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Chapter 3

Identification of checkpoints in human T-cell development using severe combined immunodeficiency stem cells


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Abstract

Background: Severe combined immunodeficiency (SCID) represents congenital disorders characterized by a deficiency of T cells due to arrested development in the thymus. Yet, the nature of these developmental blocks has remained elusive due to the difficulty of taking thymic biopsies from affected children.

Objective: Identify the stages of developmental arrest in human T-cell development caused by various major types of SCID.

Methods: Transplantation of SCID CD34+ bone marrow stem/progenitor cells into an optimized NSG xenograft mouse model, followed by detailed phenotypic and molecular characterization using flow cytometry, Ig and TCR spectratyping and deep sequencing of IGH and TRD loci.

Results: Arrests in T-cell development caused by mutations in IL2RG and IL7RA were observed at the most immature thymocytes, much earlier than expected based on gene expression profiling of human thymocyte subsets and studies with corresponding mouse mutants. TCR rearrangements were functionally required at the CD4 CD8 CD7 CD5 stage, given the developmental block and extent of rearrangements in mice transplanted with Artemis-SCID. The xenograft model used is not informative for ADA-SCID, while hypomorphic mutations lead to less severe arrests in development.

Conclusion: Transplanting CD34+ stem cells from SCID patients into a xenograft mouse model provides previously unattainable insight into human T-cell development and functionally identifies the arrests in thymic development caused by several SCID mutations.

Key messages:
- The arrests in thymic development have been unknown for most types of human SCID.
- Using a xenograft model, IL2RG- and IL7RA-SCID showed very early arrests in the thymus, followed by recombination defective SCID (Artemis), all blocking in the CD4 CD8 DN stages.
- This approach can be used for other types of SCID and help in unraveling unknown SCID cases.

Capsule summary: The nature of the blocks in T-cell development for SCID has remained elusive. Using a xenograft model, we show that different SCID-causing mutations lead to severe defects at different early developmental stages in the thymus.

Key Words: SCID, thymus, NSG, IL2Rg, IL7Ra, ADA, Artemis, T-cell development, B-cell development

Abbreviations:
ADA; adenosine deaminase, BM; bone marrow, DN; double negative, FTOC; fetal thymic organ culture, HSC; hematopoietic stem cells, HSPCs; hematopoietic stem and progenitor cells, Ig; immunoglobulin, IGH; immunoglobulin heavy chain, IL2RG; interleukin-2 receptor gamma, IL7Ra; interleukin-7 receptor alpha, ISP; immature single positive, NK cell; natural killer cell, NSG; NOD-Scid-Il2rg+ mice, PB; peripheral blood, SCID; severe combined immunodeficiency, TCR; T-cell receptor, UCB; umbilical cord blood

Introduction

Severe combined immunodeficiency (SCID) comprises a series of congenital disorders characterized by a deficiency of (functional) T cells. This can be accompanied by deficiencies in either NK or B cells or both. These different phenotypes are caused by mutations in several genes. It remains unknown where the blocks in human T-cell development reside for these different deficiencies, except for mutations in CD3ζ and ZAP70. For the three major types of human SCID that represent >80% of known SCID mutations, the stage of arrest in T-cell development is unknown. These three types are: 1, receptor signaling defects reflecting for instance mutations in IL2RG, IL7RA and JAK3, 2, recombination defects (Artemis (DCLRE1C), RAG1, RAG2, LIG4, XLF, DNA-PKcs) and 3, metabolic enzyme deficiencies (ADA, PNP).

One approach to obtain insight into human T-cell development is the careful analysis of gene expression and T-cell receptor (TCR) rearrangement of ex vivo isolated developmental subsets. While such studies have shown that murine and human T-cell development are overall very comparable, important differences in for instance the early double negative (DN) compartment and the thymus seeding progenitor have been described.

Mice with targeted mutations in key genes, including SCID-causing genes, have provided important insights in T-cell development. However, not all of these mice resemble the phenotype of SCID patients. For instance, IL7Rβ mice have a deficiency in both B and T cells, while in humans only T cells are affected. Whether genetic mouse models for SCID demonstrate comparable blocks in T-cell development to humans remains largely unanswered. Furthermore, gene expression analysis of known SCID genes in different stages of human T-cell development showed that there are differences in expression as compared to blocks observed in mice. Recent advances in xenotransplantation models now allow for better definition of human T-cell development in vivo. Importantly, these models can also be used for transplantation of human stem cells isolated from cryopreserved bone marrow (BM) samples stored in bio-banks, e.g. from SCID patients.

Using transplantation of CD34+ cells isolated from patients with proven functional null mutations in genes characteristic for each of the three major types of SCID, we aimed to study the functional roles of these genes for human T-cell development in vivo. This study provides for the first time a description of the arrests in T-cell development in SCID patients and functional insight into two important developmental checkpoints in the human thymus.
Chapter 3 Functional identification of checkpoints in human T-cell development

Methods

Isolation of human CD34+ cells

Human BM was obtained from healthy pediatric donors at the Leiden University Medical Center (LUMC, Leiden, The Netherlands). Informed consent was obtained from the parents for the use of leftover samples for research purposes. Parents / guardians and donors have consented to the donation procedure, after psychological testing and approval of the Youth Court in case of children. If the genetic research studies show any abnormalities, the parents had the opportunity to be informed. The LUMC medical ethical board (IRB) has approved the use of left over material of normal marrow for research purposes in P01.028 and P08.001. BM samples from children diagnosed with SCID were obtained according to the guidelines of the Erasmus MC. The medical ethical committees of Leiden University Medical Center and of ErasmusMC approved this study and both served as institutional review boards. Informed consent was provided according to the Declaration of Helsinki. Cells were isolated and cultivated as described previously. See the Methods section in the Online Repository for further details.

Mice

NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice were obtained from Charles River Laboratories (UK) and bred in the animal facility at the LUMC. Experimental procedures were approved by the Ethical Committee on Animal Experiments of the LUMC.

In vivo experiments were performed as described. Female mice were transplanted with CD34<sup>+</sup> cells by intravenous injection within 24 hours after irradiation with 1.91 Gy using orthovoltage X-rays.

Flow cytometry

Labelling of mononuclear cells has been described. Data were acquired on a Canto II (BD Biosciences) and analyzed using FlowJo software (Treestar, Ashland, OR, USA). Flow cytometric analysis of different stages of human B-cell development was performed as described elsewhere.

Repertoire analysis

DNA was isolated from single cell suspensions made from total thymus, spleen and BM of transplanted NSG mice using the GeneElute<sup>TM</sup> Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich, St. Louis, MO, USA). Rearrangements were analyzed using the EuroClonality/BIOMED-2 multiplex PCR protocol. Amplification of IGH rearrangements was performed as previously described. PCR products were sequenced on the Illumina MiSeq sequencer using 300 base pairs paired-end sequencing (V3 chemistry). The sequence reads containing the IGH rearrangements were uploaded in IMGT HighV-Quest. Subsequently the output files were analyzed using the IG Galaxy tool. The TRDD2-TRDD3 rearrangements were analyzed by matching the constant genomic region outside the TRDD2 and TRDD3 sequence in the amplicons, after which the length of the TRDD2-TRDD3 regions was determined. Analysis was performed using Bioperl and R.

Statistics

The Wilcoxon rank-sum test was performed when possible. P < 0.05 was considered significant and indicated in figures with *.

Results

NSG mice transplanted with HSPCs from control samples display low degree of variation

Recently, we have shown that NOD-Scid-Ig<sup>−</sup> (NSG) mice engrafted with hematopoietic stem and progenitor cells (HSPCs) isolated from umbilical cord blood and, to a lesser degree, from human BM allow robust development of myeloid and lymphoid cells, with functional B cells and T cells. First, we have transplanted NSG mice with HSPCs obtained either from umbilical cord blood (UCB) or human pediatric BM to determine the presence of different stages of human T cell development. We have transplanted 14 mice with CD34<sup>+</sup> cells isolated from cord blood from different donors and 9 mice with CD34<sup>+</sup> cells isolated from pediatric bone marrow, with cell doses ranging from 25,000 to 200,000 total cells per mouse. All stages were present in NSG mice transplanted with HSPCs obtained from UCB as well as human BM (Fig. 1A). Different DN subsets could be discriminated based on the expression of CD7 and CD5 within the CD4 CD8 DN compartment (Fig. 1B). Moreover, the relative presence of all subsets is highly comparable and showed a low degree of variation (Fig. 1A and B). Together these data demonstrate the reproducible presence of the different stages of human T-cell development. Therefore, the high robustness of the model allows us to study the effect of rare mutations in SCID on human T-cell development. In all the experiments performed hereafter, only BM-derived HSPCs and not UCB-derived HSPCs were used for transplantations as all SCID HSPCs used in this study were also BM-derived and some differences could be observed between mice engrafted with UCB-derived HSPCs compared to BM-derived HSPCs.

Figure 1: Low variation among stages of human T cell development in NSG mice transplanted with HSPCs from control umbilical cordblood and control human BM. A, B) Percentage of cells per different stage of human T cell development within thymi of NSG mice transplanted with HSPCs. A) Cells were gated within hCD45<sup>+</sup> cells or B) within hCD45<sup>+</sup>CD4<sup>+</sup>CDB (DN). Depicted is the mean and standard deviation (sd), * p < 0.05.
Chapter 3

Functional identification of checkpoints in human T-cell development

SCID humanized mice display SCID-specific phenotypes

We have engrafted NSG mice with HSPCs isolated from BM of SCID patients with mutations in different genes, including ADA-SCID, Artemis-SCID, IL7RA-SCID and IL2RG-SCID (Table 1). No T cells were detected in peripheral blood (PB) when Artemis-, IL7RA- or IL2RG-SCID HSPCs were transplanted (Fig. 2A), as expected based on the disease-defining absence of T cells. However, for ADA-SCID T, B and NK cells were readily observed (Fig. 2A-C), likely caused by complementation by secreted murine ADA, comparable to the recovery seen in ADA-SCID patients treated with PEG-ADA enzyme replacement therapy. Therefore, we focused our further study on T- and B-cell development in Artemis-, IL7RA- and IL2RG-SCID compared to controls. The results from HSPCs transplanted into NSG mice copied the phenotype found in different types of SCID patients; for Artemis-SCID we observed a deficiency of T and B cells, for IL7RA-SCID an absence of T cells, and for IL2RG-SCID a deficiency of both T and NK cells (Fig. 2A-C, Table 1). In mice transplanted with IL7RA-SCID derived HSPCs the number of NK cells is somewhat lower as compared to controls. Unfortunately clinical data on NK cells in the IL7RA-SCID patient were not available and therefore it is hard to draw conclusions from this observation, but the transplantation data are consistent with a T⁻B⁻NK⁺ phenotype. Human myeloid cells developed in all mice, although in a higher percentage and number in mice transplanted with Artemis-SCID HSPCs (Fig. 2D). Besides myeloid cells, also NK cells were present both in higher percentages and numbers, probably due to the severe T- and B-cell deficiency observed with Artemis-SCID resulting in increased development of other cell lineages. B cells were present in higher numbers in mice transplanted with ADA-SCID derived HSPCs (Fig. 2B). Since the model used is B cell-prone, this most likely reflects the higher engraftment compared to controls. The same phenotypes as observed in peripheral blood of transplanted mice were observed in the spleen and BM (Fig. 2E and F). Some CD3 expression was observed within the spleens of NSG mice transplanted with Artemis-, IL7RA- and IL2RG-SCID, however, these cells did not express TCR, CD4 or CD8 on their surface (data not shown) and likely represent staining artefacts.

Table I: Characteristics of SCID patients

<table>
<thead>
<tr>
<th>SCID</th>
<th>Mutation</th>
<th>T cells (x10⁹/L)</th>
<th>B cells (x10⁹/L)</th>
<th>NK cells (x10⁹/L)</th>
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</thead>
<tbody>
<tr>
<td>ADA</td>
<td>c.736C&gt;T, p.Gln246Stp</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Artemis</td>
<td>c.220C&gt;T, p.Arg74Stp</td>
<td>0.4⁺</td>
<td>&lt;0.01</td>
<td>0.5</td>
</tr>
<tr>
<td>IL7RA</td>
<td>c.876+1G&gt;A / c.898_902delCCTGA, p.Pro300LysfsX9</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>IL2RG</td>
<td>c.209T&gt;G, p.Met70Arg</td>
<td>8.2⁻</td>
<td>4</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>hypomorphic</td>
<td>3.4</td>
<td>1.4</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Counts of cells from different lineages from peripheral blood of patients as determined by flow cytometry and their mutation as determined by PCR. + all T cells were determined to be of maternal origin by their memory phenotype, na = not available, ⁺ all T cells were of maternal origin (proven with STR analysis on sorted cells).

Figure 2: NSG mice transplanted with SCID HSPCs show same phenotype as patients in their peripheral blood. A-D) Cells present in PB in percentage of human CD45⁺ cells over time after transplantation (left panel) or in absolute numbers at the end of the experiment (right panel); A) T cells, B) B cells, C) NK cells and D) myeloid cells. E) Absolute numbers of different cell types within spleen or F) BM. Depicted is the mean and standard deviation (sd), * p<0.05.
Artemis-SCID demonstrates a block in B-cell development before the cytoplasmic Ig\(\mu\) pre-B-II stage

To further validate the NSG xenograft model, we first investigated B cell development in NSG mice transplanted with CD34\(^+\) SCID cells and compared this to the ex vivo composition of the B-cell precursor compartment in patients. In BM of NSG mice transplanted with Artemis-SCID derived HSPCs, we observed a decrease in percentage and number of CD19\(^+\) B cells when compared to BM from control mice (Fig. 3A and B). This was not observed for the other types of SCID, which were highly comparable to mice transplanted with HSPCs obtained from healthy pediatric donors. Different stages of B-cell development were determined within the B-cell compartment\(^{20}\). B-cell precursors derived from Artemis-SCID HSPCs blocked at the CD10\(^+\) stage of B-cell development in the BM (Fig. 3C). Furthermore, almost no surface expression of IgM was detected and the percentage of B-cell precursors that expressed IgM in the cytoplasm was decreased more than 18-fold in NSG mice transplanted with Artemis HSPCs when compared to controls (Fig. 3D). There was barely any expression of cytoplasmic Ig\(\mu\) present in the B-cell precursors, therefore precursor B-cell differentiation blocks before the cytoplasmic Ig\(\mu\) pre-B-II stage. This block in B-cell development was identical to the phenotype observed in a BM aspirate of the Artemis-SCID patient (Fig. 3E). The block at the pre-B-II stage in Artemis-SCID leads to an absence of immature and mature B cells expressing IgM or IgD (Fig. 3F). We also observed a much lower percentage of B cells in spleens of NSG mice transplanted with Artemis-SCID HSPC and no mature CD20\(^+\), IgM\(^+\) or IgD\(^+\) B cells were found (Fig. 3G).

Together these data confirm a block in B-cell development for Artemis-SCID, and not for IL7RA-SCID and IL2RG-SCID. This is highly similar to the observations in the BM aspirates of the corresponding SCID patients, demonstrating that HSPC transplantation into the NSG model faithfully recapitulates human B-cell development, thereby validating the use of this model for studies of human lymphoid development.

**Artemis-SCID cells do not generate a polyclonal IGH repertoire**

We performed analysis of the immunoglobulin heavy chain (IGH) repertoire that was generated in the BM of transplanted NSG mice. Both for incomplete D-J (Fig. 4A) and complete V-D-J (Fig. 4B) rearrangements, merely a few incidental rearrangements were observed for Artemis-SCID. For IL7RA- and IL2RG-SCID a polyclonal repertoire, comparable to that observed in controls, was generated (Fig. 4A and B). To determine the repertoire diversity of IGH, we performed deep sequencing using the Illumina Miseq platform. Analysis of D to J recombination showed that a less diverse repertoire was generated in the BM of mice transplanted with Artemis-SCID derived HSPCs while this was not observed for IL7RA- and IL2RG-SCID and controls (Fig. 4C). Thus, only for a recombination defect, as exemplified by Artemis-SCID, both phenotypic and IGH rearrangement analysis indicate an arrest in B-cell development at the pre-B-II stage, which in addition led to development of a less diverse repertoire.

**Figure 3: Developmental block at the CD10\(^+\) stage for B cells in Artemis-SCID.** A) Percentage and B) number of B cells in transplanted NSG mice BM. C, D) Maturational stages of B-cell development in BM of transplanted NSG mice. E) Comparison of B-cell precursors in ex vivo BM aspirates to BM of transplanted NSG mice. F) IgM and IgD expression on non-myeloid hCD45\(^+\) cells in BM and G) spleen of transplanted NSG mice.
Chapter 3

Functional identification of checkpoints in human T-cell development

Figure 4: No polyclonal IGH repertoire was generated in developing B cells from Artemis-SCID. A) D-J and B) V-DJ rearrangement of IGH locus in total BM cells of NSG mice transplanted with SCID or control HSPCs. C) Heatmap depicting the unique junctions of D to J segments for the IGH locus in total BM cells of NSG mice transplanted with SCID or control HSPCs. Colors depict the relative length of CDR3.

Novel early blocks in T-cell development for SCID

For the vast majority of SCID patients, the blocks in T-cell development are only known by extrapolation from mouse models. Here, we directly determined the arrests in T-cell development for different types of human SCID by investigating thymi of NSG mice engrafted with SCID HSPCs. To discriminate different populations within the DN compartment, we measured expression of CD7 and CD5 within this population. Based on the observed developmental arrests at the CD4-CD8- DN stage, all types of SCID block much earlier than observed in corresponding mouse mutants (Fig. 5A). T-cell development in IL2RG-SCID was inhibited at the CD7-CD5- stage followed by IL7RA-SCID where one-third of the cells reached the subsequent CD7+CD5- DN stage (Fig. 5B), although these blocks are somewhat comparable. For Artemis-SCID the block in T-cell development was found at the DN CD7+CD5+ stage without expression of CD1a (Fig. 5B and C). As expected from these early blocks in T-cell development, there was no expression of either TCRαβ or TCRγδ on total thymocytes in mice transplanted with SCID-derived HSPCs, but readily detectable in control mice (see Fig. E1 in the Online Repository). These data demonstrate the stages of arrest in T-cell development for different types of SCID and show an absolute requirement for common-gamma type cytokine signaling at the most immature thymic stages as also illustrated by the decrease in total hCD45+ cells within thymi of these mice (Fig. 5D). We observed a high percentage of CD19+ B cells in thymi from NSG mice transplanted with HSPCs from Artemis-SCID (Fig 5E). Also for IL7RA- and IL2RG-SCID an increase in the percentage of B cells in the thymus was observed, but not as much as for Artemis-SCID. The increase in B cells that was observed was only relative and not in absolute numbers (Fig. 5F).

Importantly, we also transplanted CD34+ cells from a SCID patient with a hypomorphic mutation in IL2RG. This patient had low numbers of peripheral T cells and NSG mice transplanted with HSPCs derived from this patient indeed showed a much milder block, with only a lower percentage of DP thymocytes (Fig 5C). This demonstrates that less severe mutations lead to a less pronounced arrest in development. In addition, T cells also were detected in the peripheral blood mice transplanted with hypomorphic mutant IL2RG HSPCs (data not shown).

TCR rearrangement starts very early after thymus entry

TCR rearrangement starts at the TRD locus. Since the observed blocks in T-cell development were very early, we focused TCR repertoire analysis on the TRD locus, of which D5-D3 is rearranged first. IL7RA+ and IL2RG-SCID T-cell progenitors only showed incidental products...
of this rearrangement in accordance with the early blocks observed using flow cytometry (Fig. 6A). Using spectratyping of T-cell receptor CDR3 length distribution, Artemis-SCID T-cell progenitors showed a broad repertoire for Dδ2-Dδ3 comparable to NSG mice transplanted with control HSPCs. These spectratyping data were confirmed using next generation sequencing of the TCR Dδ2-Dδ3 locus. Again, we observed a very limited number of reads for IL7RA- and IL2RG-SCID, while the normalized read numbers of Artemis-SCID were similar to those of mice transplanted with wild type cells (Fig. 6B). For the second step of TRD rearrangement, Dδ2-Jδ1, the repertoire was more oligoclonal in Artemis-SCID T-cell progenitors, while in IL7RA- and IL2RG-SCID such rearrangements were undetectable (Fig. 6C). However, none of the different types of SCID generated a broad Dδ-Jδ or even Vδ-Jδ repertoire (Fig. 6D). TRG is rearranged after TRD and for IL2RG-SCID no rearrangement could be observed of TRG while for IL7RA-SCID merely a clonal rearrangement was observed, confirming the later block in development of IL7RA-SCID as compared to IL2RG-SCID (Fig 6E). While Artemis-SCID T-cell progenitors were able to perform the first steps of TRD rearrangement, they did not rearrange TRG and TRB loci (Fig. 6E and F). For both loci only a few incidental peaks were detected in Artemis-SCID-T cell progenitors as compared to NSG mice transplanted with control HSPCs. Judging from these clonal peaks, rearrangement of TRG is already initiated at the DN CD7+ stage and TRB at the DN CD7+CD5- stage of T-cell development.

### Discussion

SCID is a rare disease caused by different genetic defects leading to T cell deficiency that can be accompanied by deficiencies in B and/or NK cells. Here we demonstrate for the first time the arrests in T-cell development for the major types of human SCID using an in vivo model; data which typically is not available because thymic biopsies are almost never obtained. Our approach allows for comprehensive loss-of-function insight for two important developmental checkpoints; common-gamma chain cytokine signaling and the initiation of TCR rearrangements.

The arrests in T-cell development observed for the different types of SCID demonstrate a hierarchy in TCR rearrangement and the corresponding phenotypes. The earliest block in T-cell development is observed in IL2RG-SCID, followed by IL7RA-SCID, demonstrating an almost immediate requirement for common-gamma chain cytokine signaling after seeding of the thymus.

Artemis is involved in opening of the hairpin sealed coding ends during recombination. Here, we observed that Artemis-SCID cells are able to initiate rearrangement of the TRD locus as demonstrated by polyclonal Dδ2-Dδ3 and oligoclonal Dδ2-Jδ1 rearrangements. However, no complete TRC was formed. Apparently, Artemis is not needed for the first steps of TRD rearrangement, as confirmed by next generation sequencing, but is needed for complete rearrangement of TRD. An explanation would be that in absence of Artemis, the hairpin sealed coding ends are opened by other enzymes. As this process is less efficient, the chance of successful two-step rearrangement is low and only partial rearrangements would be formed, as observed here.

We here demonstrate that rearrangement of TRG is initiated at the DN CD7+ stage and TRB loci is already initiated at the DN CD7+CD5- stage of T-cell development, in contrast to previous models where TRB rearrangement was proposed at the immature single positive (ISP) or DN3 stage of development. As these DN CD7+CD5- cells were devoid of CD8α expression that marks human DN3 cells, this stage is reminiscent of the DN2 compartment in the mouse. While in the mouse rearrangement of TRB starts in the DN3 stage, in the human thymus this process seems to start earlier based on our data. In mice, mutations in Il2rg lead to reduced thymic cellularity but are permissive for thymocyte development, whereas here we observed an absolute and very early requirement for IL2RG signaling. The blocks in IL7RA-SCID and IL2RG-SCID are not completely identical, suggesting that cytokines other than IL-7 may also signal through the common-gamma chain in the early stages after thymus seeding. Cytokines that might be involved in proliferation and development in this very immature stage could be IL-2, IL-4 or IL-15. IL-15 is an important NK cell growth factor and in IL7RA deficiency this cytokine can signal normally via the IL2RG-IL15RA complex. Thus some of the CD7+CD5- DN cells observed may contain mature NK cells, rather than solely uncommitted thymocytes with T/NK cell potential. The increased percentage of B cells observed in thymus of NSG mice transplanted with Artemis-SCID HSPCs is in line with data from the mouse where very early thymic blocks also lead to increased percentages of B cells, which developed from DN...
Chapter 3 Functional identification of checkpoints in human T-cell development

Thymocytes underlying the developmental plasticity of these cells that can still develop in non-T-lineage cells in mice and men

The point at which in IL2RG deficiency blocks development has sometimes been suggested to occur before thymic seeding. Our data combined with data by Kohn et al., who showed no differences in hematopoietic stem cells (HSC) and pre-thymic progenitors of IL2RG-SCID patients indicates that this is not the case. Rather, very soon after thymic seeding, there is an absolute requirement for gc signaling (see Fig 5). Since Six et al. showed that human thymic progenitors (in contrast to mouse) express IL7Ra and IL2Rg, expression and functional requirement for gc are independently regulated.

To the best of our knowledge, only one paper has addressed the effect of human IL2RG deficiency in T cells before. Using an in vitro OP9-DL1 system, an arrest in development was shown at the CD7+CD5+CD4a stage. As no data are available on the nature of the IL2RG-SCID patient described by Six et al., it is impossible to say if the observed differences are based on differences between an in vitro model system versus in vivo transplantation data or reflect inherent differences based on different mutations with different residual signaling properties. Furthermore, the OP9-DL1 system can only address development until the earliest DP stages and is very sensitive to subtle differences in cytokines and labile contents of culture media. Development proceeds a bit further in fetal thymic organ cultures (FTOC), but fully matured T cells and thymic egress do not occur, limiting the development of human thymocytes. In the NSG model as we describe it, the thymic phenotypes are highly similar to those obtained from human thymus, underscoring the validity of this model to identify the thymic defects in human SCID patients. Another approach to model T-cell developmental blocks in SCID patients, is by measuring rearrangement of TCR loci and TREC levels in peripheral blood T cells. However, many SCID patients do not have peripheral T cells to perform these analyses on and no phenotype of thymocytes is available. The low level of TRD D2-D3 in another IL2RG-SCID patient corroborates our data described here for thymocytes, demonstrating a very early block in T-cell development.

We have chosen SCID patients with mutations that are deleterious for function as this would lead to functional null mutants and included a hypomorphic patient, who had clinically a mild SCID phenotype that was only found because of an affected cousin. This mutation indeed showed a much less severe phenotype and no clear developmental block. This helps explain the divergent findings with IL2RG-SCID thus far and, importantly, validates our approach for genotype-phenotype studies with SCID mutations.

In summary, combining the flow cytometric and molecular data obtained from the engrafted NSG thymi leads to a proposed model for the development of human thymocytes (Fig. 7), in which there is a prominent role for cytokine signaling and initiation of TCR rearrangement marks the presumed b-selection point, as Artemis deficient cells block at this stage. It was shown previously that, indeed, pTa (PTCRA) is abundantly expressed at this point in development.

In a xenograft model, it could be argued that the defects observed are not cell-intrinsic but result from incompatibilities between mouse microenvironment and human hematopoietic cells. However, in the NSG model, human T cells develop with all the developmental stages known from ex vivo removed human thymus. In addition, Parietti et al. were able to reproduce the development of human pro-thymocytes in the NSG xenograft model and it was demonstrated that human thymocytes can respond to murine MHC signals and have comparable migration on murine and human thymic slices. Furthermore, for B cells the xenogeneic environment shows a block identical to the block in development directly found in the Artemis-SCID patient and B-cell development in controls was highly comparable to normal human BM. Taken together, this validates the use of xenografted mice for studies of human lymphoid development.

There is much attention for improving thymic output after allogeneic bone marrow transplantation; for acquired immune deficiencies, and during ageing. In particular, strategies targeting the earliest human thymocytes, such as administration of IL-7, growth hormone or TSH or sex hormone ablation. Our results suggest that IL-7 combined with other early acting factors, e.g. Wnt, may be promising to restore the early human thymocyte compartment which is expected to sustain thymopoiesis by providing a pool of DN progenitor cells.

In conclusion, we here report loss-of-function data on human T-cell development, demonstrating earlier blocks in T-cell development than proposed before. The obtained insights might give opportunities for treatment of ETP-ALL by interfering with common-gamma signaling which is active in these very early stages of T-cell development. It would be of great interest to study arrests in development for other types of human SCID in this xenograft model. Finally, the xenotransplantation method we here describe may prove useful in unravelling unknown types of human SCID for which the genetic defect has remained elusive and aid diagnosis in cases with blurred phenotypes, e.g. when maternal T cells are present.
Acknowledgements

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References


Online repository material

Methods

Cryopreservation of human bone marrow-derived mononuclear cells
Mononuclear cells were isolated using Ficoll gradient separation and washed once with Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Life Technologies) and 2.5% heat-inactivated FCS (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands). Cells were resuspended in 50% RPMI/50% FCS/10% DMSO and frozen in 1 ml aliquots with a maximum of 50*10^6 cells per cryovial. Cryovials were placed in a freezing container in -80°C and relocated to liquid nitrogen storage on the following day.

Isolation of human bone marrow-derived CD34+ cells
Cryopreserved vials were thawed in a 37°C waterbath after which the cell suspension was transferred to a 15 mL conical tube. Medium (IMDM supplemented with penicillin/streptomycin and 2.5% heat-inactivated FCS) was added dropwise to the cells. Labelling and isolation of CD34+ cells was performed according to the manufacturer's protocol (CD34 Microbead Kit, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cells were centrifuged and resuspended in 300 µL Running Buffer (PBS pH 7.4, 2mM EDTA, 0.5% w/v bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA). Add DNAse I (final concentration 5 U/mL, Sigma-Aldrich, St. Louis, MO, USA) together with 100 µL FcR blocking reagent and 100 µL anti-CD34 Microbeads. Cells were not counted before labelling as we never had more than 100*10^6 mononuclear cells and with counting we would lose cells. Cells were incubated for 30 minutes in the refrigerator while shaking every 10 minutes. Isolation of CD34+ cells was performed using 2 times an LS column (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Cells were eluted in 3 mL Running Buffer using the supplied plunger. Eluted cells were centrifuged and resuspended in 500 µL StemSpan serum-free expansion medium (StemSpan-SFEM, StemCell Technologies Inc., Vancouver, BC, Canada) in the presence of 10 ng/mL stem cell factor (SCF, a gift from Amgen, Thousand Oakes, CA, USA), 20 ng/mL recombinant human thrombopoietin (rhTPO, R&D Systems, Abingdon, UK), 20 ng/mL recombinant mouse insulin-like growth factor 2 (rhIGF-2, R&D Systems) and 10 ng/mL recombinant human fibroblast growth factor-acidic (rhFGF-1, Peprotech, Rocky Hill, NJ, USA). Cells were counted using a Bürker chamber and trypan blue. Cells concentration was adjusted to 1*10^6/mL. Cells were cultured overnight in tissue-culture treated 48-well plates (Corning Incorporated, Corning, NY, USA) with 500 µL cell suspension per well. Then, cells were harvested by resuspension and transferred to a 15 mL conical tube. The well was washed by resuspension with 500 µL IMDM without phenol red (Gibco, Life Technologies, Bleiswijk, The Netherlands), which was added to the harvested cells. Cells were counted and an aliquot was taken for flow cytometry analysis to determine purity of CD34+ cells.

Transplantation into NSG mice
Cells were centrifuged and resuspended in IMDM without phenol red for intravenous injection into the tail vein of sublethally irradiated female NSG mice (200 µL/mouse). Mice were irradiated with 1.91 Gy using orthovoltage X-rays one day before injection of the cells. Mice were transplanted within 24 hours after irradiation. Peripheral blood of transplanted mice was analysed every 4 weeks and mice were sacrificed 20-22 weeks after transplantation.

Table E1: Xenograft transplantation details

<table>
<thead>
<tr>
<th>SCID</th>
<th>Number of mice transplanted</th>
<th>Transplanted number of total cells/mouse</th>
<th>Transplanted number of CD34+ cells/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADA</td>
<td>3</td>
<td>200,000</td>
<td>197,540</td>
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<tr>
<td>Artemis</td>
<td>2</td>
<td>78,000</td>
<td>73,593</td>
</tr>
<tr>
<td>IL7RA</td>
<td>1</td>
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<td>19,518</td>
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<tr>
<td>IL2RG</td>
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<td>47,150</td>
<td>38,559</td>
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<tr>
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<td>50,000</td>
<td>45,035</td>
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<tr>
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<td>33,000</td>
<td>29,555</td>
</tr>
<tr>
<td>Healthy donor</td>
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<td>200,000</td>
<td>167,480</td>
</tr>
<tr>
<td>Healthy donor</td>
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<td>100,000</td>
<td>86,740</td>
</tr>
</tbody>
</table>

Indicated are the numbers of NSG mice in which the sample was transplanted and the total mononuclear cell number and number of CD34+ cells transplanted per mouse.

Figure E1: Absence of TCR expression on SCID thymocytes. A) Expression of TCRαβ and B) TCR γδ on total hCD45+ thymocytes from transplanted NSG mice.