

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

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

Objective: We sought to identify the stages of arrest in human T-cell development caused by various major types of SCID.

Methods: We performed 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, immunoglobulin and T-cell receptor spectratyping, and deep sequencing of immunoglobulin heavy chain (*IGH*) and T-cell receptor δ (*TRD*) loci.

Results: Arrests in T-cell development caused by mutations in IL-7 receptor α (*IL7RA*) and IL-2 receptor γ (*IL2RG*) 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. T-cell receptor 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 cells. The xenograft model used is not informative for adenosine deaminase-SCID, whereas hypomorphic mutations lead to less severe arrests in development.

Conclusion: Transplanting CD34⁺ stem cells from patients with SCID into a xenograft mouse model provides previously

unattainable insight into human T-cell development and functionally identifies the arrest in thymic development caused by several SCID mutations. (*J Allergy Clin Immunol* 2015;■■■:■■■-■■■.)

Key words: SCID, thymus, NSG, *IL2RG*, *IL7RA*, adenosine deaminase, Artemis, T-cell development, B-cell development

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 natural killer (NK) cells, B cells, or both. These different phenotypes are caused by mutations in several genes.¹ It remains unknown where the blocks in human T-cell development reside for these different deficiencies, except for mutations in *CD3D*^{2,3} and ζ -associated protein of 70 kDa (*ZAP70*).⁴ For the 3 major types of human SCID that represent more than 80% of known SCID mutations, the stage of arrest in T-cell development is unknown. These 3 types are (1) receptor signaling defects reflecting, for instance, mutations in IL-2 receptor γ (*IL2RG*), IL-7 receptor α (*IL7RA*), and Janus kinase 3 (*JAK3*); (2) recombination defects (Artemis [*DCLRE1C*], recombination-activating gene 1 [*RAG1*], *RAG2*, DNA Ligase IV [*LIG4*], *XLF*, and *DNA-PKcs*), and (3) metabolic enzyme deficiencies (adenosine deaminase [*ADA*] and purine nucleoside phosphorylase [*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.⁵ Although 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.⁵⁻⁹

Mice with targeted mutations in key genes, including SCID-causing genes, have provided important insights into T-cell development.¹⁰⁻¹⁶ However, not all of these mice resemble the phenotype of patients with SCID. For instance, *Il7r*^{-/-} mice have a deficiency in both B and T cells,¹² whereas in human subjects only T cells are affected.¹⁷ Whether genetic mouse models for SCID demonstrate comparable blocks in T-cell development to human subjects 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 compared with blocks observed in mice.⁵ Recent advances in xenotransplantation models¹⁸ 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 biobanks (eg, from patients with SCID).¹⁹

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Abbreviations used

ADA:	Adenosine deaminase
BM:	Bone marrow
DN:	Double negative
HSPC:	Hematopoietic stem and progenitor cell
IGH:	Immunoglobulin heavy chain
IL2RG:	IL-2 receptor γ
IL7RA:	IL-7 receptor α
IMDM:	Iscove modified Dulbecco medium
LUMC:	Leiden University Medical Center
NK:	Natural killer
NSG:	NOD- <i>Scid</i> - <i>Il2rg</i> ^{-/-}
SCID:	Severe combined immunodeficiency
TCR:	T-cell receptor
TRD:	T-cell receptor δ
UCB:	Umbilical cord blood

Using transplantation of CD34⁺ cells isolated from patients with proved functional null mutations in genes characteristic for each of the 3 major types of SCID, we aimed to study the functional roles of these genes for human T-cell development *in vivo*. For the first time, this study provides a description of the arrests in T-cell development seen in patients with SCID and functional insight into 2 important developmental checkpoints in the human thymus.

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 parents for the use of leftover samples for research purposes. Parents/guardians and donors consented to the donation procedure after psychological testing and approval of the Youth Court in case of children. If the genetic research studies showed any abnormalities, parents had the opportunity to be informed. The LUMC medical ethical board (institutional review board) approved the use of leftover material of normal marrow for research purposes in P01.028 and P08.001. BM samples from children with a diagnosis of SCID were obtained according to the guidelines of the Erasmus MC. The medical ethics committees of Leiden University Medical Center and Erasmus MC approved this study, and both served as institutional review boards. Informed consent was provided according to the Declaration of Helsinki. Cells were isolated and cultured, as described previously.¹⁹ See the [Methods](#) section in this article's Online Repository at www.jacionline.org for further details.

Mice

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice were obtained from Charles River Laboratories (Kent, United Kingdom) 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 previously described.¹⁹ Female mice were transplanted with CD34⁺ cells by means of intravenous injection within 24 hours after irradiation with 1.91 Gy using orthovoltage x-rays.

Flow cytometry

Labeling of mononuclear cells was done as described previously.¹⁹ Data were acquired on a FACSCanto II (BD Biosciences, San Jose, Calif) and analyzed with FlowJo software (Treestar, Ashland, Ore). Flow cytometric analysis of different stages of human B-cell development was performed, as described elsewhere.^{20,21}

Repertoire analysis

DNA was isolated from single-cell suspensions made from total thymus, spleen, and BM of transplanted NSG mice by using the GeneElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, Mo). Rearrangements were analyzed by using the EuroClonality/BIOMED-2 multiplex PCR protocol.²² Amplification of immunoglobulin heavy chain (IGH) rearrangements was performed, as previously described.²³ PCR products were sequenced on the Illumina MiSeq sequencer by using 300-bp paired-end sequencing (V3 chemistry). The sequence reads containing IGH rearrangements were uploaded in IMGT HighV-Quest.²⁴ Subsequently, the output files were analyzed with the IGGalaxy tool.²⁵ The T-cell receptor δ diversity 2 (*TRDD2*-*TRDD3*) rearrangements were analyzed by matching the constant genomic region outside the *TRDD2* and *TRDD3* sequences in the amplicons, after which the length of the *TRDD2*-*TRDD3* regions was determined. Analysis was performed with BioPerl and R software.

Statistics

The Wilcoxon rank sum test was performed, when possible. A *P* value of less than .05 was considered significant.

RESULTS**NSG mice transplanted with hematopoietic stem and progenitor cells from control samples display low degree of variation**

Recently, we have shown that NOD-*Scid*-*Il2rg*^{-/-} (NSG) mice engrafted with hematopoietic stem and progenitor cells (HSPCs) isolated from umbilical cord blood (UCB) and, to a lesser degree, from human BM have robust development of myeloid and lymphoid cells, with functional B and T cells.¹⁹ First, we transplanted NSG mice with HSPCs obtained from either UCB or human pediatric BM to determine the presence of different stages of human T-cell development. We transplanted 14 mice with CD34⁺ cells isolated from cord blood from different donors and 9 mice with CD34⁺ cells isolated from pediatric BM, 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 from human BM (Fig 1, A). Different DN subsets could be discriminated based on the expression of CD7 and CD5 within the CD4⁻CD8⁻ DN compartment (Fig 1, B). Moreover, the relative presence of all subsets is highly comparable and showed a low degree of variation (Fig 1). 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 transplantation because 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 with BM-derived HSPCs.

SCID humanized mice display SCID-specific phenotypes

We have engrafted NSG mice with HSPCs isolated from BM of patients with SCID with mutations in different genes, including *ADA*-SCID, *Artemis*-SCID, *IL7RA*-SCID, and *IL2RG*-SCID (Table I). No T cells were detected in peripheral blood when *Artemis*-, *IL7RA*-, or *IL2RG*-SCID HSPCs were transplanted (Fig 2, A), as expected based on the disease-defining absence of

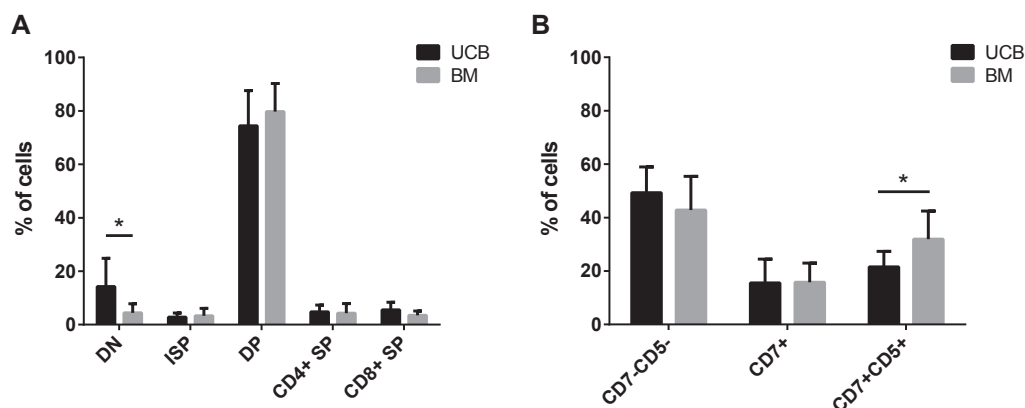


FIG 1. Low variation among stages of human T-cell development in NSG mice transplanted with HSPCs from control UCB and control human BM. Percentages of cells per different stage of human T-cell development within thymus of NSG mice transplanted with HSPCs are shown. Cells were gated within hCD45⁺ cells (**A**) or within hCD45⁺ CD4⁺ CD8⁻ cells (**B**; DN). Depicted are means and SDs. **P* < .05.

TABLE I. Characteristics of patients with SCID

SCID	Mutation	T cells ($\times 10^9/L$)	B cells ($\times 10^9/L$)	NK cells ($\times 10^9/L$)
ADA	c.736C>T, p.Gln246Stp	<0.01	<0.01	<0.01
Artemis	c.220C>T, p.Arg74Stp	0.4*	<0.01	0.5
IL7RA	c.876+1G>A / c.898_902delCCTGA, p.Pro300LysfsX9	NA	NA*	NA*
IL2RG	c.209T>G, p.Met70Arg	8.2†	4	0.06
IL2RG hypomorphic	c.655T>A, p.Tyr219Asn	3.4	1.4	0.9

Cell counts from different lineages from peripheral blood of patients, as determined by using flow cytometry, and their mutation, as determined by using PCR, are shown.

NA, Not available.

*All T cells were determined to be of maternal origin based on their memory phenotype.

†All T cells were of maternal origin (proved with short tandem repeat analysis on sorted cells).

T cells. However, for ADA-SCID, T, B, and NK cells were readily observed (Fig 2, A-C), likely caused by complementation by secreted murine ADA comparable with the recovery seen in patients with ADA-SCID treated with polyethylene glycol-conjugated ADA enzyme replacement therapy. Therefore we focused our further study on T- and B-cell development in mice receiving Artemis-, IL7RA-, and IL2RG-SCID cells compared with control animals. The results from HSPCs transplanted into NSG mice copied the phenotype found in different types of patients with SCID: for Artemis-SCID, we observed a deficiency of T and B cells; for IL7RA-SCID, we observed an absence of T cells, and for IL2RG-SCID, we observed a deficiency of both T and NK cells (Fig 2, A-C, and Table I). In mice transplanted with HSPCs from patients with IL7RA-SCID, the number of NK cells was somewhat lower as compared with that seen in control mice. Unfortunately, clinical data on NK cells in the patient with IL7RA-SCID 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 2, D). In addition to myeloid cells, NK cells were present both in higher percentages and numbers, probably because of 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 2, B). Because the model used is B-cell prone, this most likely reflects higher engraftment compared with control values. The same phenotypes

observed in peripheral blood of transplanted mice were observed in the spleen and BM (Fig 2, E and F). Some CD3 expression was observed within the spleens of NSG mice transplanted with Artemis-, IL7RA-, and IL2RG-SCID cells; however, these cells did not express TCR, CD4, or CD8 on their surface (data not shown) and likely represent staining artifacts.

Artemis-SCID demonstrates a block in B-cell development before the cytoplasmic Igμ⁺ 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 with 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 percentages and numbers of CD19⁺ B cells when compared with BM from control mice (Fig 3, A and B). This was not observed for the other types of SCID, which were highly comparable with 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 3, C). 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-SCID HSPCs when compared with those receiving control cells (Fig 3, D). There was barely any expression of cytoplasmic

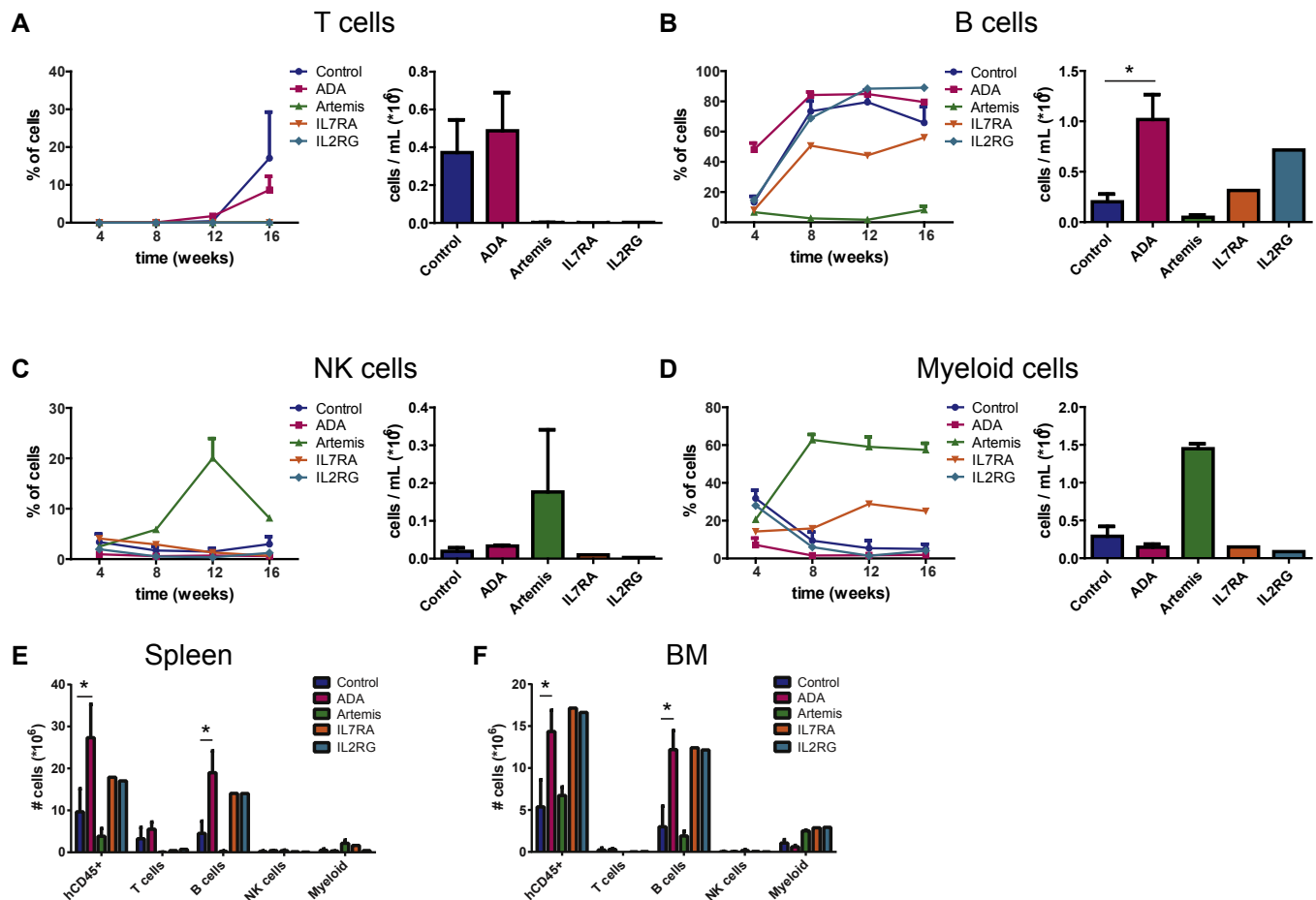


FIG 2. NSG mice transplanted with SCID HSPCs show the same phenotype as patients in their peripheral blood. **A-D**, Cells present in peripheral blood in percentages of human CD45⁺ cells over time after transplantation (*left panel*) or in absolute numbers at the end of the experiment (*right panel*). **Fig 2, A**, T cells; **Fig 2, B**, B cells; **Fig 2, C**, NK cells; and **Fig 2, D**, myeloid cells. **E** and **F**, Absolute numbers of different cell types within the spleen (**Fig 2, E**) or BM (**Fig 2, F**). Depicted are means and SDs. **P* < .05.

Igμ present in the B-cell precursors, and therefore precursor B-cell differentiation is blocked before the cytoplasmic Igμ⁺ pre-B-II stage. This block in B-cell development was identical to the phenotype observed in a BM aspirate of the patient with Artemis-SCID (**Fig 3, E**). The block at the pre-B-II stage in patients with Artemis-SCID leads to an absence of immature and mature B cells expressing IgM or IgD (**Fig 3, F**). We also observed a much lower percentage of B cells in spleens of NSG mice transplanted with Artemis-SCID HSPCs, and no mature CD20⁺, IgM⁺, or IgD⁺ B cells were found (**Fig 3, G**).

Together, these data confirm a block in B-cell development for Artemis-SCID but not for *IL7RA*-SCID and *IL2RG*-SCID. This is highly similar to the observations in the BM aspirates of the corresponding patients with SCID, demonstrating that HSPC transplantation into mice of 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 IGH repertoire that was generated in the BM of transplanted NSG mice. For both

incomplete D-J (**Fig 4, A**) and complete V-DJ (**Fig 4, B**) rearrangements, merely a few incidental rearrangements were observed for Artemis-SCID. For *IL7RA*- and *IL2RG*-SCID, a polyclonal repertoire comparable with that observed in control animals was generated (**Fig 4, A 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, although this was not observed for *IL7RA*- and *IL2RG*-SCID and control cells (**Fig 4, C**). Thus, as exemplified by Artemis-SCID, only for a recombination defect do both phenotypic and *IGH* rearrangement analysis indicate an arrest in B-cell development at the pre-B-II stage, which led to development of a less diverse repertoire.

Novel early blocks in T-cell development for SCID

For the vast majority of patients with SCID, 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

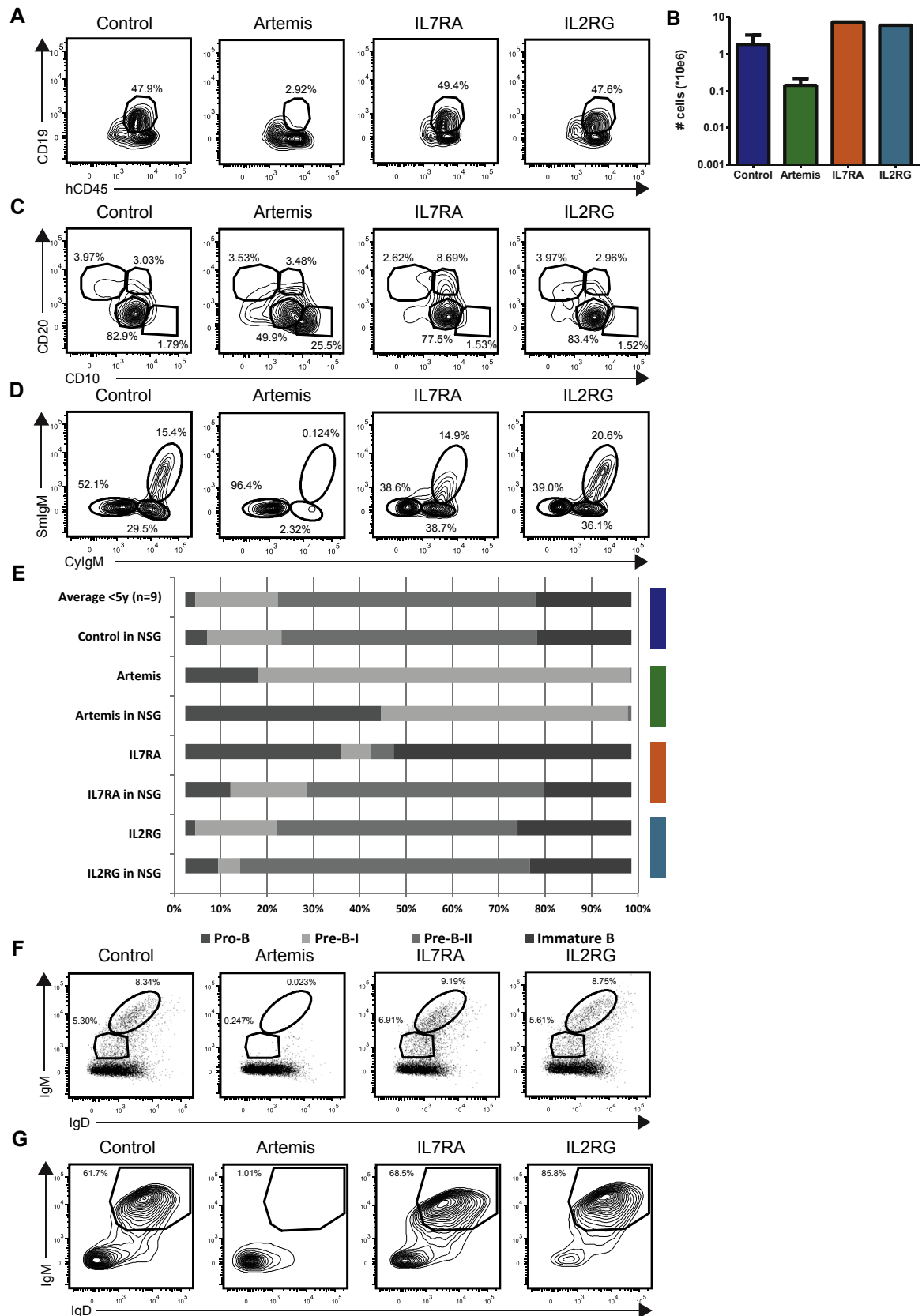


FIG 3. Developmental block at the CD10⁺ stage for B cells in Artemis-SCID cells. **A** and **B**, Percentage (**Fig 3, A**) and number (**Fig 3, B**) of B cells in transplanted NSG mice BM. **C** and **D**, Maturation stages of B-cell development in BM of transplanted NSG mice. **E**, Comparison of B-cell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

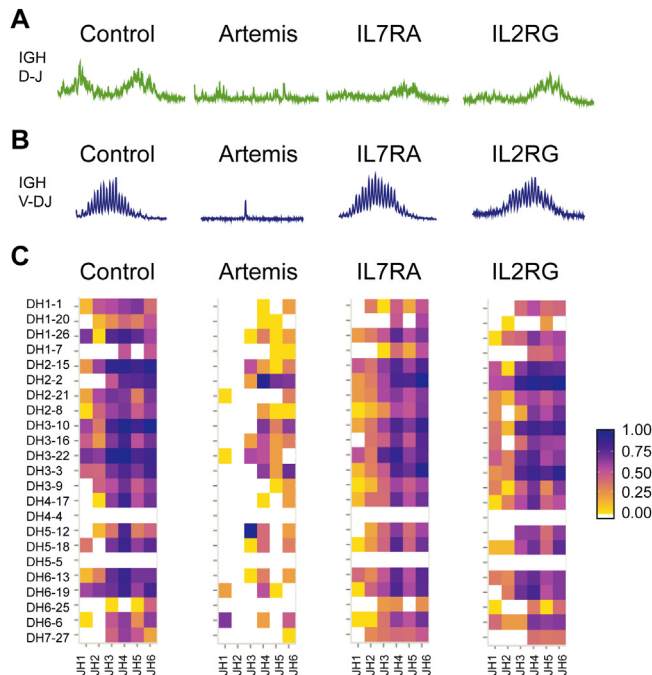


FIG 4. No polyclonal IGH repertoire was generated in developing B cells from Artemis-SCID. **A** and **B**, D-J (Fig 4, A) and V-DJ (Fig 4, B) rearrangement of IGH locus in total BM cells of NSG mice transplanted with SCID or control HSPCs. **C**, Heat map depicting unique junctions of D to J segments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

different populations within the DN compartment, we measured expression of CD7 and CD5 within this population. Based on the observed developmental arrest at the CD4⁺CD8⁺ DN stage, all types of SCID block much earlier than observed in corresponding mouse mutants (Fig 5, A). 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 5, B), 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 5, B and C). As expected from these early blocks in T-cell development, there was no expression of either TCR $\alpha\beta$ or TCR $\gamma\delta$ on total thymocytes in mice transplanted with SCID-derived HSPCs, but they were readily detectable in control mice (see Fig E1 in this article's Online Repository at www.jacionline.org). These data demonstrate the stages of arrest in T-cell development for different types of SCID and show an absolute requirement for common γ -type cytokine signaling at the most immature thymic stages, as also illustrated by the decrease in total hCD45⁺ cell numbers within the thymi of these mice (Fig 5, D). We observed a high percentage of CD19⁺ B cells in thymi from NSG mice transplanted with HSPCs from Artemis-SCID (Fig 5, E). For *IL7RA*- and *IL2RG*-SCID, an increase in the percentage of B cells in the thymus was observed also, although not as much as for Artemis-SCID. The observed increase in B-cell numbers was only relative and not in absolute numbers (Fig 5, F).

Importantly, we also transplanted CD34⁺ cells from a patient with SCID 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 showed a

much milder block, with only a lower percentage of double-positive thymocytes (Fig 5, G). 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 of mice transplanted with hypomorphic mutant *IL2RG*-SCID HSPCs (data not shown).

TCR rearrangement starts very early after thymus entry

TCR rearrangement starts at the *TRD* locus.⁵ Because the observed blocks in T-cell development were very early, we focused TCR repertoire analysis on the *TRD* locus, of which D δ 2-D δ 3 is rearranged first. *IL7RA*- and *IL2RG*-SCID T-cell progenitors only showed incidental products of this rearrangement in accordance with the early blocks observed by using flow cytometry (Fig 6, A). By using spectratyping of TCR CDR3 length distribution, Artemis-SCID T-cell progenitors showed a broad repertoire for D δ 2-D δ 3 comparable with that seen in NSG mice transplanted with control HSPCs. These spectratyping data were confirmed by using next-generation sequencing of the TCR D δ 2-D δ 3 locus. Again, we observed a very limited number of reads for *IL7RA*- and *IL2RG*-SCID, whereas the normalized read numbers for Artemis-SCID were similar to those of mice transplanted with wild-type cells (Fig 6, B).

For the second step of *TRD* rearrangement, D δ 2-J δ 1, the repertoire was more oligoclonal in Artemis-SCID T-cell progenitors, whereas in *IL7RA*- and *IL2RG*-SCID cells such rearrangements were undetectable (Fig 6, C). However, none of the different types of SCID generated a broad D δ -J δ or even V δ -J δ repertoire (Fig 6, D). *TRG* is rearranged after *TRD*,⁵ and for *IL2RG*-SCID, no rearrangement could be observed for *TRG*, whereas for *IL7RA*-SCID, merely a clonal rearrangement was observed, confirming the later block in development of *IL7RA*-SCID compared with *IL2RG*-SCID (Fig 6, E).

Although Artemis-SCID T-cell progenitors were able to perform the first steps of *TRD* rearrangement, they did not rearrange *TRG* and *TRB* loci (Fig 6, E and F). For both loci, only a few incidental peaks were detected in Artemis-SCID T-cell progenitors compared with those in 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 cells, NK cells, or both. Here we demonstrate for the first time arrests in T-cell development for the major types of human SCID using an *in vivo* model, data that typically are not available because thymic biopsy specimens are almost never obtained. Our approach allows for comprehensive loss-of-function insight for 2 important developmental checkpoints: common γ chain cytokine signaling and initiation of TCR rearrangements.

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 cells from patients with *IL2RG*-SCID,

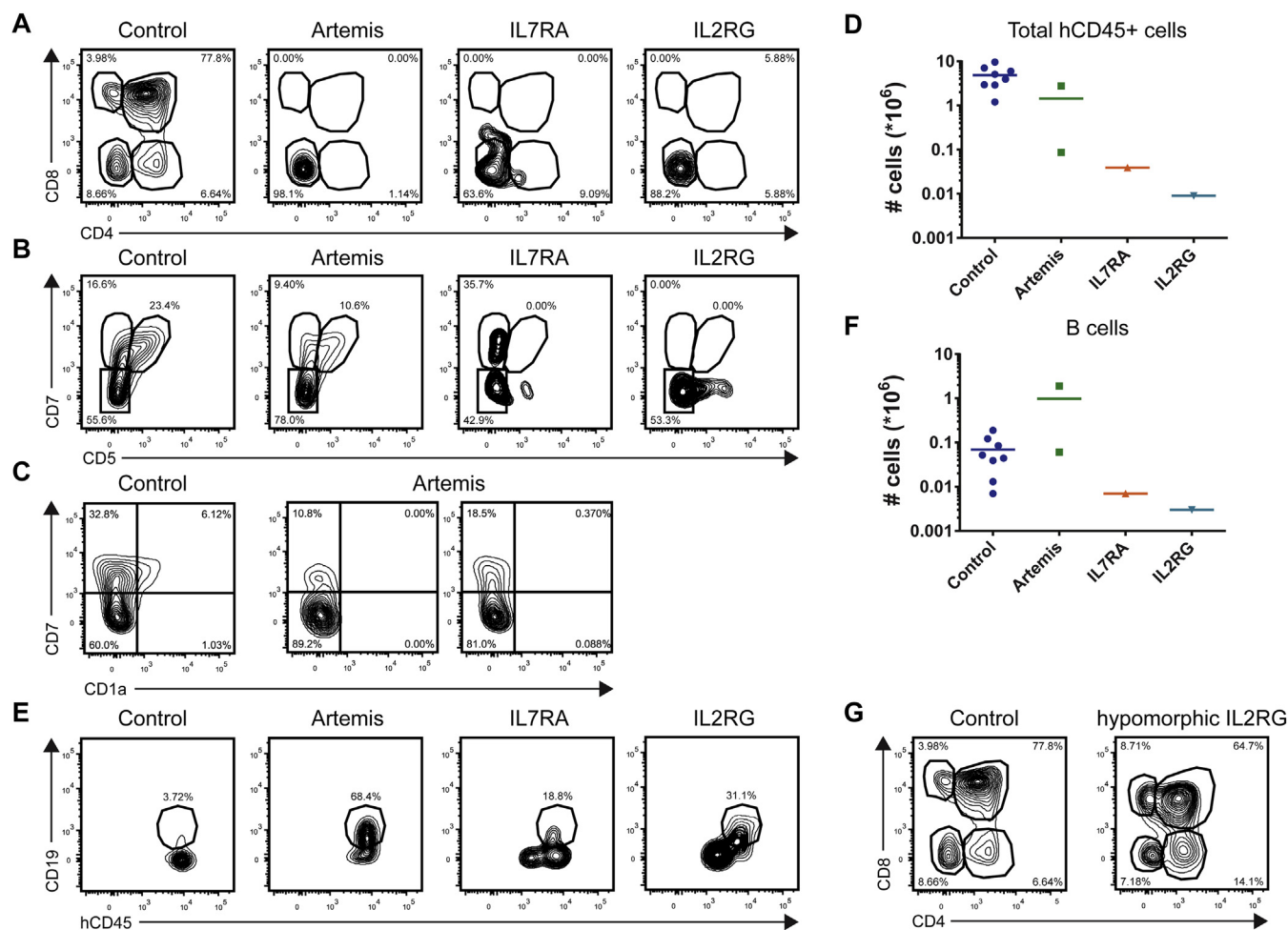


FIG 5. Early blocks in human T-cell development for different types of SCID. **A-C**, Phenotype of hCD45⁺ thymocytes (Fig 5, A) and DN thymocytes (Fig 5, B and C) in NSG mice transplanted with SCID or control HSPCs. **D**, Quantification of total human CD45⁺ cells in thymi of NSG mice transplanted with SCID or control HSPCs. **E** and **F**, Presence of CD19⁺ B cells in thymi of transplanted NSG mice. **G**, NSG mice transplanted with hypomorphic IL2RG-SCID or control HSPCs.

followed by *IL7RA*-SCID, demonstrating an almost immediate requirement for common γ chain cytokine signaling after seeding of the thymus.

Artemis is involved in opening of the hairpin sealed coding ends during recombination.²⁶ Here we observed that Artemis-SCID cells are able to initiate rearrangement of the *TRD* locus, as demonstrated by polyclonal D δ 2-D δ 3 and oligoclonal D δ 2-J δ 1 rearrangements. However, no complete TCR δ was formed. Apparently, Artemis is not needed for the first steps of *TRD* rearrangement, as confirmed by means of next-generation sequencing, but is needed for complete rearrangement of *TRD*. An explanation would be that in the absence of Artemis, the hairpin sealed coding ends are opened by other enzymes.²⁷ Because this process is less efficient, the chance of successful 2-step rearrangement is low, and only partial rearrangements would be formed, as observed here.

Here we demonstrate that rearrangement of *TRG* is initiated at the DN CD7⁺ stage and that *TRB* loci are already initiated at the DN CD7⁺CD5⁺ stage of T-cell development in contrast to previous models in which *TRB* rearrangement was proposed at the immature single-positive^{28,29} or DN3 stage of development.³⁰

Because these DN CD7⁺CD5⁺ cells were devoid of CD1a expression that marks human DN3 cells,⁸ this stage is reminiscent of the DN2 compartment in the mouse. Although in the mouse rearrangement of *TRB* starts in the DN3 stage,³¹ 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,^{10,11} whereas here we observed an absolute and very early requirement for *IL2RG* signaling. The blocks in *IL7RA*-SCID and *IL2RG*-SCID cells are not completely identical, suggesting that cytokines other than IL-7 might also signal through the common γ chain in the early steps after thymus seeding. Cytokines that might be involved in proliferation and development in this very immature stage are IL-2, IL-4, or IL-15.³²⁻³⁴ IL-15 is an important NK cell growth factor, and in patients with *IL7RA* deficiency, this cytokine can signal normally through the IL-2R γ -IL-2R β -IL-15 α complex. Thus some of the CD7⁺CD5⁺ DN cells observed might contain mature NK cells rather than solely uncommitted thymocytes with T-cell/NK cell potential.^{9,35} The increased percentage of B cells observed in thymi of NSG mice transplanted with Artemis-SCID HSPCs is in line with data from the mouse in

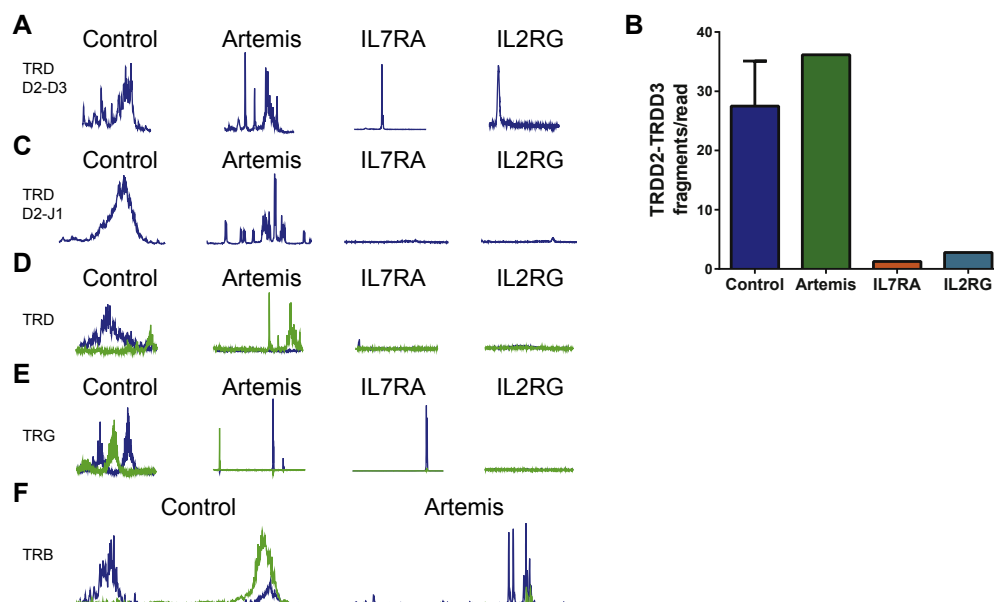


FIG 6. Hierarchical blocks in human T-cell development, as demonstrated by means of TCR repertoire analysis. **A** and **C-F**, Rearrangement of TCR loci in total thymocytes of transplanted NSG mice. **Fig 6, A**, TRD Dδ2-Dδ3; **Fig 6, C**, TRD Dδ2-Jδ1; **Fig 6, D**, TRD Dδ-Dδ (blue) and Dδ-Dδ (green); **Fig 6, E**, TRG; **Fig 6, F**, TRB. **B**, Assigned fragments of *TRDD2-TRDD3* were calculated by dividing the number of assigned reads by the number of identified fragments.

which very early thymic blocks also lead to increased percentages of B cells, which developed from DN thymocytes underlying the developmental plasticity of these cells, which can still develop in non-T-lineage cells in mice and human subjects.^{9,36}

The point at which *IL2RG* deficiency blocks development has sometimes been suggested to occur before thymic seeding. Our data combined with data by Kohn et al,³⁷ who showed no differences in hematopoietic stem cells and prethymic progenitors of patients with *IL2RG*-SCID, indicates that this is not the case. Rather, very soon after thymic seeding, there is an absolute requirement for common γ chain signaling (see Fig 5). Because Six et al³⁸ showed that human thymic progenitors (in contrast to mouse) express IL-7R α and IL-2R γ ,³⁸ expression and functional requirement for the common γ chain are independently regulated.

To the best of our knowledge, only 1 article has addressed the effect of human IL-2R γ deficiency in T cells before. By using an *in vitro* OP9-DL1 system, an arrest in development was shown at the CD7⁺CD5⁺CD1a⁻ stage.³⁹ Because no data are available on the nature of the patient with *IL2RG*-SCID described by Six et al,³⁸ it is impossible to say whether 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 double-positive stages and is very sensitive to subtle differences in cytokines and labile contents of culture media.³⁹ Development proceeds a bit further in fetal thymic organ cultures, 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 thymi, underscoring the validity of this model to identify the thymic defects in human patients with SCID. Another approach to model T-cell

developmental blocks in patients with SCID is by measuring rearrangement of TCR loci and T-cell receptor excision circle levels in peripheral blood T cells.^{40,41} However, many patients with SCID do not have peripheral T cells on which to perform these analyses, and no phenotype of thymocytes is available. The low level of *TRD* D2-D3 in another patient with *IL2RG*-SCID⁴⁰ corroborates our data described here for thymocytes, demonstrating a very early block in T-cell development.

We have chosen patients with SCID with mutations that are deleterious for function because this would lead to functional null mutants and included a hypomorphic patient who had a clinically mild SCID phenotype that was only found because of an affected cousin. This mutation 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 *TRB* rearrangement, which marks the presumed β -selection point because Artemis-deficient cells block at this stage. It was shown previously that pre T-cell antigen receptor α (*PTCRA*) is abundantly expressed at this point in development.⁵

In a xenograft model it could be argued that the defects observed are not cell intrinsic but result from incompatibilities between the 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 thymi.⁴² In addition, Parietti et al⁴³ were able to reproduce the development of human prothymocytes in the NSG xenograft model, and it was demonstrated that human thymocytes can respond to murine MHC signals and have comparable migration

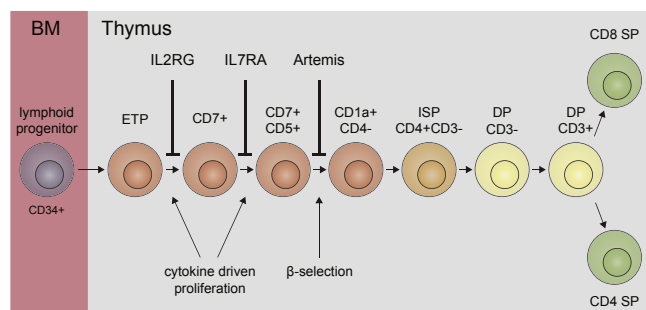


FIG 7. Proposed model for human T-cell development. Indicated are blocks in human T-cell development for different types of SCID, as observed in this study. DP, Double positive; ETP, early thymic progenitor; ISP, immature single positive; SP, single positive.

on murine and human thymic slices.⁴⁴ Furthermore, for B cells, the xenogeneic environment shows a block identical to the block in development directly found in the patient with Artemis-SCID, and B-cell development in control subjects was highly comparable with that in 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 BM transplantation for acquired immune deficiencies and during aging.⁴⁵ In particular, strategies targeting the earliest human thymocytes, such as administration of IL-7, growth hormone, or thyroid-stimulating hormone or sex hormone ablation.^{45,46} Our results suggest that IL-7 combined with other early factors, such as Wnt,⁴⁷ might 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 report loss-of-function data on human T-cell development, demonstrating earlier blocks in T-cell development than proposed before.^{5,30} The obtained insights might prove opportunities for treatment of early T-cell precursor acute lymphoblastic leukemia by interfering with common γ chain signaling, which is active in these very early stages of T-cell development. It would be of great interest to study arrest in development for other types of human SCID in this xenograft model. Finally, the xenotransplantation method we describe might prove useful in unravelling unknown types of human SCID for which the genetic defect has remained elusive and aid in diagnosis in cases with blurred phenotypes, such as when maternal T cells are present.

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Key messages

- Arrests in thymic development have been unknown for most types of human SCID.
- By using a xenograft model, *IL2RG*- and *IL7RA*-SCID showed very early arrest in the thymus, followed by recombination-defective SCID (Artemis), all of which caused blocking in the CD4⁺CD8⁺ DN stages.
- This approach can be used for other types of SCID and help in unraveling unknown cases of SCID.

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METHODS

Cryopreservation of human BM-derived mononuclear cells

Mononuclear cells were isolated by using Ficoll gradient separation and washed once with Iscove modified Dulbecco medium (IMDM) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Life Technologies, Bleiswijk, The Netherlands) and 2.5% heat-inactivated FCS (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands). Cells were resuspended in FCS/10% dimethyl sulfoxide (Greiner Bio-One B.V. and Sigma-Aldrich, respectively) or 50% RPMI/40% FCS/10% dimethyl sulfoxide and frozen in 1-mL aliquots, with a maximum of 50×10^6 cells per cryovial. Cryovials were placed in a freezing container at -80°C and relocated to liquid nitrogen storage on the following day.

Isolation of human BM-derived CD34⁺ cells

Cryopreserved vials were thawed in a 37°C water bath, 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. Labeling and isolation of CD34⁺ cells were performed according to the manufacturer's protocol (CD34 Microbead Kit; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cells were centrifuged and resuspended in 300 µL of Running Buffer (PBS pH 7.4, 2 mmol/L EDTA, 0.5% wt/vol BSA [Sigma-Aldrich]). DNase I (final concentration, 5 U/mL; Sigma-Aldrich) with 100 µL of FcR blocking reagent and 100 µL of anti-CD34 microbeads were added. Cells were not counted before labeling because 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 2 times on an LS column (Miltenyi Biotec GmbH).

Cells were eluted in 3 mL of Running Buffer by using the supplied plunger. Eluted cells were centrifuged and resuspended in 500 µL of StemSpan serum-free expansion medium (StemSpan-SFEM; STEMCELL Technologies, Vancouver, British Columbia, Canada) in the presence of 10 ng/mL stem cell factor (a gift from Amgen, Thousand Oaks, Calif), 20 ng/mL recombinant human thrombopoietin (R&D Systems, Abingdon, United Kingdom), 20 ng/mL recombinant mouse insulin-like growth factor 2 (R&D Systems), and 10 ng/mL recombinant human fibroblast growth factor-acidic (PeproTech, Rocky Hill, NJ). Cells were counted with a Bürker chamber and trypan blue stain. The cell concentration was adjusted to $1 \times 10^6/\text{mL}$.

Cells were cultured overnight in tissue culture–treated 48-well plates (Corning, Corning, NY) with 500 µL of cell suspension per well. Then cells were harvested by means of resuspension and transferred to a 15-mL conical tube. The well was washed by means of resuspension with 500 µL IMDM without phenol red (Gibco, Life Technologies), which was added to the harvested cells. Cells were counted, and an aliquot was taken for flow cytometric analysis to determine the purity of CD34⁺ cells.

Transplantation into NSG mice

Cells were centrifuged and resuspended in IMDM without phenol red for intravenous injection into the tail veins of sublethally irradiated female NSG mice (200 µL per mouse). Mice were irradiated with 1.91 Gy by using orthovoltage x-rays 1 day before cell injection. Mice were transplanted within 24 hours after irradiation. Peripheral blood of transplanted mice was analyzed every 4 weeks, and mice were killed 20 to 22 weeks after transplantation.

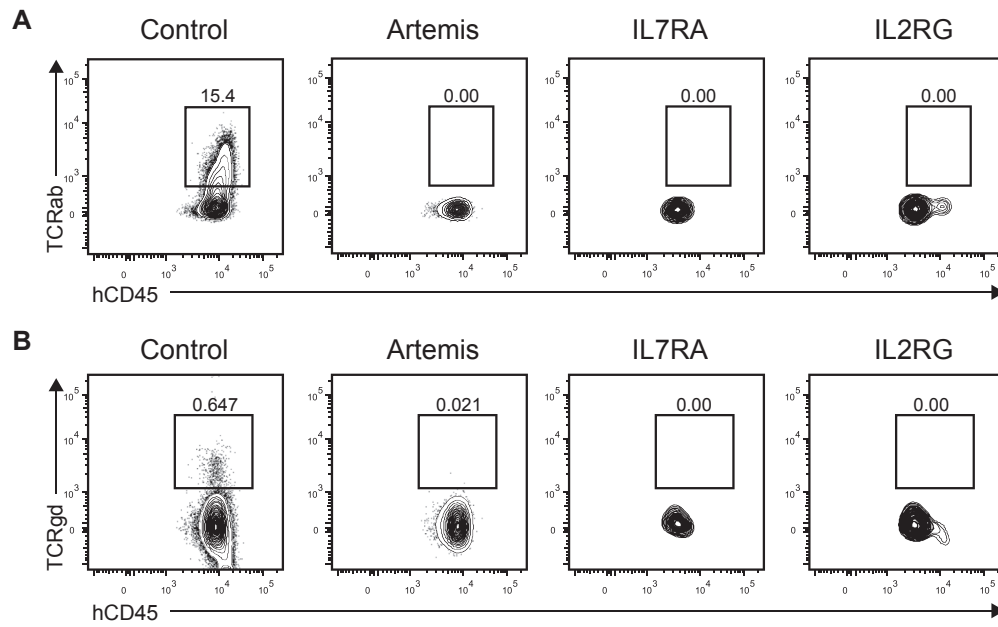


FIG E1. Absence of TCR expression on SCID thymocytes. Expression of TCR $\alpha\beta$ (**A**) and TCR $\gamma\delta$ (**B**) on total hCD45⁺ thymocytes from transplanted NSG mice.

TABLE E1. Xenograft transplantation details

SCID	No. of mice transplanted	Transplanted no. of total cells per mouse	Transplanted no. of CD34⁺ cells per mouse
<i>ADA</i>	3	200,000	197,540
<i>Artemis</i>	2	78,000	73,593
<i>IL7RA</i>	1	22,000	19,518
<i>IL2RG</i>	1	47,150	38,559
<i>IL2RG</i> hypomorphic	4	50,000	45,035
Healthy donor	1	33,000	29,555
Healthy donor	4	200,000	167,480
Healthy donor	3	100,000	86,740

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