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 IgVκ repertoire. Complementation of bone marrow progenitors from the patient with the *BLNK* gene and transplantation into NOD/SCID/γcko mice allowed the complete restoration of B-cell differentiation and a normal usage of the IgVκ genes. (J Allergy Clin Immunol 2014;134:145-54.)

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[1] and mediates selection of the Igμ chain repertoire,[2] which leads to the counter-selection of self-reactive Igμ chains.[3] 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.[4] 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,[4] and, most recently, the p85a subunit of PI3K.[5]

Four patients with *BLNK* defects have been reported to date,[4,6,7] 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/γcko (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 g/mL) (Lymphoprep, Axis-Shield, Oslo, Norway). $CD34^+$ hematopoietic progenitor cells were positively selected by using the human Miltenyi indirect CD34 Microbead Kit (Miltenyi Biotech, Bergisch Gladbach, Germany), as previously described.[8]

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, $CD34^+$ 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).

Analysis of the IgVH and IgVκ repertoires

A high proportion (>70%) of sorted $CD34^+$ 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.[9]

Transduction of human $CD34^+$ cells and transplantation into NSG mice

$CD34^+$ cells purified from the patient's BM were cultured as previously described[8] 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). 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 $CD34^+$ cells transduced with the BLNK vector (*BLNK*-TD1 and *BLNK*-TD2), and one mouse received the patient's $CD34^+$ 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 $CD45^+$ cells) was determined by flow cytometry. In the *BLNK*-TD1 and *BLNK*-TD2 mice, the percentages of human $CD45^+$ 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 $CD45^+$ cells accounted for 4.5% of the BM cells and 0.5% of the spleen cells. For repertoire analysis, human $CD45^+CD19^+IgM^-$ cells and $CD45^+CD19^+IgM^+$ cells were sorted from the BM of *BLNK*-TD2, and human $CD45^+CD19^+IgM^-$ 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 χ^2 test (Cochran-Mantel-Haenszel test) and were considered significant when P values were ≤.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. $CD19^+CD20^+$ 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 $CD34^-CD19^+$ pre-BII/immature/mature B cells (0.4% vs 10.5% in the control subject), whereas $CD34^+CD19^+$ 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 $CD19^+$ cells were $CD34^+$ 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 $CD22^{bright}$, 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 $CD19^+CD22^+$ 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