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Human Lymphoid Development in the Absence of Common γ-Chain Receptor Signaling

Lisa A. Kohn,* Christopher S. Seet,**† Jessica Scholes, † Felicia Codrea, ‡ Rebecca Chan,* Sania Zaidi-Merchant,* Yuhua Zhu,* Satiro De Oliveira,§ Neena Kapoor,* Ami Shah,* Hisham Abdel-Azim,* Donald B. Kohn,*§ and Gay M. Crooks*§

Despite the power of model systems to reveal basic immunologic mechanisms, critical differences exist between species that necessitate the direct study of human cells. Illustrating this point is the difference in phenotype between patients with SCID caused by mutations affecting the common γ-chain (γc) cytokine signaling pathway and mice with similar mutations. Although in both species, null mutations in either IL-2RG (which encodes γc), or its direct downstream signaling partner JAK3, result in T and NK cell deficiency, an associated B cell deficiency is seen in mice but not in humans with these genetic defects. In this study, we applied recent data that have revised our understanding of the earliest stages of lymphoid commitment in human bone marrow (BM) to determine the requirement for signaling through IL-2RG and JAK3 in normal development of human lymphoid progenitors. BM samples from SCID patients with IL-2RG (n = 3) or JAK3 deficiency (n = 2), which produce similar “T-NK-B+γ” clinical phenotypes, were compared with normal BM and umbilical cord blood as well as BM from children on enzyme treatment for adenosine deaminase–deficient SCID (n = 2). In both IL-2RG– and JAK3-SCID patients, the early stages of lymphoid commitment from hematopoietic stem cells were present with development of lymphoid-primed multipotent progenitors, common lymphoid progenitors and B cell progenitors, normal expression patterns of IL-7RA and TLSPR, and the DNA recombination genes DNTT and RAG1. Thus, in humans, signaling through the γc pathway is not required for prethymic lymphoid commitment or for DNA rearrangement. The Journal of Immunology, 2014, 192: 000–000.

Even combined immunodeficiency is a rare genetic syndrome characterized by a profound deficiency in functional T lymphoid cells and variable degrees of B cell deficiency or dysfunction (1). Multiple genetic mutations can cause this syndrome, several of which cause defects in cytokine signaling. Depending on the specific mutation, normal production of B lymphocytes and/or NK cells also is affected. The most common form of SCID, X-linked SCID (X-SCID) (2–7), is caused by null mutations in the IL-2RG gene, whose protein product is a type I cytokine receptor known as the common γ-chain (γc) (7). γc is a cytokine receptor subunit that forms a complex with the ligand-specific receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 to provide a common signaling chain for these receptors (8–13). Mutations of IL-2RG therefore result in a complex immunologic phenotype because of an inability to differentiate or function in response to multiple lymphoid cytokines (8). Human SCID caused by deficiency in γc is characterized by an absence of peripheral T and NK cells and present but functionally impaired B cells (8, 14).

After cytokine ligand stimulation of one of its partner receptor chains, dimerization of γc activates the hematopoietic-restricted tyrosine kinase JAK3/STAT pathway (15–17). Disruption of the gene encoding JAK3 causes an autosomal form of SCID with an otherwise identical clinical phenotype to X-SCID (18–20). Although IL-2RG and JAK3 null mutations in mice produce the same profound deficiency of T and NK cells seen in humans, a major species-specific difference is seen in B cell development. Whereas humans with mutations in IL-2RG or JAK3 have normal numbers of circulating B cells (18, 21), mice with similar mutations are unable to develop B cells (22–26). The lack of B cell development in mice with defective γc signaling has been specifically attributed to an inability to respond to IL-7, because mice deficient in IL-7Rγc, the IL-7 ligand binding partner to γc, are similarly unable to develop B cells (27). Further demonstrating that IL-7 requirements for B lymphopoiesis are different between the two species, patients with IL-7Rγc defects have T cell deficiency but normal numbers of B cells (27, 28).

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Address correspondence and reprint requests to Dr. Gay M. Crooks, Department of Pathology and Laboratory Medicine, University of California, 2014 TLSB, 610 Charles E. Young Drive, East, Los Angeles, CA 90095-7239. E-mail address: GCrooks@mednet.ucla.edu

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Abbreviations used in this article: ADA, adenosine deaminase; B2M, β2-microglobulin; BM, bone marrow; γc, common γ-chain; CB, cord blood; C/EBP, early B cell factor 1; FSC, forward light scatter; K562, knockdown; LMPF, lymphoid-primed multipotent progenitor; SSC, side scatter; TSLPR, thymic stromal lymphopoietin receptor; X-SCID, X-linked SCID.
All stages of hematopoiesis, from early progenitors through many categories of mature lymphoid cells, have been examined in mice that are null for IL-2RG, IL-7Rα, or IL-7 (29); however, examination of how these genes affect lymphoid commitment in humans has been lacking, as patients with these mutations are rare, access to bone marrow (BM) is difficult and the identification of the early lymphoid progenitor stages in normal human BM has lagged behind that of mice (30).

The stepwise process of differentiation from multipotent hematopoietic stem cells (HSC) into functional lymphocytes initially proceeds through specific progenitor stages with progressively more limited lineage potential. Upregulation of CD10 expression on CD34+ progenitor cells has long been assumed to herald the onset of lymphoid commitment in human hematopoiesis (31). We have recently identified a lymphoid progenitor stage in human BM (32) that precedes the previously described CD34+CD10+ common lymphoid progenitor (CLP) in lineage commitment from HSC. This progenitor lacks CD10 expression and is marked by high expression of the homing molecule L-selectin (CD62L). The CD34+linnegCD10neg expression was functionally similar to the lymphoid-primed multipotent progenitor (LMP) identified in murine BM (33) in that they possess full lymphoid (T, B, and NK), dendritic, and some myeloid (mostly monocytic) potential but lack myelopoietic potential. The CD34+linnegCD10neg progenitor identified in human BM (32) has been termed the CLP (CD34+linnegCD10neg). We now apply the CD34+linnegCD10neg progenitor identified in human BM (32) to analyses of BM from patients with SCID to define the impact of γc and Jak3 deficiency on development of the first stages of human lymphopoiesis.

Materials and Methods

Collection of BM, cord blood, and peripheral blood cells

Human BM was collected from patients with SCID at University of California, Los Angeles, and Children’s Hospital of Los Angeles under informed consent through protocols approved by the University of California, Los Angeles, and Children’s Hospital of Los Angeles Institutional Review Boards. Normal human adult BM was obtained from healthy donors as anonymous waste material from normal deliveries at University of California, Los Angeles.

Cell isolation and analysis

Mononuclear cells were isolated by density gradient centrifugation using Ficoll–Paque (GE Healthcare). Samples were enriched for CD34+ cells by CD34+ progenitor cells has long been assumed to herald the onset of lymphoid commitment in human hematopoiesis (31). We have recently identified a lymphoid progenitor stage in human BM (32) that precedes the previously described CD34+CD10+ common lymphoid progenitor (CLP) in lineage commitment from HSC. This progenitor lacks CD10 expression and is marked by high expression of the homing molecule L-selectin (CD62L). The CD34+linnegCD10neg expression was functionally similar to the lymphoid-primed multipotent progenitor (LMP) identified in murine BM (33) in that they possess full lymphoid (T, B, and NK), dendritic, and some myeloid (mostly monocytic) potential but lack myelopoietic potential. The CD34+linnegCD10neg progenitor identified in human BM (32) has been termed the CLP (CD34+linnegCD10neg). We now apply the CD34+linnegCD10neg progenitor identified in human BM (32) to analyses of BM from patients with SCID to define the impact of γc and Jak3 deficiency on development of the first stages of human lymphopoiesis.

Results

Clinical and immunologic characteristics of subjects

Clinical data of subjects with SCID and healthy control subjects who provided BM samples are presented in Table I. BM samples from three male infants with IL-2RG deficiency (aged 2 wk–3 mo), and one female and one male infant with Jak3-deficient SCID (both aged 3 mo) were analyzed to assess the effects of γc pathway signaling defects. BM samples from three adults and a 6-y-old child were used as healthy donor controls. For closer age-matched controls, we also examined marrow from two children (ages 3 and 21 mo) with adenosine deaminase (ADA) deficiency (ADA-SCID) both of whom were on polyethylene glycol–ADA enzyme replacement therapy with partial immune recovery at the time of BM collection and flow cytometry analysis (38). Analysis of umbilical CB was included as a reflection of normal newborn hematopoiesis.

As expected based on findings in peripheral blood, BM aspirates from patients with IL-2RG– and Jak3-deficient SCID showed an almost complete absence of contaminating T lymphoid cells (Fig. 1A). Of note, in three of the five patients whose peripheral blood was analyzed, maternal engraftment of T lymphocytes was detected by fluorescent in situ hybridization. Consistent with maternal engraftment, the few CD3+ T cells detectable in SCID marrow aspirates were predominantly mature CD45RO+ cells, in contrast to normal adult BM and CB in which naive CD45RAaT cells predominated (Fig. 1B). Concordant with their peripheral blood phenotype, NK cells were barely detectable (<1%) in the BM of SCID patients (Fig. 1C).

Human lymphoid commitment is not dependent on IL-2RG/Jak3 signaling

We have previously shown in human BM that IL-2RG transcription is significantly upregulated during differentiation of HSC into LMPP (CD34+LinnegCD10negCD45RA+CD62L+), and expression continues to increase between the LMPP and CLP. The LMPP can be expressed by negative controls using the florescent minus one technique (36).

Quantitative PCR analysis

After isolation of cells by flow cytometry, RNA was extracted with a Qiagen RNEasy Microkit (Qiagen) and reverse transcribed with Omniscript RT, Oligo DT, random hexamers, and RnAguard (Pharmacia Biotech). An ABI Via7 was used for real-time PCR with TaqMan Gene Expression Mastermix and TaqMan probe–based gene expression analysis (probes; Hs00172121_m1 [β2M], Hs00172743_m1 [DNTT], Hs00929694_m1 [EF1], Hs00233515_m1 [CD2], Hs01062241_m1 [CD3E], and Hs00843250_m1 [β2-microglobulin (B2M)]) (Life Technologies). Each biologic sample was assayed in technical triplicate. The optimal reference gene for BM populations was previously determined to be B2M (32, 37), and B2M expression was detected in all populations and samples tested (Supplemental Fig. 1). Quantitative PCR results were normalized through the use of the change-in-cycling-threshold methods (ΔACT).

Statistical analysis

F Prism version 5 (GraphPad Software) was used for statistical analysis and graphic generation. Flow cytometry data were analyzed with FlowJo software.

Results

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of immunophenotypic HSC based on expression of the progenitor Ag CD34 and absence of CD38 and other lineage-specific Ags (CD34^lin^-CD38^- cells) was similar in normal BM and SCID BM samples (Fig. 2A, Supplemental Table I). The CD10^-LMPP and CD10^-CLP populations were both readily detectable in BM from infants with IL-2RG-deficient SCID and JAK3-deficient SCID as well as infants on treatment for ADA-deficient SCID (Fig. 2B, 2C, Supplemental Table I). Thus, although IL-2RG is expressed in the earliest stages of human lymphoid commitment, signaling through IL-2RG/JAK3 is not required to generate these progenitors. As we have previously noted, the profile of lymphoid progenitors in umbilical CB was markedly different to that of all normal and SCID BM samples, with no clear population of immunophenotypic LMPP and a relatively low frequency of immunophenotypic CLP.

Consistent with BM from healthy infants, CD34^+ progenitor cells from all IL-2RG- and JAK3-deficient SCID patients were heavily skewed toward B cell commitment, consisting of mostly fully B cell–committed CD34^+CD10^-CD19^- progenitors and, to a lesser extent, the more immature CD34^+CD10^-CD19^- CLP population (which has predominantly B cell potential but also can generate small numbers of NK and T cells) (Fig. 3A) (32, 39–41). Surface expression of IL-7Rα (the receptor chain that binds IL-7 and partners with the signaling domain IL-2RG) was detected on CLPs from normal BM and BM from all SCID patients (IL-2RG, JAK3, and treated ADA) (Fig. 3B) (42). IL-7Rα’s alternative dimerization and signaling partner, TSLPR (gene name CRLF2), was also detected on CLPs from all normal and SCID samples examined (Fig. 3C). Thus, signaling through γc or its downstream partner JAK3 is not required for normal generation of B lymphoid progenitors. γc-deficient B lymphoid progenitors, however, possess normal expression of both surface IL-7Rα and its alternative signaling partner TSLP and are thus capable of responding to TSLP.

B cell differentiation is maintained in the absence of IL-2RG and JAK3 signaling

Despite the lack of functional γc signaling, IL-2RG– and JAK3-deficient lymphoid progenitors were able to produce immunophenotypically late stages of B cell differentiation, as shown by
expression of CD24, CD20, and surface IgM (Fig. 4A–C). On the basis of a recently described phenotype for human B-1 cells (CD20+CD3−CD43+CD27+) (34), few of the B cells in BM of SCID patients were of the B1 cell lineage (Fig. 4D, 4E).

To determine whether γc signaling is required for induction of expression of genes involved in DNA rearrangement, specific subsets of cells representing each stage of differentiation were isolated by flow cytometry and analyzed for gene expression by quantitative PCR. Consistent with their normal counterparts, DNTT (also known as TDT) (a DNA polymerase that catalyzes the addition of deoxynucleotides in pre-B and pre-T lymphocytes during early differentiation generating Ag receptor diversity by synthesizing non–germ-line elements during VDJ rearrangement) and RAG1 (activator of Ig V-D-J recombination) were expressed normally in SCID BM, with absent expression in HSC, onset of expression at low levels at the LMPP stage and then further up-regulation in CLP and B cell progenitors (Fig. 5A, 5B).

EBF1, a transcription factor critical for B cell differentiation that is a direct target of Stat-5–mediated IL-7R signaling (43) and whose overexpression can partially rescue B lineage potential in IL-7 knockout (KO) mice (29, 44, 45), was upregulated in CLP and subsequent stages of B cell differentiation in both SCID BM and in normal BM (Fig. 5C). Of note, although EBF1 was not expressed in normal adult LMPPs in our previous studies (32) or in this study (in either the adult or the 6-y-old subject), we were able to detect EBF1 expression in LMPPs from all IL-2RG–deficient and JAK3-deficient SCID BM samples, demonstrating that expression of this B cell specific transcription factor occurred at an earlier stage of lymphoid differentiation in these IL-2RG– and

**FIGURE 2.** Lack of IL-2RG/JAK3 signaling does not block early lymphoid commitment. (A) CD34 and CD38 expression on CD34+–enriched, DAPI-negative, lineage-negative (lin−) hematopoietic cells (lineage includes CD3, CD14, CD19, CD56, and glycophorin a). HSC (defined as CD34+ DAPI−lin−CD34+neg) gating shown. (B) CLPs (defined as CD34+DAPI−neg lin−CD34+5RA+CD10+−) are detected within CD34+lin− cells from all sources. (C) LMPPs (defined as CD34+DAPI−lin−CD10−negCD45RA+ CD62Lhi) are detected within CD34+CD10−neglin− hematopoietic cells from all BM samples but not normal CB. Numbers in parentheses for each SCID sample refer to information in Table I.

**FIGURE 3.** IL-7Rα and TSLPR are expressed in the absence of IL-2RG/JAK3 signaling. (A) CD10 and CD19 expression on CD34+ hematopoietic progenitor cells. Cell surface expression (shown as black histogram) of IL-7Rα (B) and TSLPR (C) on cells gated as CLPs (CD34+ CD10+CD19−neg). Unstained controls shown as gray. Numbers in parentheses for each SCID sample refer to information in Table I.
JAK3-deficient SCID patients. However, it should be noted that in BM from infants with ADA-deficient SCID on treatment with polyethylene glycol–ADA, LMPP also expressed EBF1, suggesting that the discrepancy of results with normal BM may be due to the very young age of the SCID patients rather than their genetic mutations.

We have previously shown that CD2, which encodes a T and NK cell specific cell surface adhesion molecule, is normally upregulated in LMPP but is not detectable in either HSC or CLP (32). Similar to normal BM, CD2 was also exclusively detected in the LMPP of BM from patients with SCID (two of three tested with IL-2RG deficiency and the sole patient tested with JAK3 deficiency) (Fig. 5D), providing further support that the potential for T and NK differentiation is not restricted at the LMPP stage but disrupts later stages of lineage-specific differentiation in the marrow and thymus (Fig. 6).

Discussion

Although the phenotype of lymphocytes in the peripheral blood of patients with SCID has been well described, studies of how the various genetic defects that lead to this life-threatening syndrome affect the earliest stages of lymphoid commitment have been lacking. Access to BM samples from untreated patients with SCID is challenging; these conditions are extremely rare, and unless detected early through newborn screening, infants typically present with life-threatening infections, requiring urgent HSC transplantation or gene therapy to survive, after which the fundamental hematopoietic abnormalities are permanently corrected. In addition, although numerous studies on murine BM have been reported over the past two decades, there has relatively little information on the identification of normal human lymphoid progenitors. Of the studies on human lymphopoiesis that do exist, almost all have used umbilical CB rather than postnatal BM. It is now clear from the current paper and from previous published data that important differences in immunophenotype and function exist between BM and CB stem cells and progenitors (32, 46–48). It should not be surprising that the types of progenitors that circulate transiently in neonatal CB, and the signaling requirements of those progenitors, are different from progenitors that reside in BM to provide steady-state postnatal hematopoiesis. Thus, measurement of progenitors in abnormal BM requires direct comparison with normal BM.

Age-matched controls are particularly challenging for studies on patients with SCID, almost all of whom are infants. Although HSCs maintain the potential to differentiate into lymphocytes throughout life, lymphoid output decreases relative to myeloid differentiation in later life (49). The frequency of B lymphoid...
progenitors is highest in infants, falling between early childhood (<10 y) and later childhood and adulthood (10–60 y), with the lowest levels in elderly adults (>70 y) (50–53). In this paper, we were able to include BM from a healthy 6-y-old child as well as BM samples from healthy adults as normal controls for comparison with BM from infants with IL-2RG– and JAK3-deficient SCID. As additional controls, we included BM from two younger patients (3 and 22 mo) on enzyme treatment for ADA deficiency, a form of SCID in which g, signaling is normal, who showed partial recovery of T and B cell numbers by the time of marrow analysis.

Of note, although the LMPP and CLP immunophenotypes were very similar in normal and all types of SCID marrow, significant differences were seen between all the BM samples and those from CB, emphasizing the important influence of ontogeny and tissue source on immunophenotypic identification of lymphoid progenitors (47). Specifically, the CD34+linnegCD10+ CLP BM phenotype is very rare in this hematopoietic source.

FIGURE 5. Gene expression for B cell commitment and DNA rearrangement is maintained in the absence of IL-2RG/ JAK3 signaling. Quantitative PCR analysis of gene expression in HSC, LMPP, and CLP from BM of IL-2RG– SCID (n = 3), JAK3-SCID (n = 1), ADA-SCID (n = 2), and NBM (n = 2, one adult and one 6-y-old child), presented relative to expression of B cell progenitors (BP). Recombination activating gene 1 (RAG1) (A), DNA nucleotidyltransferase (DNTT, also known as TDT) (B), early B cell factor 1 (EBF1) (C), and cluster of differentiation 2 (CD2) (D). Equivalent numbers of HSC (CD34+linnegCD38neg), LMPP (CD34+linnegCD45RA+CD10negCD62Lhi), CLP (CD34+linnegCD10+), and BP (CD34+CD10+CD19+) were tested. Gene expression is shown in the sequence HSC, LMPP, CLP, and BP; *, no expression was detectable in these populations. Each biologic sample was assayed in technical triplicate; error bars represent SEM of the means of biologic samples.

Early B cell factor 1 (EBF1) is a transcription factor critical for the early stages of B lineage commitment (54). In mice, IL-7 has been shown to regulate expression of EBF1; lack of EBF expression is seen in IL-7Ra–/– or IL-7–/– mice, and EBF1 overexpression can partially abrogate the B cell deficiency seen with these IL-7 signaling defects (29, 44, 45, 55). In our previous studies and in the normal controls for the current paper, we consistently found EBF upregulation occurs first at the CLP stage when IL-7Ra is also first expressed (32) with persistent expression in fully B cell–committed CD34+CD19+ progenitors. Although LMPP in normal BM possess B cell potential, transcriptional evidence of B lymphoid commitment (EBF and PAX5) is not normally detectable at this earlier stage of lymphoid development (32). In contrast, in IL-2RG– and JAK3-deficient SCID patients, EBF expression was upregulated earlier in lymphoid commitment at the LMPP stage, a finding that could suggest an early skewing toward B lymphoid commitment in g, I-bearing multipotent progenitors that lack T and NK potential. However, it is also possible that this difference is a function of age rather than signaling effects, because LMPPs in the ADA patients also expressed EBF. It would thus appear that the B lineage predominance that occurs in normal infancy originates at the LMPP stage.

We have shown that, consistent with its earlier stage of lymphoid commitment, the LMPP population has greater NK and T cell potential than CD34+linnegCD10+ “CLPs,” a population which is mostly committed to the B cell lineage (32). Consistent with its greater T and NK potential, CD3e and CD2, two markers shared between cells of these lineages, are exclusively expressed in CD34+CD10+CD62Lhi LMPP (32). Experimental restrictions make it difficult to definitively prove the identity of the BM precursors that normally seed the human thymus, but from numerous mouse models and more limited studies of human thymic progenitors, it appears that more than one marrow progenitor, and even HSC, can migrate to the thymus and initiate T cell differentiation (56–62). It is likely that the human CD34+CD10+CD62Lhi LMPP population represents one such lymphoid progenitor that seeds the human thymus, given the finding of a similar immunophenotypic subset among human thymocytes and strong in vitro T cell potential (31, 32, 40, 41). The presence in IL-2RG– and JAK3-deficient BM of apparently normal LMPPs that express CD2 suggests that the impact of g, signaling on T cell...
but significantly upregulated in CLP. In normal BM, not affected in T-NK-B+ SCID.

EBF as activating pathways that induce B lymphopoiesis in the absence of IL-7. FLT3 expression has been used to discriminate murine LMPP from HSC by flow cytometry. However, in humans, although FLT3 mRNA is increased in LMPP, FLT3 protein is detectable at similar levels in HSC and LMPP (32). Because both RNA and protein expression of FLT3 are significantly downregulated in CLP and B cell progenitors (32), it is likely that if FL-induced signaling is responsible for B lymphoid differentiation in the setting of γc deficiency, this effect is predominantly at the LMPP stage, rather than on more B cell–committed progenitors such as CD34⁺CD10⁺ CLP and CD34⁺CD19⁺ B cell progenitors.

TSLP binds to IL-7Rα and activates Stat5 signaling through the TSLPR chain, independently from γc (69, 70). As with γc deficiency, B cell deficiency is seen in mice but not humans with IL-7Rα deficiency. However, TSLP KO mice have normal lymphopoiesis demonstrating that TSLP is redundant in mice when γc signaling is normal. Nonetheless, murine studies show that TSLP has a nonredundant role in the setting of γc deficiency; TSLPR/γc double-KO mice exhibit more severe lymphopenia than γc−/− mice, and TSLP administration can partially rescue lymphopenia in γc-deficient mice (71). It has been postulated that the normal B cell numbers seen in human γc deficiency could be because of alternative signaling from TSLP through the IL-7Rα/TSLPR complex. Although our data do not answer this possibility directly, the finding that surface TSLPR and IL-7Rα were readily detectable on CLP and B cell progenitors demonstrates that TSLP induced signaling in these lymphoid progenitors is at least possible.

Although B cells in patients with IL-2RG-SCID have undergone normal V(D)J Ig rearrangement (72, 73), most Jκ remain in germ-line configuration. The T cell defects present in all types of SCID would be expected to contribute to the functional defects of B cells. However, clinical data of immune reconstitution following transplantation show that an indirect effect on B cells is insufficient to fully explain the functional defects in γc-deficient B cells. In a large follow-up study of patients with SCID who had undergone nonmyeloablative transplantation (74), in the subset of patients with γc mutations, Ig deficiency was not corrected in the absence of donor B cell chimerism, even if T cell reconstitution was present. Thus, intrinsic functional defects exist in γc-deficient B cells. Of the cytokines that enlist the γc-associated receptors, IL-4 deficiency is known to be required for mature B cell function, and thus, impaired signaling through IL-4Rα/γc dimers could account for the B cell functional defects in human X-SCID (75, 76).

In summary, in view of the normal generation of both LMPP and CLP in the presence of IL-2RG and JAK3 deficiency, and the subsequent robust production of B cells, we conclude that γc signaling is not required for lymphoid commitment in humans. The normal expression of DNTT and RAG1 demonstrate that γc signaling is also not required for DNA recombination. The profound lymphoid defects seen in these patients therefore result from blocks to differentiation downstream from the multipotent lymphoid progenitor stage, as NK cells differentiate from more committed progenitors in the marrow and as T cell commitment is initiated and sustained in the thymus.

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The authors have no financial conflicts of interest.
LYMPHOID PROGENITORS IN γC AND Jak3-BDEFICIENT SCID MARROW

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