CD4 ratio. In addition, a delay in development was observed in some but not all mice confirming previous data obtained from *in vitro* studies[57]. Together with an accumulation of $CD3^-$ DP cells this study indicates three different mechanisms by which *LMO2* overexpression affects human T-cell development.

WAS patients do have low T-cell numbers in their peripheral blood, which have reduced functionality, and it is thought that this decrease is caused by diminished thymic output[61]. As WAS protein (WASP) deficiency affects migrational properties[62], it might be that the arrest in T-cell development is leaky and caused by the lowered capacity to migrate through the thymus during development. It would be of interest to determine the stage of arrest in human T-cell development for WAS patients. Knowledge on the stage of arrest might provide more insights into the mechanisms of ectopic expression of LMO2 on T-ALL development in these patients and whether this is comparable to the mechanisms in X-linked SCID.

In a gene therapy trial for X-linked chronic granulomatous disease (X-CGD), using a gammaretroviral vector driving expression of the transgene by the LTR, insertional mutagenesis in *EVI1* resulted in the development of myelodysplasia. X-CGD can be caused by a mutation in *GP91phox* that affects the phagocytic capacity of neutrophils. These cells belong to the myeloid lineage and therefore myeloid cells that are transduced with the correct version of *GP91phox* will most probably have a selective advantage over their non-transduced counterparts. In WAS, all lineages are affected and in the above described gene therapy trials for WAS patients did develop both T-ALL and acute myeloid leukemia (AML) due to insertional mutagenesis. Leukemia of B cells and/or NK cells has not been observed in the gene therapy trial for WAS. Also in the X-linked SCID trials, in which beside the T cells also the NK cells are affected by IL2RG deficiency, leukemia in NK cells was not observed. It could be that the selective advantage for B cells and NK cells over untransduced cells is not as great as for T cells and myeloid cells whereby they experience less selective pressure leading to lower changes for development of clonal outgrowth of B cells and NK cells.

Assessment of genotoxicity of preclinical viral vectors

As leukemia development by insertional mutagenesis was not observed in preclinical models, new screening methods for genotoxicity have been developed. The *in vitro* immortalization (IVIM) assay screens for clonal outgrowth of transduced lineage-negative murine BM cells using a limiting dilution assay[63]. Using this assay, different designs of vectors can be screened in a preclinical stage to determine their genotoxicity in a relatively short period. However, it is a myeloid skewed assay and thereby it is unknown how accurate the predictions will be for development of T-ALL due to the stringent selective pressure during T-cell development, which might be different from the limiting dilution used in this assay. A different assay makes use of lineage-negative BM cells of tumor-prone *Cdkn2a*$^{-/-}$ mice that are then transduced with different designs of viral vectors and transplanted in wildtype mice[64]. The advantage of this model is the *in vivo* readout by tumor development, although the selective advantage over uncorrected cells, as present in a gene therapy setting, is not there. Both assays make use of murine BM-derived cells, but as indicated, differences exist between human and murine lymphoid development. In **chapter 6** it was demonstrated that the humanized NSG model can be used to study the effects of LMO2 overexpression in human cells. The same model might be used to study insertional mutagenesis in human cells. Experiments to assess genotoxicity of viral vectors in NSG mice have been performed by transplanting $CD34^+$ cells that were transduced by an LTR-driven gammaretroviral vector in these mice[65]. Presence of dominant clones was observed in transplanted NSG mice, however, no tumor development was observed as was in the *Cdkn2a*$^{-/-}$ mouse model. It was speculated that longer duration (more than 6 months) of experiments would be needed or secondary transplantations to observe

insertional mutagenesis caused leukemias. In conclusion, there is currently no accurate model to determine genotoxicity of integrating viral vectors in human cells in a preclinical setting that will be predictive for the clinical setting, at least for lymphoid leukemias.

Up to now, insertional mutagenesis in gene therapy trials has only been observed when gammaretroviral constructs driving expression of the transgene from the viral LTR were used. During preclinical testing in mouse models insertional mutagenesis leading to development of T-ALL has not been observed. As described in **chapter 1**, clinical trials for primary immunodeficiencies are currently based on using selfinactivating (SIN) vectors that drive expression of the transgene from in internal promoter thereby decreasing enhancer activities on nearby genes. Recently, alpharetroviral vectors have been demonstrated to be successful in correcting X-linked chronic granulatomous disease (CGD) in preclinical models[66]. These vectors have a more safe integration profile than both gammaretroviral and lentiviral vectors, which lowers the chance of insertional mutagenesis[67]. However, viral vectors need integration in the host genome for stable expression and this remains random whereby the chance of insertional mutagenesis will always remain.

Targeted strategies for gene correction

Correcting the genetic defect itself instead of expressing a correct version of the affected gene would allow for physiological expression and normal regulation of the gene without the need for random integration of a viral vector. This strategy is being tested by many different labs using different technologies. Genovese *et al.*, have used zinc finger nucleases (ZFNs), which generate a site-specific double strand break (DSB) in the DNA together with an integrase-defective lentiviral vector (IDLV) encoding a gene targeting construct for *IL2RG*[68]. Hereby, they corrected the IL2RG mutation in HSCs from an X-linked SCID patient and after transplantation in NSG mice there was restored development of both T cells and NK cells. Another approach makes use of patient-derived induced pluripotent stem cells (iPSCs) combined with transcription activator-like effector nucleases (TALENs) to correct the mutation in *IL2RG*[69]. *In vitro* it was shown that corrected cells could differentiate into both T cells and NK cells while the uncorrected cells could not. The advantage of these types of approaches is the site-specific correction thereby circumventing the chance for insertional mutagenesis. However, the number of cells that can be specifically targeted by these strategies at this moment remains low as compared to viral transduction, which is used currently in gene therapy clinical trials. Another repair approach utilizes the CRISPR/Cas9 system in which guide RNAs target the Cas9 endonucleases to the gene of interest, which is mutated, to generate DSBs[70]. When the correct version of the gene is introduced as well, this can be used as template to repair the DSB by homologous recombination thereby correcting the mutation. This has not been used for SCID yet, but proof of principle has been obtained by correcting a mutation in *CFTR*, which can cause cystic fibrosis, in organoid cultures and thereby restoring functionality[71]. The CRISPR/Cas9 system is easier to adapt for different targets, by changing the sequence of the guide RNA, and results in comparable or higher targeting efficiencies than ZFNs or TALENs and therefore is a promising

tool for future site-specific gene therapy[72]. However, to obtain enough cells for transplantation into patients after site-specific gene correction, even higher targeting efficiencies or better stem cell expansion protocols will be needed.

Future perspectives

Knowledge on human T-cell development lags behind that of murine T-cell development. Using humanized mouse models, the role of genes on human T-cell development can now be studied in an *in vivo* setting by transplantation of human null mutants and other patient samples, which will provide new insights. As SCID is a rare disease, the availability of human SCID derived HSPCs is limited. Potentially, this could be circumvented by exploiting the CRISPR/Cas9 system to induce DSBs in genes of interest in cord blood-derived $CD34^+$ cells. Repair of these DSBs can occur via non-homologous end-joining (NHEJ), leaving insertion/deletion (indel) mutations that can lead to frameshift mutations and premature stopcodons[73]. Introduction of the targeting sequence together with Cas9 and a reporter gene can be performed by lentiviral transduction[74]. In addition, the function of genes that are known to be important in murine T-cell development and of which the role in human T-cell development is unknown, such as *TCF7* deficiency[75], could be tested using this system. Screening for knockouts is easy when the targeted gene normally results in a protein expressed on the cell surface. These HSPCs could then be sorted after transduction and transplanted in immunodeficient mice. The most preferable model could be the NSGW41; these mice do not need to be irradiated, which could otherwise also affect transplanted HSPCs. As described, without irradiation a better estimation of repopulation capacity can be made. When the targeted gene does not result in a surface-expressed protein, clones would have to be grown to be screened on DNA level. Currently, there are no protocols available that would allow for this expansion while maintaining repopulating capacity of HSPCs.

Furthermore, the above described NSGW41 will be very useful for better characterization of the "true" long-term repopulating (LT)-HSC. The $Lin^-CD34^+CD38^-CD90^+CD45RA^-CD49f^+$ have a LT-HSC frequency of 1 in 10.5 cells[8]. In mice the expression of c-Kit is used for characterization of the HSC compartment. Currently, there is also indication that expression of this receptor on human HSC would confer more potent repopulation activity[15]. Another marker used for isolation of more primitive HSPCs is CD133 of which the expression is, in contrast to CD38, less influenced during culture[76]. Sorting of different fractions of the hematopoietic progenitor compartment and transplantation into non-irradiated NSGW41 mice might help in the search for the "true" LT-HSC and more differentiated progenitors and their developmental potential, which might result in a more defined human hematopoietic developmental hierarchy. As transplantation of single cells remains challenging, barcoding technology[77] (and **chapter 5**) could be used to determine whether progenitors give multi-lineage output or are more restricted. More insights towards the phenotype of human multipotent progenitors and their lineage offspring would be of great interest as also here differences remain between mice and man[78]. As described above, Dick and coworkers have performed many studies on the identification of the human LT-HSC and their multipotent offspring. As the T-cell potential was not studied in their *in vivo* experiments, it remains difficult to determine presence of common progenitors for different lymphoid lineages and when this potential diverges.

An impracticality of the existing data on human T-cell development is the different antibodies that have been used by different labs when studying human T-cell development. Combining these antibody panels would allow for integration and better comparison of existing datasets. However, antibody panels for flow cytometry are limited to a maximum of 17 antibodies per panel[79]. Therefore, integration of data would only be able if multiple panels would be designed, which will need to have substantial overlap to be able to compare data from different tubes. Recently, a new technology has been developed combining flow cytometry with mass spectrometry, which is called cytometry by time-of-flight (CyTOF)[80, 81]. Instead of labelling antibodies with a fluorochrome, they are labelled with rare earth metal isotopes. As there is no spectral overlap, compensation of signals is not needed and currently more than 40 parameters can be measured simultaneously which in the future might extend up to 100 parameters[82]. This technique would allow for the inclusion of most of the markers used by different research groups, in order to generate a comprehensive overview of human T-cell development. Especially, more insight in the DN compartment would be very useful to better stage arrests in development for different types of SCID and to better define ETP-ALL, a type of T-ALL of very immature phenotype with poor prognosis[83]. Potential new populations of progenitors could then be sorted with their defining markers using regular flow cytometry and their developmental potential could be studied using the OP9-DL1 coculture system[84]. Furthermore, these sorted populations could be used for expression analysis and analysis of rearrangement of TCR loci, as has been performed previously for the known populations of human T-cell development[17]. Together this might provide more insights into signaling networks regulating TCR rearrangements and may provide targets for treatment of ETP-ALL. Insights in the earliest progenitor within the human thymus might provide insights towards the phenotype of the elusive human thymus seeding progenitor, for which many phenotypes have been proposed so far[85-88].

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