

# The BLNK adaptor protein has a nonredundant role in human B-cell differentiation

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**Background:** Expression of the pre-B-cell receptor (pre-BCR) by pre-BII cells constitutes a crucial checkpoint in B-cell differentiation. Mutations that affect the pre-B-cell receptor result in early B-cell differentiation blockades that lead to primary B-cell immunodeficiencies. BLNK adaptor protein has a key role in the pre-B-cell receptor signaling cascade, as illustrated by the abnormal B-cell development in the 4 patients with *BLNK* gene defects reported to date. However, the BLNK protein's precise function in human B-cell differentiation has not been completely specified.

**Methods:** B-cell development, including IgVH and Vk chain repertoires analysis, was studied in the bone marrow of a new case of BLNK deficiency *in vitro* and *in vivo*.

**Results:** Here, we report on a patient with agammaglobulinemia, with a total absence of circulating B cells. We detected a homozygous mutation in *BLNK*, which leads to the complete abrogation of BLNK protein expression. In the bone marrow, we identified a severe differentiation blockade at the pre-BI- to pre-BII-cell transition. IgVH gene rearrangements and selection of the IgH repertoire were normal, whereas the patient's pre-BI cells showed very restricted usage of the IgVk repertoire. Complementation of bone marrow progenitors from the patient with the *BLNK* gene and transplantation into NOD/SCID/γck mice allowed the complete restoration of B-cell differentiation and a normal usage of the IgVk genes. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

**Key words:** Agammaglobulinemia, B-lymphocyte, lymphopoiesis, BLNK protein, gene rearrangement, pre-B-cell receptor, SCID-hu mouse, gene therapy

During murine B-cell differentiation in the bone marrow (BM), expression of the pre-B-cell receptor (pre-BCR) by pre-BII cells constitutes a crucial checkpoint in B-cell development. The pre-BCR controls pre-BII-cell proliferation and differentiation<sup>1</sup> and mediates selection of the Igμ chain repertoire,<sup>2</sup> which leads to the counter-selection of self-reactive Igμ chains.<sup>3</sup> The pre-BCR is composed of 2 Igμ chains, 2 surrogate light chains, and the Igα-Igβ signaling molecules. Engagement of the pre-BCR results in the activation of several protein tyrosine kinases, including Bruton tyrosine kinase (Btk). Activated Btk and PLCγ2 bind to the BLNK/SLP65 adaptor protein and enable Btk to phosphorylate PLCγ2. Signaling through this complex results in the expression of the RAG proteins, which induces IgG recombination and promotes pre-BII-cell differentiation.

In humans, mutations that affect the pre-BCR's structure or signaling cascade result in severe B-cell differentiation blockades at the pre-BI- to pre-BII-cell transition, which, in turn, lead to primary B-cell immunodeficiencies.<sup>4</sup> The hallmarks of these primary B-cell immunodeficiencies include low or null peripheral B-cell counts, severe hypo- or agammaglobulinemia, and recurrent bacterial infections in early life. Approximately 85% of these patients have X-linked agammaglobulinemia disease and present *BTK* gene mutations. Approximately half of the remaining patients present autosomal recessive agammaglobulinemia caused by mutations that variously affect the Igμ chain, the Igα and Igβ signaling molecules, the λ5 chain of the surrogate light chain, the BLNK adaptor protein,<sup>4</sup> and, most recently, the p85a subunit of PI3K.<sup>5</sup>

Four patients with *BLNK* defects have been reported to date,<sup>4,6,7</sup> but the precise function of the BLNK protein in human B-cell development has not yet been determined. Here, we describe a new case of *BLNK* deficiency and provide a detailed molecular and cellular characterization of the B-cell differentiation blockade. Moreover, *BLNK* gene transfer into the patient's BM cells and subsequent transplantation into NOD/SCID/γck (NSG) mice enabled us to restore B-cell differentiation; this finding demonstrates the direct and nonredundant involvement of the BLNK protein, in pre-BII-cell differentiation and Ig light chain gene rearrangements in humans.

## METHODS

### Patient samples and cell preparations

Peripheral blood mononuclear cells and mononuclear BM cells were harvested from the patient after the provision of written informed consent. The constitution of a collection of BM samples from healthy controls and patients

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**Abbreviations used**

BCR: B-cell receptor  
 BM: Bone marrow  
 Btk: Bruton tyrosine kinase  
 INSERM: Institut National de la Santé et de la Recherche Médicale  
 NSG: NOD/SCID/γcko  
 RF: Reading frame

had been approved by the regional investigational review board (reference, DC 2011-1338), and the collection was used in accordance with French legislation and ethical guidelines. Peripheral blood mononuclear cells also were obtained from the patient's parents. Control mononuclear BM cells correspond to the unused residue of an allogeneic hematopoietic stem cell harvest from a healthy adult donor. Mononuclear BM cells were isolated by Ficoll-Hypaque density gradient centrifugation ( $d = 1.077 \text{ g/mL}$ ) (Lymphoprep, Axis-Shield, Oslo, Norway). CD34<sup>+</sup> hematopoietic progenitor cells were positively selected by using the human Miltenyi indirect CD34 Microbead Kit (Miltenyi Biotech, Bergisch Gladbach, Germany), as previously described.<sup>8</sup>

**Flow cytometry analysis and cell sorting**

Monoclonal antibodies against CD45 (HI30), CD34 (8G12), CD19 (HIB19), CD22 (HIB22), IgM (G20-127), IgD (IA6-2), BLNK (2B11), BLNK isotype (MDPC-177), and 7-aminoactinomycin D (7AAD) were obtained from BD Biosciences (San José, Calif). For BLNK and IgM intracellular staining, cells were fixed and permeabilized by using the BD Cytofix and BD Phosflow reagents from BD Biosciences. After staining, the cells were analyzed on an 8-color FACSCanto II cytometer (BD Biosciences) with gating on viable, 7AAD-negative cells. The data were processed by using FlowJo software (Treestar, Ashland, Ore). For repertoire analysis, CD34<sup>+</sup> cells were sorted on an ARIA II system (BD Biosciences).

**Mutation detection**

Genomic DNA was purified from peripheral blood mononuclear cells by using the DNA Blood Minikit (Qiagen, Courtaboeuf, France). After amplification, PCR products were purified by using Qiaquick PCR (Qiagen). DNA was sequenced by MWG-Biotech (Ebersberg, Germany) by using *BLNK*-specific primers (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

**Analysis of the IgVH and IgVk repertoires**

A high proportion (>70%) of sorted CD34<sup>+</sup> patient's cells expressed CD19 and, therefore, are referred to henceforth as pre-BI cells. Total RNA was extracted from these pre-BI cells by using the RNeasy Plus Microkit (Qiagen). RNA was reverse-transcribed by using the Super Script II Reverse Transcriptase Kit (Life Technologies, Carlsbad, NM), according to the manufacturer's instructions. After RT-PCR (by using VH1, VH3, or VH4 with CH1mu primers and Vk consensus with Ck primers) (Table E1), sequences were analyzed by using IgBLAST (NCBI). IgH CDR3 length was determined by counting amino acid residues between positions 94 and 102. Position 102 corresponds to a conserved tryptophan in all JH segments. D segments were identified according to criteria by Corbett et al.<sup>9</sup>

**Transduction of human CD34<sup>+</sup> cells and transplantation into NSG mice**

CD34<sup>+</sup> cells purified from the patient's BM were cultured as previously described<sup>8</sup> and transduced with a lentiviral construct that contained the human *BLNK* at a multiplicity of infection of 100. *BLNK* mRNA expression was monitored in transduced cells by using a quantitative PCR with a TaqMan probe (Hs00179459m1; Life Technologies) (see Fig E1 in this article's Online

Repository at [www.jacionline.org](http://www.jacionline.org)). *BLNK* transcript level was normalized against human endogenous GAPDH.

Nonobese diabetic severe combined immunodeficiency gamma NOD/SCID/γcko (NSG) mice (strain NOD.CgPrkdcscid Il2rgtm1Wj/SzJ, obtained from Charles River Laboratories, St Germain sur l'Arbresle, France) were housed in a specific pathogen-free facility. On the day before transplantation, 6-week-old mice were irradiated at 3 Gy with a cesium 137 source. Two mice received the patient's CD34<sup>+</sup> cells transduced with the *BLNK* vector (*BLNK*-TD1 and *BLNK*-TD2), and one mouse received the patient's CD34<sup>+</sup> cells transduced with the empty vector (mock). Twelve weeks after transplantation, NSG recipients were analyzed. Cells were harvested from femurs and spleens, and human cell engraftment (evaluated with the percentage of human CD45<sup>+</sup> cells) was determined by flow cytometry. In the *BLNK*-TD1 and *BLNK*-TD2 mice, the percentages of human CD45<sup>+</sup> cells were 21% and 4.3%, respectively, in the BM, and were 0.9% and 1%, respectively, in the spleen. For the mock mouse, human CD45<sup>+</sup> cells accounted for 4.5% of the BM cells and 0.5% of the spleen cells. For repertoire analysis, human CD45<sup>+</sup>CD19<sup>+</sup>IgM<sup>−</sup> cells and CD45<sup>+</sup>CD19<sup>+</sup>IgM<sup>+</sup> cells were sorted from the BM of *BLNK*-TD2, and human CD45<sup>+</sup>CD19<sup>+</sup>IgM<sup>−</sup> cells were sorted from the BM of the mock recipient on an ARIA II system (BD Biosciences). All experiments and procedures were performed in compliance with the French Ministry of Agriculture's regulations on animal experiments and had been approved by the regional animal care and use committee (reference, P2.IAS.065.09).

**Statistical analysis**

Differences in IgH, IgK gene, and CDR3 length usages were analyzed with a  $\chi^2$  test (Cochran-Mantel-Haenszel test) and were considered significant when  $P$  values were  $\leq 0.05$ .

**RESULTS****Blockade of B-cell differentiation in the patient's BM**

The patient was a boy born into a nonconsanguineous family. During childhood, he had recurrent otitis and lung infections. Agammaglobulinemia was diagnosed at the age of 6 years. CD19<sup>+</sup>CD20<sup>+</sup> B cells were absent in the peripheral blood, and no immunoglobulin could be detected in the serum. Immunoglobulin replacement therapy was initiated at that time and is ongoing. This observation prompted us to analyze B-cell differentiation in the patient's BM. Flow cytometry analysis revealed an almost complete absence of CD34<sup>+</sup>CD19<sup>+</sup> pre-BII/immature/mature B cells (0.4% vs 10.5% in the control subject), whereas CD34<sup>+</sup>CD19<sup>+</sup> pre-BI-cell frequency was elevated (4.9% vs 0.7% in the control subject) (Fig 1, A). In the patient's BM, 92% of the CD19<sup>+</sup> cells were CD34<sup>+</sup> compared with 6% in the control, a proportion within the range of values observed in samples from healthy children. Most of the patient's cells expressed a low level of CD22, whereas control cells were CD22<sup>bright</sup>, underlying the immature stage of patient's cells (Fig 1, B). Cell surface IgMs were not found on the patient's B cells (data not shown), whereas cytoplasmic IgMs were detected on fewer than 6% of the patient's CD19<sup>+</sup>CD22<sup>+</sup> B cells (compared with 55.7% for control cells) (Fig 1, B). These data indicate that the patient presented a B-cell differentiation blockade between the pre-BI and the pre-BII stages.

**The absence of BLNK expression in the patient's B cells due to a *BLNK* gene mutation**

Direct sequencing of the patient's DNA PCR products revealed a homozygous C-to-T substitution in codon 282 of exon 12 of the *BLNK* gene (g.844C>T), which results in the replacement of the

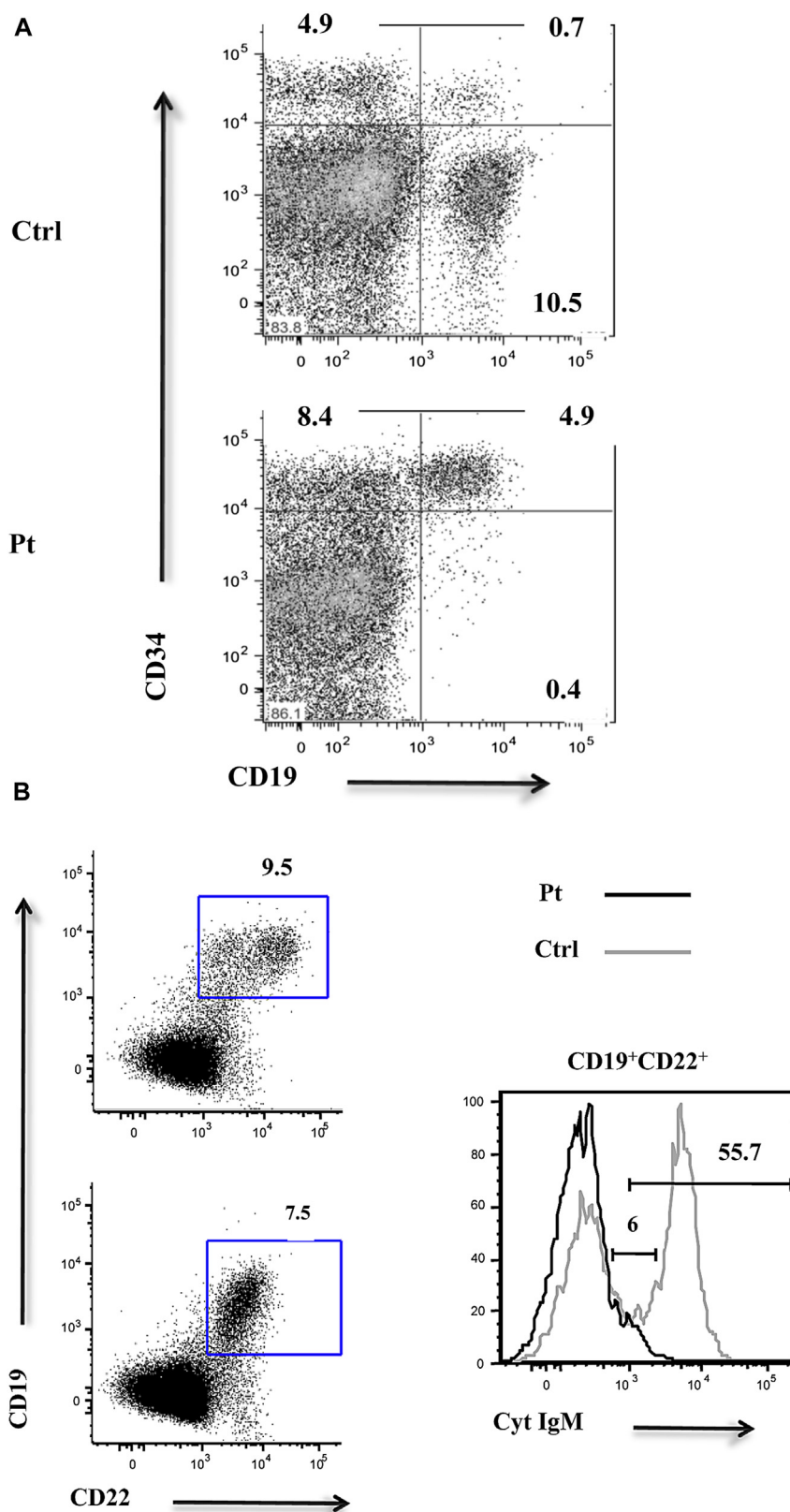


**FIG 1.** Analysis of bone marrow B-cell development in a patient with agammaglobulinemia. Bone marrow mononuclear cells harvested from the patient (12 years old) and from a control subject (20 years old) were analyzed by flow cytometry for cell surface expression of CD34 and CD19 (**A**); CD19, CD22, and cytoplasmic IgM (**B**).

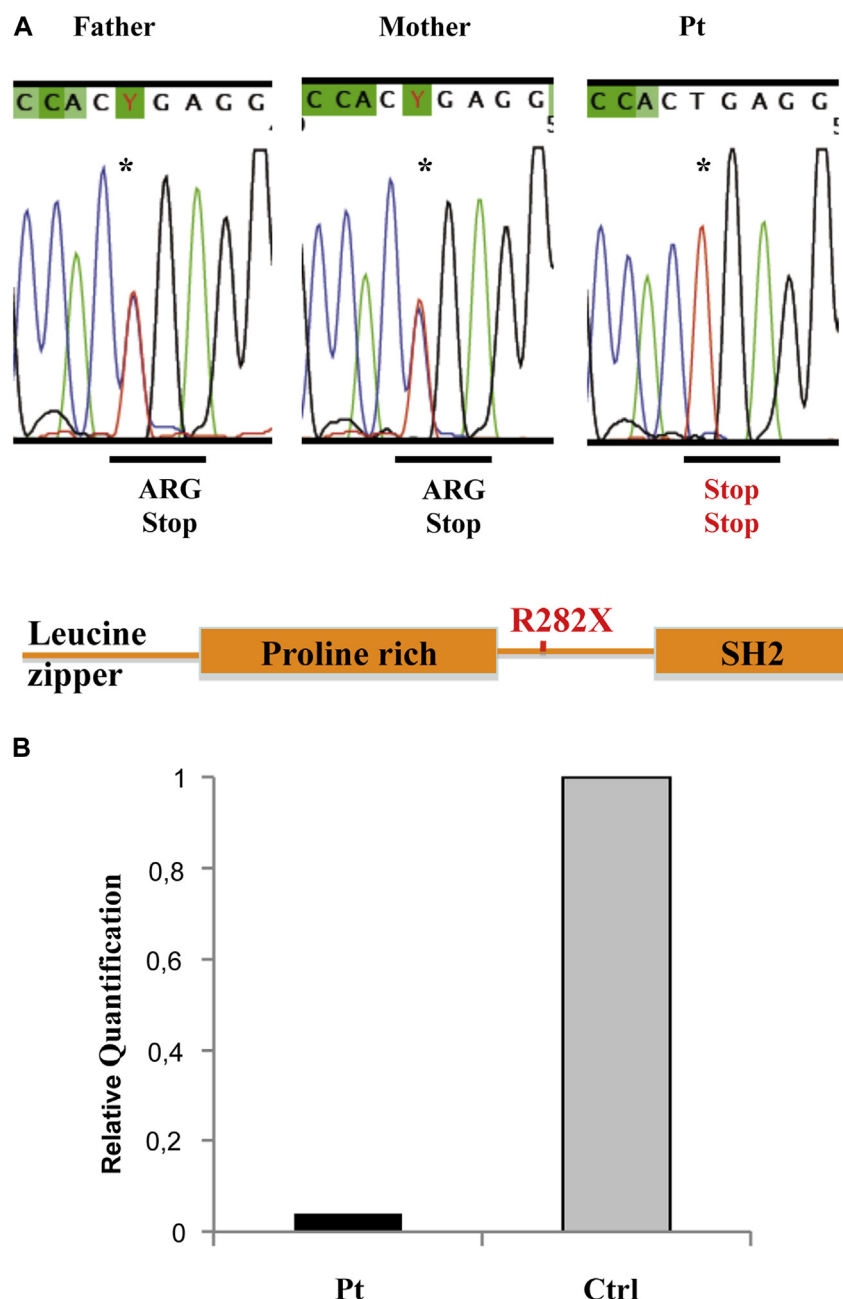


**FIG 2.** BLNK mutation in a patient with agammaglobulinemia. Electropherograms of genomic DNA from the patient and his parents by using BLNK-specific oligonucleotides. A homozygous g.844C>T substitution was found in the patient's CD34<sup>+</sup> cells (**A**, top panel). This led to a p.R282X mutation in the BLNK protein (**A**, bottom panel) affecting levels of *BLNK* transcripts (**B**) and BLNK protein expression as assessed by flow cytometry analysis (**C**). Light gray represents the isotype-matched control for BLNK staining.

wild-type arginine by a premature stop codon between the BLNK protein's "proline-rich" and "SH2" regions (Fig 2, A). It is noteworthy that the patient's parents were heterozygous for this mutation. The homozygous mutation resulted in very weak production of BLNK mRNA transcripts; this was likely due to nonsense-mediated mRNA decay (Fig 2, B) and explained the lack of the BLNK protein in CD34<sup>+</sup>CD19<sup>-</sup> and CD34<sup>+</sup>CD19<sup>+</sup> BM subpopulations as observed by flow cytometry (Fig 2, C). Thus, the homozygote pR.282X mutation identified in the *BLNK* gene of the patient induced a complete absence of the BLNK protein in B cells.

### IgH expression and selection independent of BLNK expression

To characterize IgVH gene expression in the patient's BLNK-deficient pre-BI cells, Igμ transcripts for the 3 major VH families (VH1, VH3, and VH4) were cloned and sequenced. One hundred six IgVH sequences were obtained and compared with control pre-BI sequences.<sup>10</sup> Analysis of the 3 VH1, VH3, and VH4 families, and the CDR3 length did not reveal any statistically significant difference between the patient's cells and control cells ( $P = .63$  for VH1,  $P = .15$  for VH3,  $P = .64$  for VH4,  $P = .44$  for IgVH

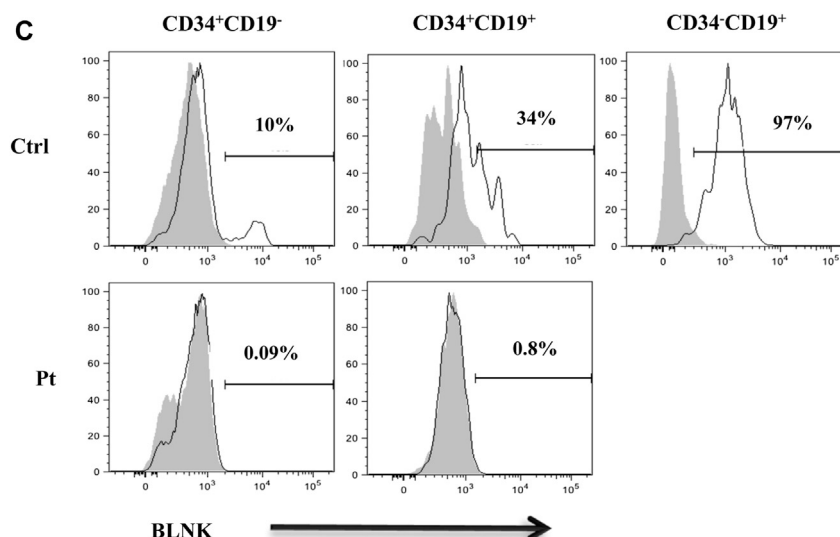


FIG 2. (Continued)

CDR3 length) (Fig 3, A and B). To determine whether BLNK expression influences CDR3 selection, CDR3 frame analysis was performed in BLNK-deficient pre-BI cells and control pre-BI cells. In each sample, approximately two-thirds of the IgVH sequences were found to be in frame, which indicated the presence of an Igμ-mediated selection in the patient's BLNK-deficient pre-BI cells (Table I). An analysis of the diversity heavy (DH) reading frame (RF) usage revealed that IgVH that contained CDR3s with hydrophilic (RF2) or hydrophobic (RF3) RFs were prevailing in the patient's pre-BI cells as in control pre-BI cells (Fig 3, C for selected DH genes). Altogether, these results indicated that Igμ-mediated selection may occur in the absence of BLNK expression.

### Restricted IgVκ repertoire in BLNK-deficient pre-BI cells

Analysis of the IgVκ repertoire in BLNK-deficient pre-BI cells was performed by using Vκ-Cκ consensus oligonucleotides. Of the 44 sequences analyzed, only 22 were different, and 80% of these sequences used a single Vκ gene (Vκ2.30) combined preferentially with the Jκ2 gene (Table II). Half of the sequences were found to be in frame, and most of them contained very long N diversity sequences. When compared with IgVκ repertoires from control pre-BI cells, the proportion of in-frame sequences was identical (Table I); however, as expected, restricted usage of the Vκ2.30 gene was not observed in control cells because Vκ2.30 gene was used in only 18.2% of the sequences.<sup>10</sup> Vκ usage was statistically highly different between the patient and the control ( $P = 9.3 \times 10^{-3}$ ). Moreover, there were no signs of extensive Vκ-Jκ rearrangements that normally mediate receptor editing because Jκ3 to 5 were not preferentially used in BLNK-deficient pre-BI cells (Table II). Analysis of our results suggests that BLNK deficiency is associated with a restricted IgVκ repertoire in the patient's pre-BI cells.

### Restoration of B-cell differentiation after gene therapy in NSG mice

To formally prove the *BLNK* mutation's involvement in the patient's phenotype, *BLNK* cDNA was transferred in CD34<sup>+</sup>

hematopoietic progenitors harvested from the patient. The expression of *BLNK* transgene was evaluated by quantitative RT-PCR in transduced and mock-transduced cells (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) before injection of these cells into irradiated NSG mice. Twelve weeks after transplantation, more than 80% of the human CD45<sup>+</sup> cells belonged to the B-cell lineage (as assessed by CD19 expression) in the BM of the 3 recipients mice (Fig 4). Interestingly, 6% and 8% of the CD19<sup>+</sup> BM cells from the 2 *BLNK*-recipient mice expressed surface IgM (compared with 1.7% for the mock recipient mouse) (Fig 4). Moreover, in the spleen, all the CD19<sup>+</sup> B cells from the *BLNK*-recipient mice expressed surface IgM, whereas B cells were not detected in the corresponding cell sample from the mock mouse (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Surface IgD were not detected (data not shown), which indicated that CD19<sup>+</sup>IgM<sup>+</sup> cells likely corresponded to immature/T1B cells. It is noteworthy that human IgM was detected in the serum of one of the *BLNK*-recipient mice (data not shown). These data indicate that the patient's BM phenotype could be rescued by *BLNK* complementation. This constitutes the first demonstration of the direct, nonredundant implication of the BLNK protein in the pre-BI to pre-BII transition during human B-cell differentiation.

### Reconstitution of the patient's IgVκ repertoire after gene therapy in NSG mice

We further analyzed IgVκ repertoires in reconstituted NSG mice (Fig 5). The analysis of IgVκ sequences in purified CD45<sup>+</sup>CD19<sup>+</sup>IgM<sup>+</sup> BM cells from the mock mouse confirmed the restricted usage of the IgVκ-2-30 gene (83% of sequences) as observed in the patient's CD34<sup>+</sup> BM pre-BI cells (Table II). Interestingly, most of the IgVκ sequences from the mock mouse were found to be identical to those of the patient's CD34<sup>+</sup> cells (underlined sequences in Table II). Amplification of IgVκ sequences from sorted CD19<sup>+</sup>IgM<sup>+</sup> and CD19<sup>+</sup>IgM<sup>+</sup> BM cells from the *BLNK*-TD2-recipient mouse revealed a decrease of Vκ2-30 gene usage to 21% and 11% in CD19<sup>+</sup>IgM<sup>+</sup> and CD19<sup>+</sup>IgM<sup>+</sup> cells, respectively, associated with a much more

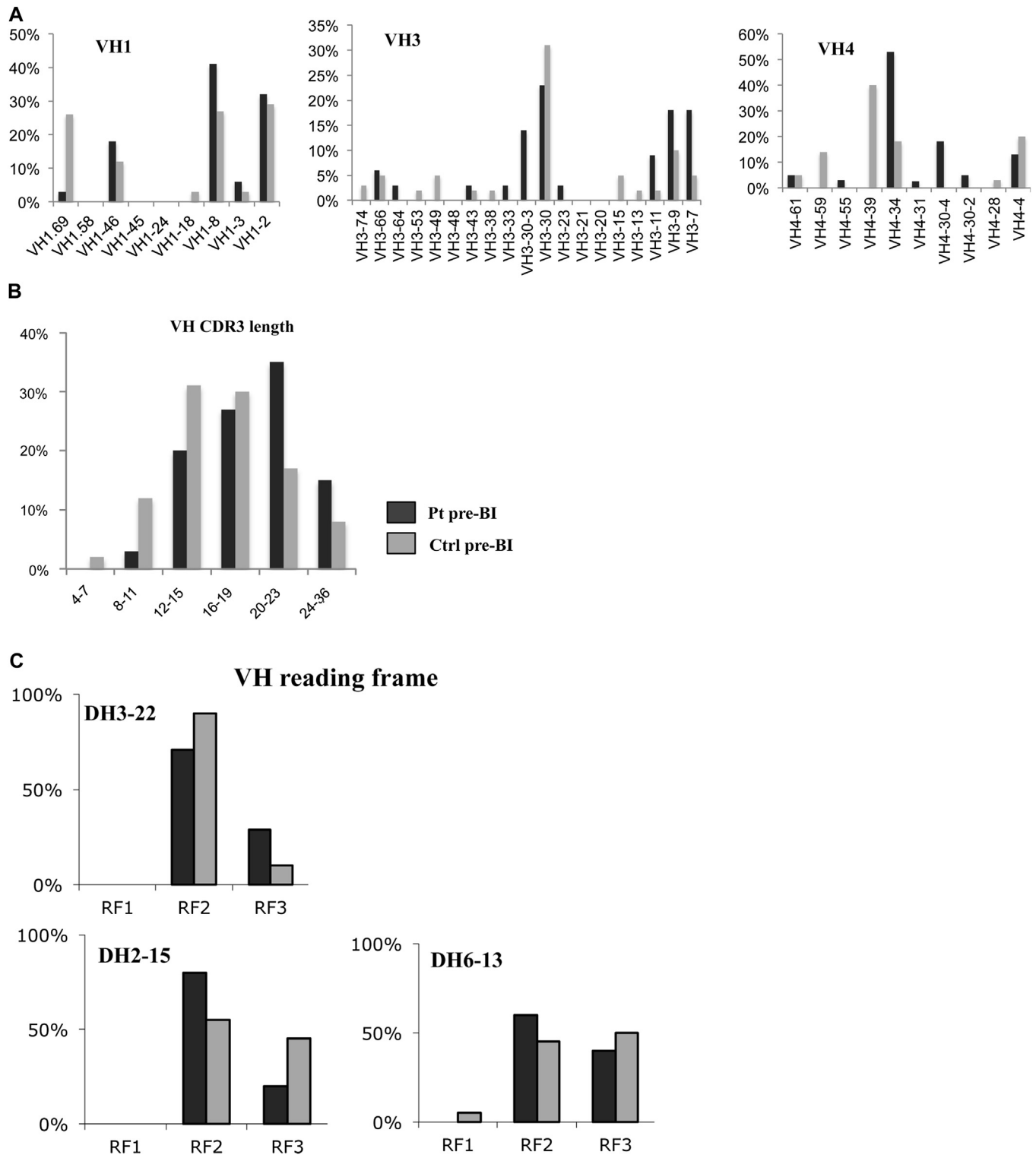


**FIG 3.** Normal IgVH repertoire in the patient's pre-BI cells. V<sub>H</sub>-D<sub>H</sub>-J<sub>H</sub> sequences from the patient's (black bars, 106 sequences) and control pre-BI cells (gray bars, 117 sequences) are represented: VH1, VH3, and VH4 gene usage (**A**). IgVH CDR3 length in amino acids (**B**). Reading frame usage in the IgVH CDR3s is shown for some D segments (14, 5, and 5 patient's pre-BI sequences analyzed for DH3-22, DH2-15, and DH6-13, respectively) (**C**).

diverse IgV $\kappa$  repertoire ( $P = 1.8 \times 10^{-3}$  and  $P = 3.1 \times 10^{-6}$ , respectively) and normalization of J $\kappa$  gene usage (Fig 5). Moreover, 70% of the IgV $\kappa$  sequences from CD19<sup>+</sup>IgM<sup>+</sup> cells were

found to be in frame, which indicated that BCR selection had occurred (data not shown). These results indicated that restoration of BLNK expression allowed the reconstitution of a normal IgV $\kappa$

**TABLE I.** IgVH CDR3 RF analysis of the patient's and the control's pre-BI cells

| CDR3   | Patient |    | Control |    |
|--------|---------|----|---------|----|
|        | Out     | In | Out     | In |
| VH (%) | 20      | 80 | 33      | 67 |
| Vk (%) | 50      | 50 | 45      | 55 |

repertoire in immature B cells. Analysis of our data shows for the first time that BLNK signaling is required for the initiation of IgVκ gene rearrangements at the pre-BII-cell stage.

## DISCUSSION

To date, 4 patients with *BLNK* mutations have been described.<sup>4,6,7</sup> BM was analyzed in only one of these cases and contained few CD19<sup>+</sup> cells, mostly CD34<sup>+</sup>. Here, we report on a new case of *BLNK* deficiency characterized by agammaglobulinemia and the total absence of circulating B cells. The *BLNK* gene mutation introduces a premature stop codon that results in the abrogation of BLNK protein expression. In the BM, a high frequency of pre-BI cells and an almost absence of more mature B cells were observed. Accumulation of pre-BI cells has already been described in patients affected by other B-cell differentiation blockades, such as RAG or Artemis<sup>11,12</sup> (and our unpublished observation). In the patient, the few mononuclear cells that display the CD34<sup>+</sup>CD19<sup>+</sup> pre-BII/immature/mature B phenotype may correspond to either (i) cells that express a rearranged IgVκ gene, probably the Vk2.30, capable of binding to an Igμ chain to form a functional BCR, or (ii) pre-BI cells that have

attempted to further differentiate. The low expression of CD22 and CD45 (data not shown) and the high level of CD10 (data not shown) observed in this cell population compared with control cells argue in favor of the latter explanation and is in accordance with the pre-BI cell phenotype described by van Lochem et al,<sup>13</sup> Hystad et al,<sup>14</sup> and our unpublished observations. Moreover, the long N diversity sequences observed at the Vκ to Jκ junctions are indicative of IgVκ gene rearrangements that occur at the pre-BI stage (ie, when the terminal deoxynucleotidyl transferase is fully active).

The present study is the first to have included complementation assays of BLNK-deficient human cells with a view to formally confirming BLNK's role in B-cell development. We demonstrated that *BLNK*-corrected CD34<sup>+</sup> cells were able to differentiate up to the immature B-cell stage *in vivo* because human CD19<sup>+</sup>IgM<sup>+</sup>IgD<sup>-</sup> cells were found both in the BM and in the spleen in the recipient NSG mice. Moreover, these cells have the capacity to develop into plasma cells as documented by the Ig secretion. In contrast to Igμ<sup>-/-</sup> deficiency (in which only one-third of the IgVH sequences were found to be in frame in pre-BI cells<sup>10</sup>), we observed normal selection of the IgH repertoire. Therefore, our results indicate that the absence of BLNK expression (and thus of pre-BCR signaling) does not prevent IgVH expression and selection. One can legitimately hypothesize that the binding of newly formed Igμ chains to surrogate light chains is sufficient to select in-frame IgVH CDR3s in pre-BI cells.

BLNK deficiency was associated with a very restricted (mainly Vk2.30 gene usage) unselected IgVκ repertoire. When compared with IgVκ repertoires in normal pre-BI cells, the proportion of in-frame sequences was identical; however, restricted usage of the Vk2.30 gene was never observed in normal pre-BI cells.<sup>10</sup>

**TABLE II.** Restricted IgVκ repertoire in patient's pre-BI cells

| Vk gene | Vk 3' end sequence      | N diversity                                           | Jk 5' end sequence          | No. of reads |
|---------|-------------------------|-------------------------------------------------------|-----------------------------|--------------|
| 3-15*   | CAGCAGTATAATACTGGCCTCC  | GGTAAAAATGA                                           | ACGTTCCGGCCAAGGGACCAAGG     | 1            |
| 1D-37†  | CAACGGACTTACAATGCCCTC   | GGA                                                   | TGTACACTTTTGCCAGGGGACCAAGC  | 2            |
| 7-3     | CTGCAGAGTAAGAATTTT      | GG                                                    | ATTCAATTTTCGGCCCTGGGACCAAAG | 3            |
| 1D-39   | CAACAGAGTTACAGTACCCCTCC | G                                                     | ACTTTTCGGCGGAGGGACCAAGG     | 4            |
| 2-30    | ATGCAAGGTACACACTGGCCTCC | GTTAATTTTCGGAAGAAAACCCCGG<br>TTAATTTATGGTGGTCACATGTGG | GTACACTTTTGCCAGGGGACCAAGC   | 2            |
| 2-30    | ATGCAAGGTACACAC         | GTACTTACCCTCATCACCCACATCA                             | TTTGGCCAGGGGACCAAGC         | 2            |
| 2-30    | ATGCAAGGTACACACTGGCC    | GAGGCCCTTTCTTTAT                                      | TTTGGCCAGGGGACCAAGC         | 2            |
| 2-30    | ATGCAAGGT               | GAGAGGGGG                                             | ACTTTTGCCAGGGGACCAAGC       | 2            |
| 2-30    | ATGCAAGGTACACACTGGCC    | CCTCCAGCT                                             | TGTACACTTTTGCCAGGGGACCAAGC  | 2            |
| 2-30    | ATGCAAGGTACACACTGGCCTCC |                                                       | GTACACTTTTGCCAGGGGACCAAGC   | 2            |
| 2-30    | ATGCAAGGTACACACTGGCCT   | CCGGGG                                                | CACTTTTGCCAGGGGACCAAGC      | 2            |
| 2-30    | ATGCAAGGTACACACTGGCCTCC | GAGGGA                                                | TGTACACTTTTGCCAGGGGACCAAGC  | 2            |
| 2-30    | ATGCAAGGTACACACTGGC     | TAAGT                                                 | ACTTTTGCCAGGGGACCAAGC       | 2            |
| 2-30    | ATGCAAGGTACACACTGGCCTCC | GGG                                                   | GCACTTTTGGCAGGGGACCAAGC     | 2            |
| 2-30    | ATGCAAGGTACACACTGG      | GA                                                    | GTACACTTTTGCCAGGGGACCAAGC   | 2            |
| 2-30    | ATGCAAGGTACACACTGGCCTCC | GGTATAACTCAT                                          | TTTCGGCCCTGGGACCAAAG        | 3            |
| 2-30    | ATGCAAGGTACACACTGGCCCCC | GATGAGGGGG                                            | ATTCATTTTCGGCCCTGGGACCAAAG  | 3            |
| 2-30    | ATGCAAGGTACACACTG       | AGG                                                   | TTCATTTTCGGCCCTGGGACCAAAG   | 3            |
| 2-30    | ATGCAAGGTACACACTGGCCT   | GATTCCCCGACCTTA                                       | GCTCACTTTTCGGCGGAGGGACCAAGG | 4            |
| 2-30    | ATGCAAGGTACACACTGGCCTCC | GAGAACC                                               | CTCACTTTTCGGCGGAGGGACCAAGG  | 4            |
| 2-30    | ATGCAAGGTACACACTGGCCT   | ACGAGGTAC                                             | TTCGGCGGARGGACCAAGG         | 4            |
| 2-30    | ATGCAAGGTACACACTGGCCT   |                                                       |                             | ?            |

\*Boldface sequences correspond to in-frame sequences.

†Underlined sequences are also found in CD19<sup>+</sup>IgM<sup>-</sup> cells isolated from the BM of the NSG mouse injected with nontransduced CD34<sup>+</sup> patient's cells (see Fig 5).

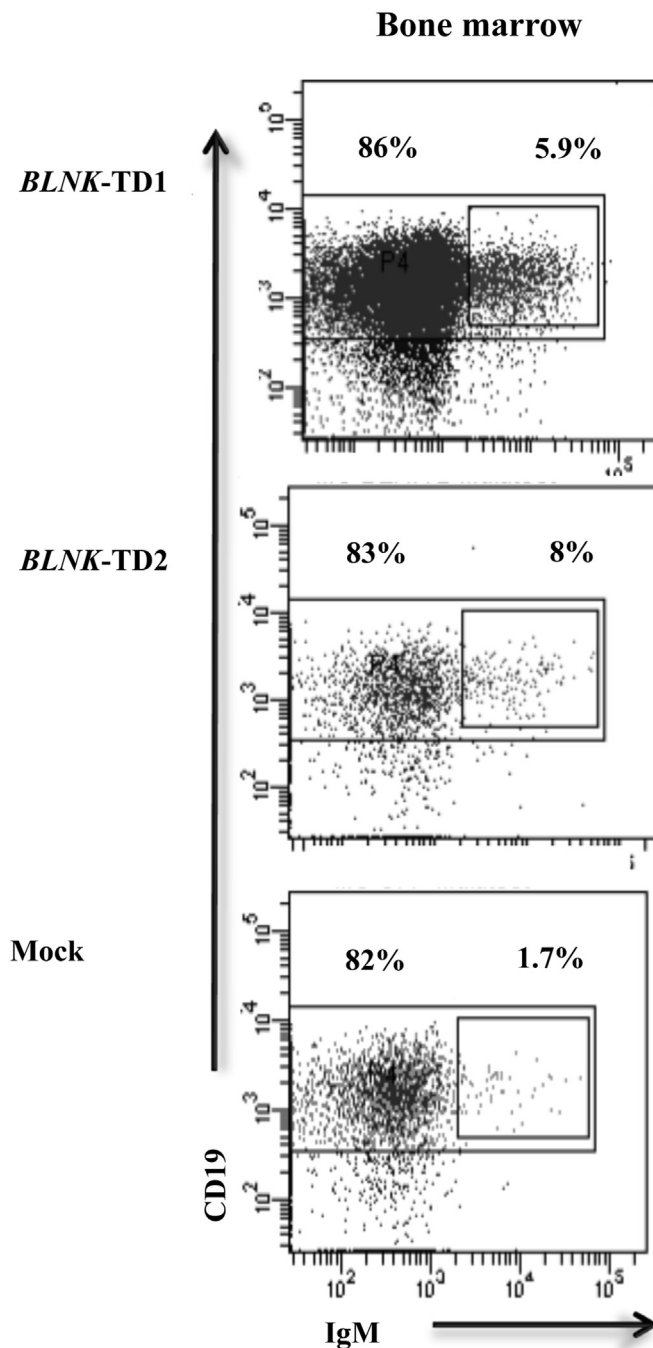


**FIG 4.** Ectopic expression of BLNK in the patient's CD34<sup>+</sup> cells leads to B-cell reconstitution *in vivo*. Patient's BM CD34<sup>+</sup> cells were transduced *ex vivo* with either a lentiviral vector that contained *BLNK* cDNA or the empty lentiviral vector (mock) before injection into NSG mice ( $n = 2$ , *BLNK*-TD1 and *BLNK*-TD2 for *BLNK* lentiviral vector;  $n = 1$ , for the empty vector). Twelve weeks after transplantation, human CD45<sup>+</sup> cells from the BM were analyzed for CD19 and IgM expression by flow cytometry.

Moreover, because Jk3 to 5 were not preferentially used in BLNK-deficient pre-BI cells, there was no sign of the ongoing Vk-Jk rearrangements (receptor editing), as reported for Ig $\mu$ <sup>-/-</sup> pre-BI cells.<sup>10</sup> It remains to be determined whether the observed Vk2-30 expression corresponds to a preferential access to this gene within the Vk locus. The gene therapy approach used to

induce BLNK protein expression in the BLNK deficient's cells allowed diversification of both the IgVk repertoire and Jk gene usage. Furthermore, 70% of the IgVk sequences from CD19<sup>+</sup>IgM<sup>+</sup> cells were found to be in frame, which indicated that BCR selection had occurred. Thus, our results showed that the restricted IgVk repertoire in the patient's pre-BI cells was due to BLNK deficiency and deficient pre-BCR signaling because pre-BCR signaling has been shown to induce upregulation of IRF4/8 and other factors needed for Ig light chain gene rearrangements.<sup>15,16</sup>

The B-cell differentiation defects observed in the absence of BLNK expression are more severe in humans than in mice. Indeed, in *Blnk*<sup>-/-</sup> mice, B-cell development is altered at the pre-BII to immature B-cell stages,<sup>17,18</sup> and a normally rare population of large pre-BCR-expressing cells dominates the BM.<sup>19</sup> Moreover, decreased serum IgM and IgG3 titers, a poor IgM immune response, and a normal IgG immune response have been observed in *Blnk*<sup>-/-</sup> mice.<sup>18</sup> Although the issue of the IgVk repertoire has not been analyzed in these studies, the normal IgG production observed during immune responses suggests that usage of the IgVk genes is not restricted. The comparison of the BLNK deficiency in humans and mice clearly suggests the presence of additional adaptors that can substitute for murine BLNK in the pre-BCR's signaling cascade. Alternatively, impaired pre-BCR signaling in *Blnk*<sup>-/-</sup> mice may be compensated for by other external stimuli. Among these stimuli, IL-7 is one candidate that appears not to be essential for proper B-cell production in human, whereas it is strictly required for B-cell differentiation in adult mice.<sup>20-23</sup> Although the exact explanation remains to be found, pre-BCR functioning appears to be very different in the 2 species, which highlights the lack of predictability of murine models for human B-cell deficiencies.

In terms of tumor development, some *Blnk* deficient mice were shown to develop spontaneously pre-B-cell tumors and pre-B-cell lymphomas, but the frequencies reported varied from 4% to 18% in the long-term follow-up. However, this observation suggests a role of BLNK as a tumor suppressor gene.<sup>24-26</sup> Decreased or absence of *BLNK* expression has also been reported in childhood B-cell acute lymphoblastic leukemia<sup>27-29</sup> at a variable frequency (from less than 1% to 50%). In these cases, BCR-ABL1 translocations induce a constitutive activation of the Jak3/STAT5 pathway, which, in turn, increases the levels of the pre-BCR and drives proliferation and survival of malignant pre-B cells. Thus, alteration in pre-BCR signaling pathway such as mutations in the BLNK adaptor protein could facilitate pre-B-cell transformation.<sup>30</sup> However, the implication of BLNK deficiency as a primary event in pre-B-cell transformation remains unclear because no case of pre-B-cell leukemia has been reported in the 5 young patients with BLNK deficiency reported so far. In conclusion, our results indicate that BLNK has a nonredundant key function in human B-cell development by regulating the pre-BI to pre-BII transition and Ig light chain gene rearrangements.

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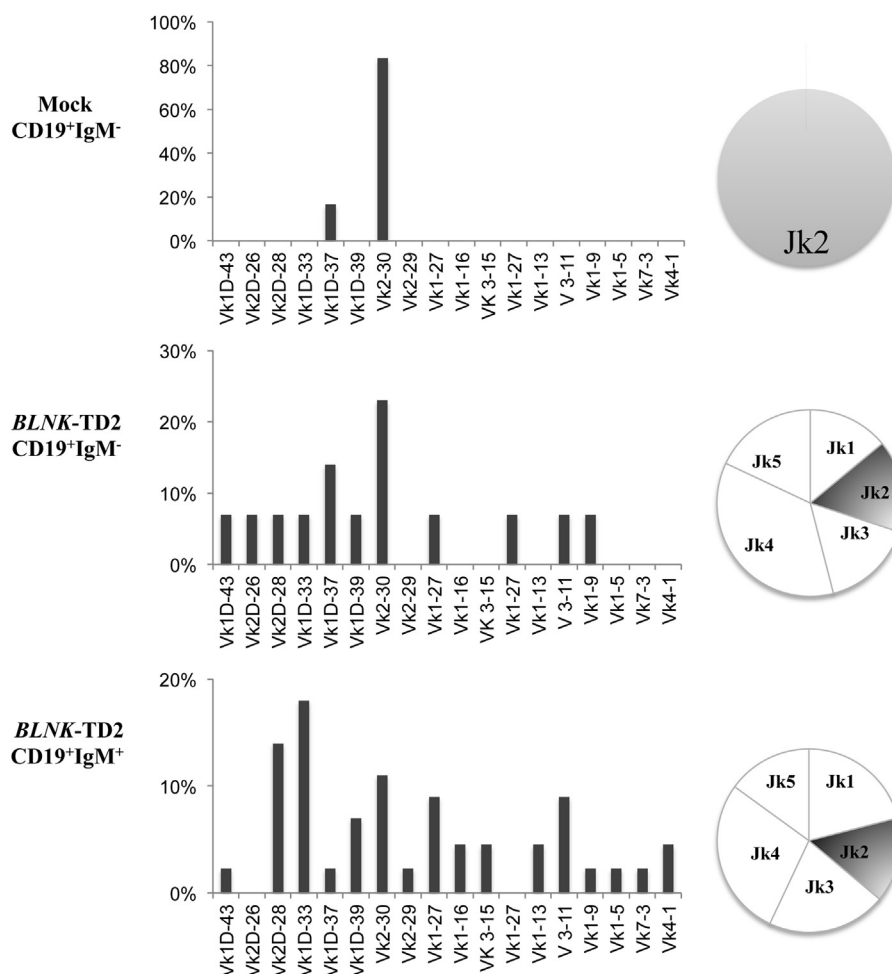


**FIG 5.** Reconstitution of the IgVκ repertoire after BLNK expression in the patient's cells. IgVκ repertoires were obtained from sorted CD19<sup>+</sup>IgM<sup>-</sup> cells from the mock mouse (*top*, 6/26 different sequences), sorted CD19<sup>+</sup>IgM<sup>-</sup> cells (*middle*, 14/51 different sequences) and CD19<sup>+</sup>IgM<sup>+</sup> cells (*bottom*, 44/61 different sequences) from the BLNK-TD2 mouse. Vκ usage (*left*) and Jκ usage (*right*) are indicated. The Vκ genes are represented in the 5'-to-3' direction on the Igκ locus.

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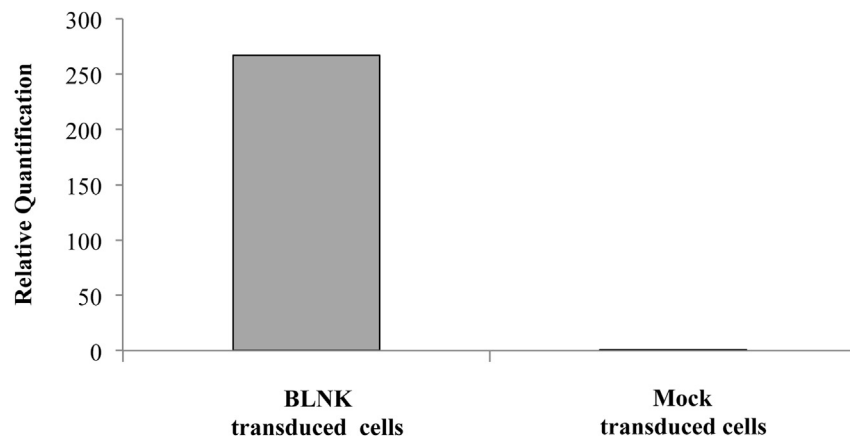


**FIG E1.** Detection of *BLNK* transcripts in the patient's transduced or mock-transduced CD34<sup>+</sup> cells.

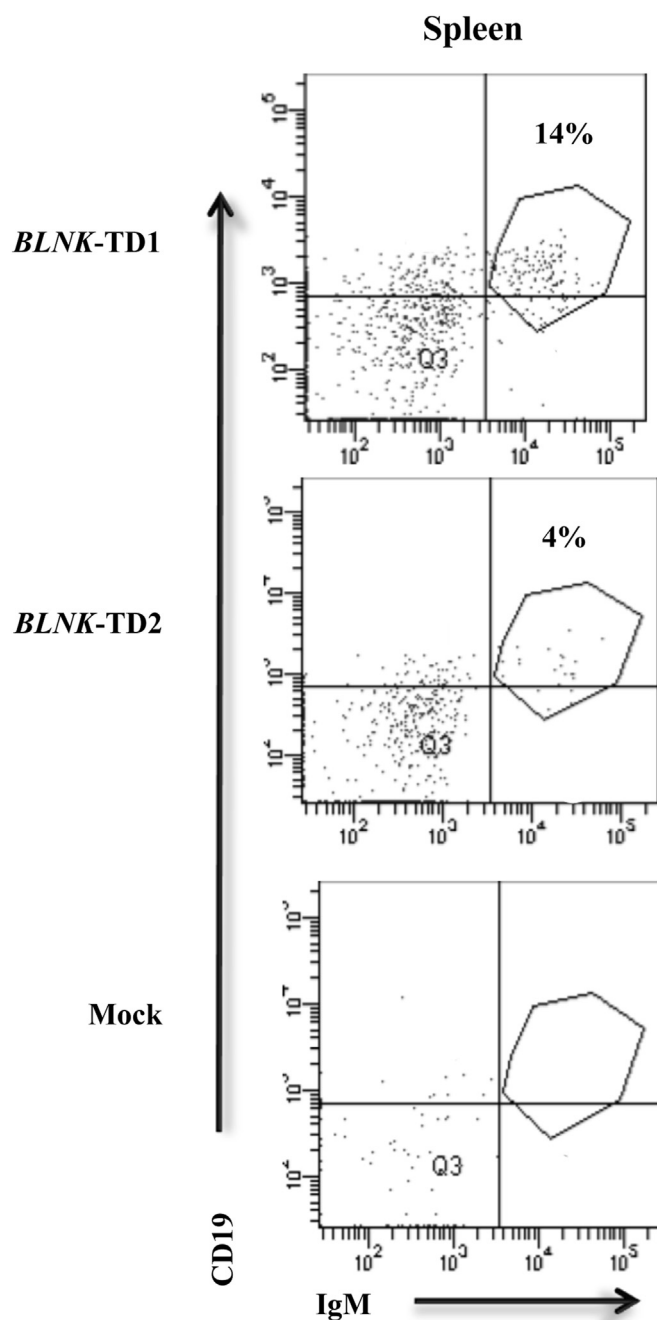


**FIG E2.** Ectopic expression of BLNK in the patient's CD34<sup>+</sup> cells leads to B-cell reconstitution *in vivo*. Patient BM CD34<sup>+</sup> cells were transduced *ex vivo* with either a lentiviral vector that contained *BLNK* cDNA or the empty lentiviral vector (mock) before injection into NSG mice (n = 2, *BLNK*-TD1 and *BLNK*-TD2 for *BLNK* lentiviral vector; n = 1, for the empty vector). Twelve weeks after transplantation, we analyzed by flow cytometry the expression of CD19 and IgM in the human CD45<sup>+</sup> spleen population.

**TABLE E1.** Oligonucleotides sequences

| Oligonucleotides | 5' → 3'                                      |
|------------------|----------------------------------------------|
| Blnk Fw1         | ATTCGTGTCTCTTACAATTACCAAAC                   |
| Blnk Rev1        | GATTTCAGTATTTCTGAGATGCC                      |
| Blnk Fw2         | TGTATTTCGAGAAGAGTAGGAGAC                     |
| Blnk Rev2        | CTTCTCATCCAGGTAACAGAGA                       |
| Blnk Fw3         | CAGCTTCTAGCATCGGCTTTG                        |
| Blnk Rev3        | ACTACATCTCCACTGTAGTCAA                       |
| Blnk Fw4         | GAAGGCAGGTGCGAAATGT                          |
| Blnk Rev4        | GCGGCTGACCACCAGCATGTAAT                      |
| Blnk Fw5         | GGCAGGAATGGTCCTGACGG                         |
| Blnk Rev5        | CCTCAAGCAGCAGCCAGGTT                         |
| Blnk Fw6         | CTGGATCTCAATGGCTTCCAC                        |
| Blnk Rev6        | GGGCAGTCAATAGCAGGTTGT                        |
| Blnk Fw7         | CTCCCATACAGTCAACTATGAA                       |
| Blnk Rev7        | GCACTGTCAATTTCTGAGCCTC                       |
| Blnk Fw8         | GAGTGTGAATTTTCTCAATGGC                       |
| Blnk Rev8        | GTGATTACTCTTCATAGTTGATCT                     |
| Blnk Fw9         | CAGAGAGGCCAGCCTCAGTG                         |
| Blnk Rev9        | ATGAAGTCAACAGGGCAGTGG                        |
| Blnk Fw10        | GCTCCATTTTGTCTTCGTTGTG                       |
| Blnk Rev11       | GAGTTTCATGTTTACAATTGCC                       |
| Blnk Fw12        | TTGGGATGAGGACAGACATTTTC                      |
| Blnk Rev13       | CAGGCCTATCAGACTCTAGGA                        |
| Blnk Fw14        | GGGATAGGCATAGAAAGAGTTC                       |
| Blnk Rev14       | CTTCTTCTCAGAAGACATGCTC                       |
| Blnk Fw15        | GGTGTAATGTAGAGGCCTGTC                        |
| Blnk Rev15       | GAGCCACTGCACTCCAGTGAA                        |
| Blnk Fw16        | GGAATGTAATATAGGTGTTCTTCA                     |
| Blnk Rev16       | CTCCTCATCTCTAGCATCTATTA                      |
| Blnk Fw17        | GAGAGAAGATGGGAGGAGTGG                        |
| Blnk Rev17       | AGTGTCTGAAGCAATGGTATTGATC                    |
| Blnk Fw          | AAGTTTAAACCCATGGACAAGCTTAATA<br>AAATAACCGTCC |
| Blnk Rev         | TTGTTTAAACTCATTATGAACTTTAACT<br>GCATACTTCAG  |
| VH1 Fw           | ATGGACTGGACCTGGAGGGTC                        |
| VH3 Fw           | ATGGAGTTTGGGCTGAGCTGG                        |
| VH4 Fw           | ATGAAACACCTGTGGTTCTT                         |
| CH1mu Rev        | AATTCTCACAGGAGACGA                           |
| Vκ cons. Fw      | ATGACCCAGTCTCCATCCTCCCTG                     |
| Cκ Rev           | CGGGAAGATGAAGACAGATG                         |