

Differential expression of CD21 identifies developmentally and functionally distinct subsets of human transitional B cells

Santi Suryani,^{1,2} David A. Fulcher,³ Brigitte Santner-Nanan,⁴ Ralph Nanan,⁴ Melanie Wong,⁵ Peter J. Shaw,⁶ John Gibson,⁷ Andrew Williams,⁵ and Stuart G. Tangye^{1,2}

¹Immunology and Inflammation Group, Garvan Institute of Medical Research, Darlinghurst; ²St Vincent's Clinical School, University of New South Wales, Kensington; ³Department of Immunology, Institute of Clinical Pathology and Medical Research, Westmead Hospital, Westmead; ⁴Discipline of Paediatrics, Nepean Clinical School, University of Sydney, Penrith; ⁵Department of Immunology and Allergy, Children's Hospital at Westmead, Westmead; ⁶Paediatrics & Child Health, Children's Hospital; and ⁷Blood and Marrow Transplantation Program, Institute of Haematology, Royal Prince Alfred Hospital, Sydney, Australia

The transitional stage of B-cell development represents an important step where autoreactive cells are deleted, allowing the generation of a mature functional B-cell repertoire. In mice, 3 subsets of transitional B cells have been identified. In contrast, most studies of human transitional B cells have focused on a single subset defined as CD24^{hi}CD38^{hi} B cells. Here, we have identified 2 subsets of human transitional B cells based on the differential expression of CD21. CD21^{hi} transitional cells displayed higher expres-

sion of CD23, CD44, and IgD, and exhibited greater proliferation and Ig secretion in vitro than CD21^{lo} transitional B cells. In contrast, the CD21^{lo} subset expressed elevated levels of *LEF1*, a transcription factor highly expressed by immature lymphocytes, and produced higher amounts of autoreactive Ab. These phenotypic, functional, and molecular features suggest that CD21^{lo} transitional B cells are less mature than the CD21^{hi} subset. This was confirmed by analyzing X-linked agammaglobulinemia patients and the ki-

netics of B-cell reconstitution after stem cell transplantation, which revealed that the development of CD21^{lo} transitional B cells preceded that of CD21^{hi} transitional cells. These findings provide important insights into the process of human B-cell development and have implications for understanding the processes underlying perturbed B-cell maturation in autoimmune and immunodeficient conditions. (Blood. 2010;115:519-529)

Introduction

B-cell development begins in the bone marrow (BM) from pluripotent hematopoietic stem cells (HSCs), which progress through the pro- and pre-B-cell stages. Pre-B cells continue the developmental process by initiating Ig variable gene rearrangement and becoming an immature B-cell expressing a functional IgM B-cell receptor (BCR) capable of binding specific antigens. Before exiting the BM, immature B cells undergo negative selection to prevent self-reactive cells from entering in the periphery. After this first selection checkpoint, immature B cells enter the periphery as transitional B cells to continue the maturation process.¹⁻³

The transitional stage of B-cell development represents a second stage where self-reactive B cells are purged from the mature B-cell repertoire.⁴ Indeed, approximately 40% of transitional cells in human peripheral blood (PB) are self-reactive; consequently, only approximately 50% develop into mature B cells.^{3,5,6} In mice, 2 classification systems have been described to distinguish subsets of transitional B cells. Loder et al defined 2 subsets: transitional type 1 (T1) and type 2 (T2), with them having CD24^{hi}CD21^{lo}CD23^{lo}IgM^{hi}IgD^{lo} and CD24^{hi}CD21^{hi}CD23^{hi}IgM^{hi}IgD^{hi} phenotypes, respectively.⁷ Alternatively, Allman et al identified 3 subsets of transitional cells as AA4⁺CD23⁻IgM^{hi} (T1), AA4⁺CD23⁺IgM^{hi} (T2), and AA4⁺CD23⁺IgM^{lo} (T3).⁸ More recently, T3 cells have been proposed as being an anergic population that does not give rise to mature B cells.^{9,10} Each of these different

stages of transitional B-cell development is differentially dependent on signals delivered through the BCR and cytokine receptors. Thus, mutations in *syk*, *baff*, or *baff-r* arrest B-cell development at the T1 to T2 stage,¹¹⁻¹³ whereas mutations in *btb*, *lyn*, *vav*, *BLNK*, and *cd45* prevent maturation of T2 B cells into mature B cells.^{7,8,14-19}

Most of our knowledge of B-cell development has been derived from studies in mice, whereas several of the corresponding processes in humans remain incompletely defined. Because mutations in key genes (eg, *BTK*, *BLNK*, and *CD45*) result in profound differences in B-cell development in mice and humans,^{17,20-22} examination of human B-cell ontogeny is necessary to understand fully the cellular and molecular features and requirements of this process.

Human transitional B cells were first described as CD10⁺ or CD24⁺⁺CD38⁺⁺ B cells, which can be found at low frequencies in the blood and lymphoid tissues but are increased in cord blood (CB).^{5,23-25} Attempts have been made to separate further transitional B cells into T1-like and T2-like subsets based on the differential expression of IgD and CD38²⁴ or CD24 and CD38.²⁶ However, the delineation of subsets of transitional B cells according to these criteria is rather subjective, and the developmental and functional relationship between putative human transitional B-cell subsets remains unknown. Therefore, it is pertinent to identify markers that can better segregate transitional subpopulations to

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Table 1. BTK mutations in XLA patients

XLA patient	Mutation (DNA)	Amino acid change
XLA 1	c.1742G>C	p.TRP581SER
XLA 2	c.494G>A	p.CYS165TYR
XLA 3	c.494G>A	p.CYS165TYR
XLA 4	c.463G>A	p.CYS155ARG
XLA 5	c.82C>A	p.ARG28SER

Nucleotide 1 corresponds to the A of the ATG-translation initiation codon. XLA 1 has been previously described.²⁸

improve our understanding of the biology of human B-cell development.

We report the identification of human transitional B-cell subsets based on the differential expression of CD21, (ie, CD21^{lo} and CD21^{hi}). Detailed phenotypic, functional, molecular, and ontogenic assessment of these subsets demonstrated that the development of CD21^{lo} transitional B cells preceded that of CD21^{hi} transitional B cells, suggesting that the CD21^{lo} subset represents the precursor of CD21^{hi} transitional B cells. These findings provide important insights into human B-cell development and have implications for understanding the processes underlying perturbed B-cell maturation in autoimmune and immunodeficient conditions.

Methods

mAbs and reagents

The following monoclonal antibodies (mAbs) were used: fluorescein isothiocyanate (FITC)-conjugated anti-CD21 (Beckman Coulter); biotinylated anti-CD27 and anti-CD38, phycoerythrin-Cy7 (PE-Cy7) anti-CD20, PE-anti-CD23, PE-anti-B-cell activating factor belonging to the TNF family (BAFF) receptor (BAFF-R; eBioscience); biotinylated anti-CD44, PE-anti-CD27, allophycocyanin-anti-CD10, streptavidin-peridinin chlorophyll protein; PE-, FITC-, allophycocyanin-, and PE-Cy7 IgG1 isotype control mAb (BD Biosciences PharMingen), Pacific Blue-anti-CD21 (Exbio), FITC-anti-Bcl2 (Dako North America), biotinylated anti-IgD (Southern Biotechnology), and FITC-anti-CD24 (Caltag). Recombinant human CD40L has been described.²⁷ BAFF, interleukin-13 (IL-13), and IL-21 were from PeprTech; F(ab')₂ fragments of goat anti-human Ig were from Jackson Laboratories; IL-4 and IL-10 were provided by Dr Rene de Waal Malefyt (DNAX Research, Palo Alto, CA); CpG2006 was from Prologo.

Isolation of B-cell subsets

CB and PB were collected from healthy donors, patients with X-linked agammaglobulinemia (XLA; Table 1), or those recovering from HSC transplantation (HSCT). All studies were approved by institutional ethics review committees of all participating institutions, and informed consent was obtained in accordance with the Declaration of Helsinki. Normal spleens were obtained through the Australian Red Cross Blood Service. Mononuclear cells (MNCs) were prepared as previously described.²⁷ Total human B cells were isolated by negative isolation, labeled with mAb specific for CD20, CD10, CD21, and CD27, and then sorted into subsets of CD21^{lo} transitional (CD20⁺CD27⁻CD10⁺CD21^{lo}), CD21^{hi} transitional (CD20⁺CD27⁻CD10⁺CD21^{hi}), naive (CD20⁺CD27⁻CD10⁻), and memory (CD20⁺CD27⁺CD10⁻) B cells (FACS Aria, BD Biosciences).

Immunofluorescence staining

Five- and six-color flow-cytometric analysis was performed to characterize human transitional B-cell subsets. MNCs were labeled with anti-CD20, -CD10, -CD21, and -CD27 to resolve CD21^{lo} and CD21^{hi} transitional B cells, as well as an mAb to the protein of interest or control mAbs. To detect Bcl-2, cells were fixed in 2% formaldehyde and permeabilized and

stained in phosphate-buffered saline-0.5% saponin with anti-Bcl-2 mAb. Single-color compensation controls were run for all experiments. Samples were acquired on a FACSCanto II (BD Immunohistochemistry Systems) and analyzed using FlowJo (TreeStar).

Rhodamine assay

PB B cells were labeled with anti-CD20, -CD10, -CD21, and -CD27 mAbs, washed in phosphate-buffered saline, and then stained with rhodamine 123 (R123, 12.5 μM; Sigma-Aldrich).²⁹ Samples were collected at various time points, fixed with 1% formaldehyde, and analyzed for extrusion of R123.

KREC assay

Total genomic DNA was extracted from sorted B-cell subsets. The ratio of κ recombination excision circles (KREC) joints (signal joint) to the Jκ-Cκ recombination genomic joints (coding joint) was determined as previously described.^{30,31}

RNA isolation and microarray

Total RNA was extracted from CB and PB B-cell subsets. cRNA was synthesized and amplified using biotin-labeled ribonucleotide and T7 polymerase (Affymetrix). Biotinylated cRNA was then hybridized to Human Genome U133 Plus 2.0 Affymetrix Arrays. The resulting data were analyzed by GeneSpring Software, Version 7.3.1. Detailed descriptions of each microarray experiment are provided at www.ncbi.nlm.nih.gov/geo (accession no. GSE17186).

Quantitative PCR

Polymerase chain reaction (PCR) primers (Integrated DNA Technologies) were designed using the Roche UPL primer design program. Primer sequences and Roche UPL probes are as follows: *Glyceraldehyde 3-phosphate dehydrogenase*, UPL probe 60, 5' ctctgctcctctgttcgac, 3' acgacaaatccgttgactc; *Lymphoid enhancing binding factor-1* (LEF-1), UPL probe 17, 5' cagatgtcaactccaacaagg, 3' ggagacaaggataaaaagttaggg; KIAA1199, UPL probe 43, 5' ggaccagatgctggaagaag, 3' cttgaagccaacgaatgc. Amplification reactions were performed using the Roche LightCycler 480 Probe Master Mix with the following conditions: denaturation at -95°C for 10 minutes; amplification, 45 cycles at 95°C for 10 seconds, 65°C for 30 seconds, and 72°C for 5 seconds, and cooling at 40°C for 30 seconds. All reactions were standardized to glyceraldehyde 3-phosphate dehydrogenase.

In vitro culture

Sorted B-cell subsets (5-15 × 10³ cells) were cultured in media alone or with combinations of CD40L (1:200), CpG2006 (1 μg/mL), BAFF (500 ng/mL), anti-Ig (2.5 μg/mL), IL-4 (100 U/mL), IL-10 (100 U/mL), IL-13 (10 ng/mL), or IL-21 (50 ng/mL). To investigate survival, the cells were cultured for a total of 96 hours. At different times, a known number of CaliBRITE beads (BD Biosciences) were added to culture wells before harvesting, and the number of viable B cells was calculated as a function of the ratio of beads to live cells. For proliferation, cells were cultured for 5 days, and the incorporation of [³H]thymidine (0.5 μCi; MP Biomedicals) was determined during the final 18 hours.

Enzyme-linked immunosorbent assays

B-cell subsets (5-10 × 10³/well per 200 μL) were cultured in medium alone or with CpG alone, CD40L alone, or in combination with IL-21. Supernatants were harvested on day 12, and secretion of IgM, IgA and IgG was determined by enzyme-linked immunosorbent assay.^{27,32} Auto-reactivity was measured by antinuclear antibody (ANA) enzyme-linked immunosorbent assay as previously described⁵ except that bound IgM was detected with biotinylated goat F(ab')₂ antihuman IgM Ab (Southern Biotechnology) followed by horseradish peroxidase-streptavidin (Jackson ImmunoResearch).

Statistical analyses

All data were analyzed using GraphPad Prism software by repeated-measures analysis of variance and Tukey multiple comparison posttest when significance differences ($P < .05$) between groups were detected.

Results

Identification of 2 human transitional B-cell subsets

Human transitional B cells can be identified either by high expression of CD24 and CD38²³⁻²⁵ (Figure 1A left panel) or expression of CD10 (Figure 1A right panel).^{24,25} For the purposes of this study, we identified human transitional B cell as CD20⁺CD27⁻CD10⁺ cells, and naive and memory B cells as CD20⁺CD27⁻ and CD20⁺CD27⁺ cells, respectively (Figure 1B left panel). A previous study of HIV-infected persons noted differential expression of CD21 on CD10⁺ transitional B cells.³³ Based on this, we divided human transitional B cells into subsets corresponding to CD21^{lo} and CD21^{hi} populations (Figure 1B right panel). To ensure that the CD21^{lo} and CD21^{hi} transitional B-cell populations indeed represented distinct subsets, their phenotype was determined. IgD, CD44, and BAFF-R were consistently higher on CD21^{hi} transitional B cells compared with CD21^{lo} transitional cells (Figure 1C; supplemental Figure 1A-C, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). When multiple donors were examined, there was a clear trend that the level of CD23 (expressed as mean fluorescence intensity [MFI]) and frequency of CD23⁺ cells were greater for CD21^{hi} compared with CD21^{lo} transitional B cells (ie, MFI: 532 ± 109 vs MFI: 171 ± 27 and 62.6% ± 7.0% vs 23.2% ± 2.7%, respectively; mean ± SEM [n = 8]; Figure 1C; supplemental Figure 1A-C). However, it is also clear that CD23 expression on CD21^{lo} and CD21^{hi} transitional B cells is heterogeneous. Thus, it was not feasible to isolate transitional B-cell subsets according to the differential expression of CD23 because the CD23⁺ subset of CD10⁺ transitional cells would include not only the CD21^{hi} population but also some of the CD21^{lo} transitional cells (Figure 1C; supplemental Figure 1A-C). Expression of CD10 on CD21^{lo} and CD21^{hi} transitional B cells was similar (Figure 1C). In contrast to CD10 and those markers presented in Figure 1C, expression of CD5, CD9, CD20, CD24, CD38, CXCR4, TACI, and BCMA on CD21^{lo} and CD21^{hi} transitional B cells was comparable (supplemental Figure 2). These phenotypic differences were noted irrespective of whether the transitional B cells were isolated from human CB, PB, or spleen (Figure 1C; supplemental Figure 2).

When the frequencies of B-cell subsets were enumerated, it was found that CB contained the greatest proportion of total transitional B cells (28.3% ± 5.4% of all B cells; n = 6) followed by PB (10.6% ± 1.0%; n = 35), whereas the spleen contained the lowest frequency (4.3% ± 1.9%; n = 7). The highest frequency of CD21^{lo} transitional B cells was detected in the spleen (67.6% ± 6.7% of all transitional B cells), whereas they were least represented in PB (24.7% ± 1.9%) (Figure 1D). Accordingly, CD21^{hi} transitional B cells were most abundant in PB (Figure 1D). In contrast, transitional B cells in CB were composed equally of both the CD21^{lo} and CD21^{hi} subsets (Figure 1D).

Human transitional, naive, and memory B cells can be resolved from one another by their differential expression of the ABCB1 transporter and their relative abilities to extrude the dye R123.²⁹ We confirmed this finding by demonstrating that naive B cells extruded R123, whereas memory B cells could not (Figure 1E). Both subsets

of transitional B cells extruded R123 to a comparable level, however not as efficiently as naive B cells (Figure 1E). The comparable extrusion of R123 by CD21^{lo} and CD21^{hi} transitional B cells was mirrored by the similar expression of *ABCB1* mRNA in these cells as determined by microarray analysis (not shown).

We determined the replication history of the 2 transitional subsets using a genomic DNA PCR assay that measures KRECs.³⁰ This analysis revealed that the in vivo proliferative activity of memory B cells exceeded that of naive cells by more than 2-fold (mean number of cell divisions ± SEM: 8.5 ± 1.2 and 3.60 ± 0.39, respectively, n = 4, $P < .01$; Figure 1F).^{30,31} The in vivo proliferation of memory B cells also significantly exceeded that of both subsets of transitional B cells ($P < .001$; Figure 1F). The proliferation of both subsets of transitional B cells (<1.5 divisions in vivo) was less than naive B cells, and there was no significant difference between the in vivo replicative history of the CD21^{lo} and CD21^{hi} transitional B cells (Figure 1F; $P > .05$). Thus, both the CD21^{lo} and CD21^{hi} subsets of transitional cells correspond to recently generated quiescent B cells.

CD21^{lo} and CD21^{hi} transitional B-cell subsets exhibit a unique gene expression profile

To demonstrate further that the CD21^{lo} and CD21^{hi} transitional B cells were distinct subsets, we performed microarray analysis on PB and CB B-cell subsets. We identified 6 genes that differed in their level of expression between CD21^{lo} and CD21^{hi} transitional B-cell subsets by more than 2-fold. *LEF-1*, *Deltex-1* (*DTX1*), *KIAA1199*, *A Kinase Anchor Protein 12* (*AKAP12*), and 1 unknown gene (Affymetrix probe 237187_at) were expressed at the highest levels in CD21^{lo} transitional B cells, followed by the CD21^{hi} transitional population, whereas expression in naive and memory B cells was even lower or extinguished (Figure 2A). In contrast, expression of another unknown gene (239287_at) was lowest in CD21^{lo} transitional B cells, increased in CD21^{hi} transitional B cells, and further up-regulated in naive B cells (Figure 2A). Quantitative PCR analysis confirmed that *LEF1* and *KIAA1199* were expressed at significantly higher levels in CD21^{lo} transitional B cells compared with the CD21^{hi} transitional, naive, and memory B-cell subsets ($P < .001$; Figure 2B). Collectively, this phenotypic and molecular analysis of human B cells suggests that CD10⁺CD21^{lo} and CD10⁺CD21^{hi} B cells corresponds to 2 separable subsets of transitional cells that differ not only from each other but also other mature B-cell subsets, ie, naive and memory cells.

In vitro analysis of survival of human B-cell subsets

The microarray analysis also revealed that expression of Bcl-2 was lower in CD21^{lo} transitional B cells compared with the CD21^{hi} subset and was increased further in naive and memory B cells (Figure 3A). These differences were confirmed by intracellular staining and flow cytometry (Figure 3B). This analysis revealed significantly higher expression of Bcl-2 protein in both naive and memory B cells relative to CD21^{lo} and CD21^{hi} transitional B cells ($P < .001$). The differential expression of Bcl-2 among B-cell subsets appeared to be unique for proapoptotic and antiapoptotic molecules because *BAD*, *BAX*, *BID*, and *BCLXL* were detected at similar levels in the transitional, naive, and memory cells in both CB and PB (Figure 3A). We next determined whether differential expression of Bcl-2 correlated with in vitro survival of human B-cell subsets. There was a trend that CD21^{hi} transitional B cells survived better than CD21^{lo} transitional cells (Figure 3C left), but this difference was not statistically significant. However, consistent with their increased expression of Bcl-2, survival of naive and memory B cells was significantly greater than that of both transitional B-cell

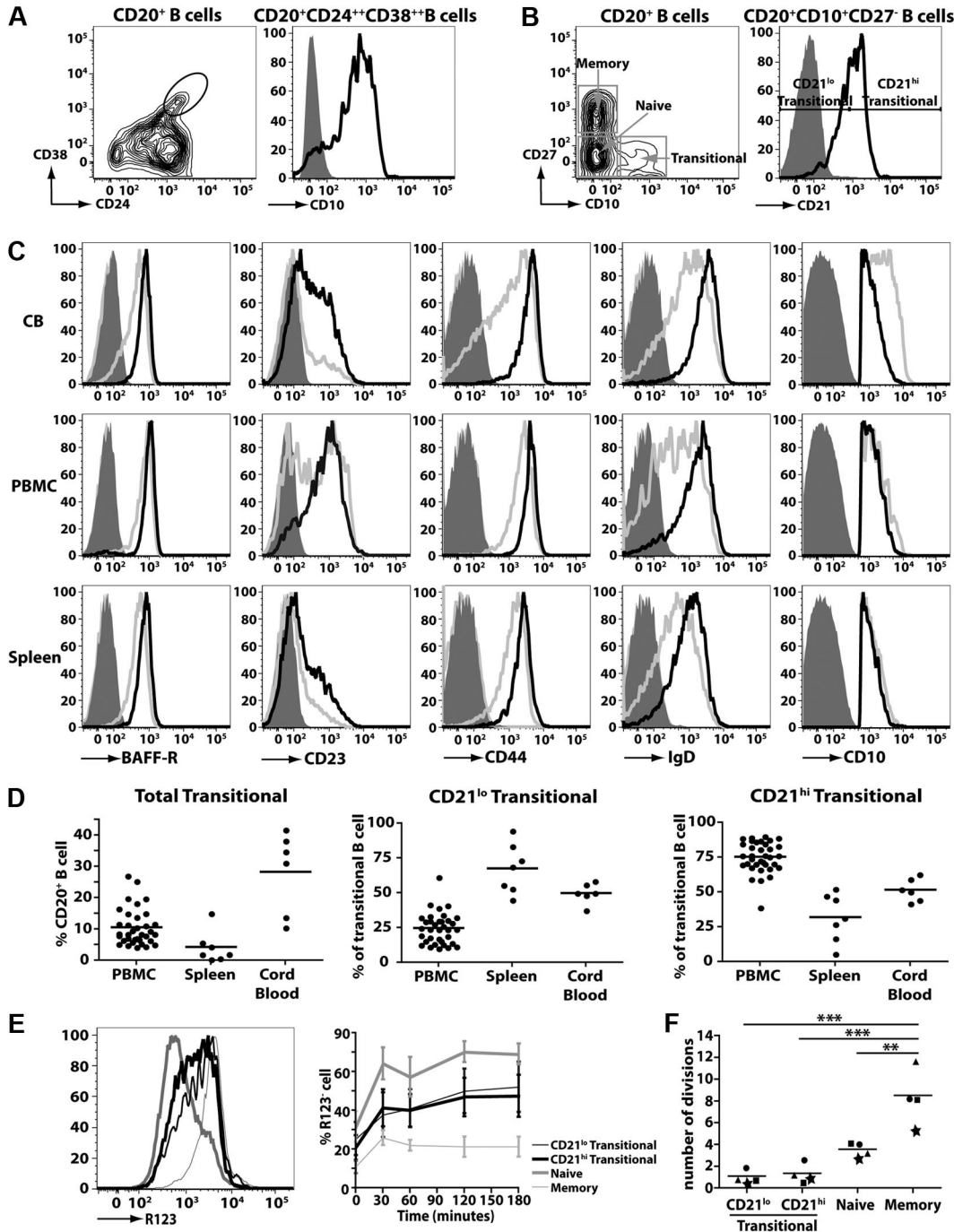
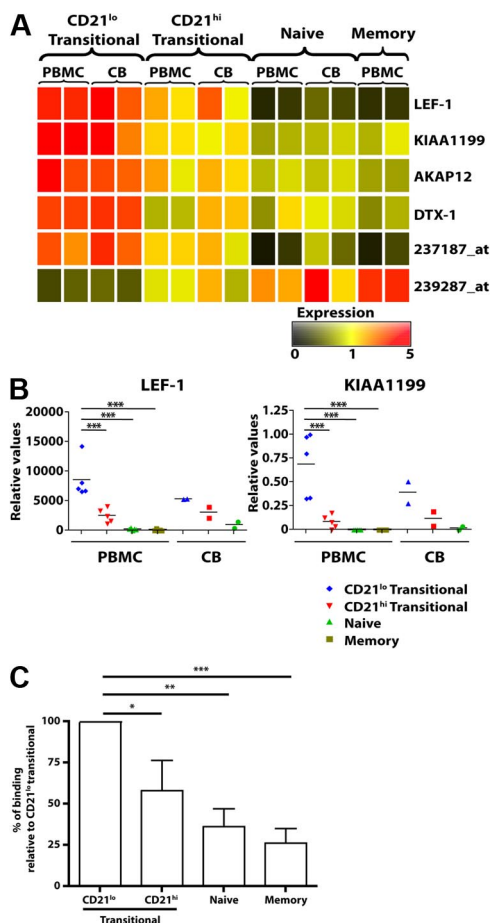


Figure 1. Identification of 2 subsets of human transitional B cells. (A-B) Peripheral blood mononuclear cells (PBMCs) were stained with mAbs against CD20, CD24, CD38, and CD10. (A) Left panel: Expression of CD24 and CD38 on CD20⁺ B cells; transitional B cells were initially defined as CD24^{hi}CD38^{hi} cells. Right panel: Expression of CD10 on CD24^{hi}CD38^{hi} B cells. (B) PBMCs were stained with mAbs specific for CD20, CD27, and CD10 to identify transitional (CD20⁺CD27⁻CD10⁻), naive (CD20⁺CD27⁻CD10⁻), and memory (CD20⁺CD27⁺CD10⁻) B cells (left panel). The transitional B-cell population was further divided into CD21^{lo} and CD21^{hi} subsets (right panel). (C) MNCs from CB, PB, and spleen were stained with mAbs against CD10, CD20, CD27, and CD21. Expression of BAFF-R, CD23, CD44, IgD, and CD10 on CD21^{lo} (gray line) and CD21^{hi} (black line) transitional B-cell subsets was determined by flow cytometry. The corresponding isotype control is represented by the light gray filled and dark gray filled histograms for CD21^{lo} and CD21^{hi} transitional B cells, respectively. Note that, for the depiction of the CD10 histograms, the solid gray overlay represents the fluorescence of all lymphocytes after labeling with an isotype control mAb. The plots are representative of more than 5 experiments using cells from different donors. (D) The frequencies of total transitional B cells as well as CD21^{lo} and CD21^{hi} subsets in PB (n = 35), spleen (n = 7), and CB (n = 6). The results are expressed (left to right) as percentage of CD20⁺ B cells, and percentage of CD20⁺CD27⁻CD10⁺ transitional B cells. (E) PB B cells were labeled with mAbs against CD10, CD21, and CD27, together with R123, and then incubated at 37°C. Samples were collected at various times and analyzed for extrusion of R123. Left panel: Representative data for R123 extrusion after 60 minutes. Right panel: Kinetics of extrusion of R123 (expressed as the percentage of R123⁻ cells; mean ± SEM, n = 4). (F) The replication history of each subset was determined by KREC analysis.^{30,31} This was performed on B-cell subsets isolated from 4 unrelated donors; each symbol (●, ■, *, ▲) represents an individual donor, whereas the horizontal line represents the mean. ***P* < .01. ****P* < .001.

populations (ie, ~ 24, 40, and 50 hours; naive vs transitional: *P* < .05; memory vs transitional: *P* < .001). This pattern of survival was also seen for splenic B-cell subsets (Figure 3C right). We then questioned

whether survival of transitional B cells could be improved by exogenous factors. Unexpectedly, stimulation with BAFF, a crucial survival factor for murine B cells,³⁴ had no effect on either subset of human transitional



B cells (Figure 3D left). In contrast, anti-Ig improved survival of both transitional subsets, with the greatest effect being on CD21^{hi} transitional B cells (Figure 3D right).

CD21^{hi} transitional B cells are functionally more mature than CD21^{lo} transitional B cells

We next investigated the functionality of the CD21^{lo} and CD21^{hi} transitional B-cell subsets and compared their responses with those of naive and memory cells. Stimulation with CD40L resulted in greater [³H]thymidine incorporation by all B-cell subsets compared with that occurring in unstimulated B cells (Table 2). Although stimulation through the BCR failed to induce proliferation of any B-cell subset, it synergized with CD40L resulting in a proliferative response exceeding that induced by CD40L alone by 2- to 4-fold (Table 2). Notably, proliferation of CD40L or CD40L/anti-Ig-stimulated CD21^{lo} transitional B cells was consistently and substantially less than that of CD21^{hi} transitional B cells, whereas proliferation of CD21^{hi} transitional B cells resembled the naive subset

(Table 2). We determined the effect of cytokines on proliferation of the transitional B-cell subsets. Although addition of IL-4, IL-10, IL-13, or IL-21 improved proliferation of all CD40L-stimulated B-cell subsets, the magnitude of the response by CD21^{lo} transitional B cells was always less than that of CD21^{hi} transitional and naive B cells (Table 2).

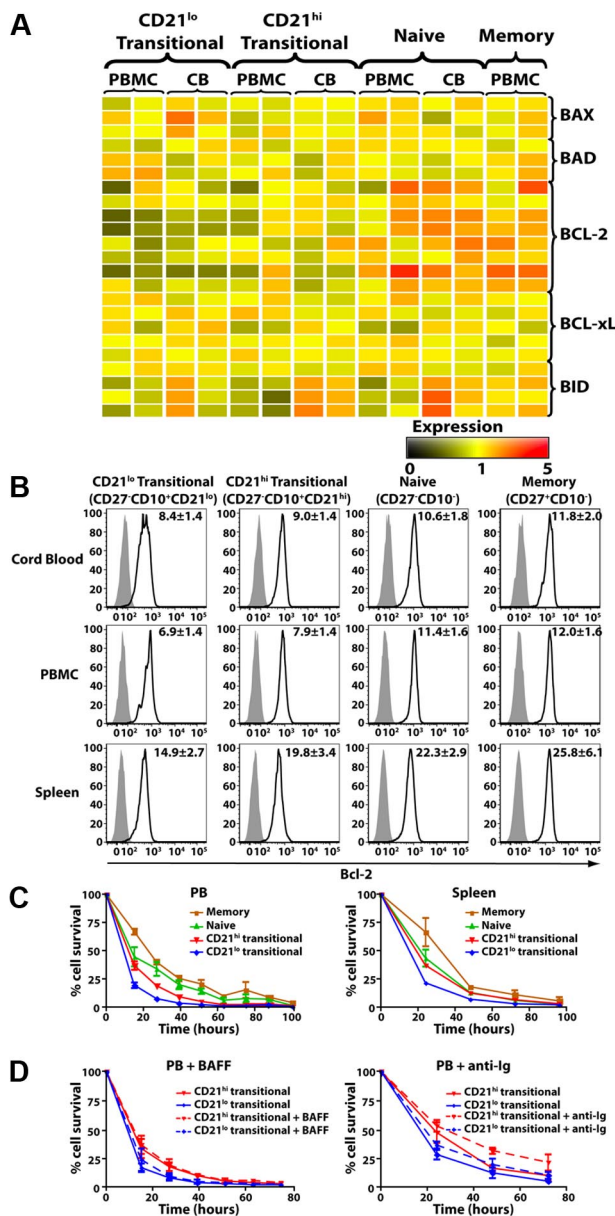


Table 2. CD21^{lo} transitional B cells undergo less proliferation than CD21^{hi} transitional B cells

	CPM			
	CD21 ^{lo} transitional	CD21 ^{hi} transitional	Naive	Memory
Culture conditions #1				
Unstimulated	225 ± 23	247 ± 41	280 ± 48	376 ± 113
Anti-Ig	232 ± 31	296 ± 49	366 ± 72	383 ± 51
CD40L	462 ± 91	781 ± 175	749 ± 203	3316 ± 716
Anti-Ig + CD40L	1022 ± 84	2715 ± 758	2493 ± 473	3697 ± 1008
Culture conditions #2				
Unstimulated	395 ± 24	515 ± 172	565 ± 283	997 ± 79
CD40L	4845 ± 704	7782 ± 1247	6362 ± 990	12 614 ± 2435
+ IL-4	22 036 ± 1982	31 959 ± 7314	21 646 ± 5580	15754 ± 798
+ IL-10	12 761 ± 1602	17 488 ± 3793	17 321 ± 3723	37 831 ± 6266
+ IL-13	15 450 ± 340	25 869 ± 5423	19 498 ± 5110	16 201 ± 2384
+ IL-21	4845 ± 704	7782 ± 1247	6362 ± 990	88 415 ± 2435

Subsets of CD21^{lo} transitional, CD21^{hi} transitional, naive, and memory B cells were purified from the PB of healthy donors and then cultured ($5-15 \times 10^3$ cells per 100 μ L per well) in the absence or presence of either F(ab')₂ fragments of anti-Ig (2.5 μ g/mL), CD40L (1/200 dilution of membrane preparation), or anti-Ig plus CD40L (Culture conditions #1); or CD40L alone or together with IL-4 (100 U/mL), IL-10 (100 U/mL), IL-13 (10 ng/mL), or IL-21 (50 ng/mL; Culture conditions #2). Proliferation was assessed by pulsing each culture well with 0.5 μ Ci of [³H]thymidine and determining its incorporation during the final 18 hours of a 5-day culture. The results represent the mean \pm SEM of replicate cultures (3 independent experiments for anti-Ig/CD40L and 2 independent experiments for cytokine).

To assess Ig secretion, B-cell subsets were stimulated with CpG, CD40L, or CpG/CD40L in the absence or presence of IL-21, a potent inducer of human B-cell differentiation.^{32,35,36} Both transitional subsets secreted low amounts of IgM but neither IgG nor IgA in response to CD40L alone or CpG with or without IL-21 (Table 3). CD40L/CpG increased IgM secretion and induced secretion of low amounts of IgG and IgA by CD21^{lo} and CD21^{hi} transitional B-cell subsets (Table 3). Under these conditions, the levels of Ig secreted by CD21^{hi} transitional B cells were 2- to 5-fold greater than CD21^{lo} transitional B cells. These results demonstrate that CD21^{lo} transitional B cells are functionally less mature than the CD21^{hi} subset. In contrast to proliferation, differentiation of

CD21^{hi} transitional B cells into Ig-secreting cells was inferior to that of naive B cells. However, both subsets of transitional B cells secreted comparable amounts of IgM, IgG, and IgA in response to stimulation with CD40L/IL-21, with or without CpG (Table 3).

Increased production of autoreactive IgM antibodies by CD21^{lo} transitional B cells

A previous study discovered that more than 40% of human transitional B cells expressed an autoreactive BCR.⁵ It was therefore of interest to determine whether the 2 transitional subsets described here produce different levels of autoreactive Ab. B-cell

Table 3. Ig secretion by transitional B-cell subsets

	Ig concentration, ng/mL			
	CD21 ^{lo} transitional	CD21 ^{hi} transitional	Naive	Memory
IgM				
Unstimulated	< 5	< 5	< 5	5.3 ± 1.4
CpG	15.5 ± 6.7	25.8 ± 19.3	33.3 ± 10.3	1205 ± 165
+ IL-21	4.6 ± 2.8	22.3 ± 17.8	18.7 ± 4.5	1083 ± 549
CD40L	9.1 ± 1.2	6.5 ± 3.0	17.2 ± 5.9	184 ± 63
+ IL-21	8232 ± 1498	8128 ± 781	13 815 ± 1228	30 318 ± 9380
+ CpG	1030 ± 142	1826 ± 438	4817 ± 2282	7784 ± 3834
+ CpG + IL-21	13 742 ± 3354	16 567 ± 5956	20 879 ± 3776	30 034 ± 324
IgG				
Unstimulated	< 5	< 5	< 5	< 5
CpG	< 5	< 5	< 5	39.0 ± 20.1
+ IL-21	< 5	< 5	< 5	148.8 ± 94.7
CD40L	< 5	< 5	< 5	10.8 ± 5.8
+ IL-21	645 ± 97	904 ± 465	620 ± 204	2853 ± 568
+ CpG	7.8 ± 3.4	20.1 ± 8.1	54.6 ± 13.6	114.8 ± 20.9
+ CpG + IL-21	762 ± 215	983 ± 372	1082 ± 206	2950 ± 1245
IgA				
Unstimulated	< 5	< 5	< 5	< 5
CpG	< 5	< 5	< 5	585 ± 296
+ IL-21	< 5	7.2 ± 4.2	10.3 ± 6.4	638 ± 184
CD40L	< 5	< 5	< 5	90 ± 68
+ IL-21	612 ± 368	719 ± 224	644 ± 312	19 898 ± 5429
+ CpG	5.8 ± 3.1	36.3 ± 30.6	686 ± 285	6270 ± 2883
+ CpG + IL-21	585 ± 205	1051 ± 288	1919 ± 714	49 607 ± 22 512

B-cell subsets were purified from the PB of different healthy donors and then cultured ($5-10 \times 10^3$ cells per 200 μ L per well) in the absence or presence of CpG (10 ng/mL), CD40L, or CpG plus CD40L without or with IL-21. After 12 days, supernatants were harvested and the amount of secreted IgM, IgG, and IgA was determined by Ig heavy chain-specific ELISAs. The results represent the mean \pm SEM of 3 independent experiments.

subsets were stimulated with CD40L/IL-21, and the level of IgM ANA was determined. CD21^{lo} transitional B cells produced IgM that contained the greatest amount of ANA, and this amount was significantly higher than the self-reactive IgM produced by CD21^{hi} transitional cells ($P < .05$; Figure 2C). The levels of self-reactive Ab produced by naive ($P < .01$) and memory ($P < .001$) B cells were also significantly less than CD21^{lo} transitional B cells (Figure 2C). Thus, whereas previous studies elegantly established that transitional B cells are enriched for self-reactive B cells,⁵ our findings demonstrate greater autoreactivity within the CD21^{lo} subset of transitional B cells.

CD21^{lo}CD10⁺ B cells represent the predominant population in cases of B-cell deficiency

To gain further insight into the relationship between CD21^{lo} and CD21^{hi} transitional B cells, we analyzed the B-cell compartment of patients with XLA resulting from mutations in *BTK*. XLA patients have a block in B-cell development in the BM resulting from impaired BCR signaling²¹ such that the frequency of PB B cells was 0.1% plus or minus 0.1% ($n = 5$; range, 0.03%-0.27%), compared with 10.1% plus or minus 2.8% in normal healthy persons (Figure 4). Although the absolute number of B cells was drastically diminished in these patients, most of these had a transitional phenotype (XLA, 76.1% \pm 14.6% of all B cells; healthy donors, 9.9% \pm 1.9%; Figure 4). Furthermore, most transitional B cells in XLA belonged to the CD21^{lo} subset ($\sim 75\%$). This contrasts with normal donors, in whom only approximately 25% of transitional B cells belong to this subset. These findings provide strong evidence that CD21^{lo}CD10⁺ and CD21^{hi}CD10⁺ B cells are developmentally distinct populations of transitional B cells.

CD21^{lo} transitional B cells are the first B cells to appear in the periphery after HSCT

The data derived from the analysis of XLA patients suggested that the generation of CD21^{lo} transitional B cells precedes that of CD21^{hi} transitional B cells. An extension of this is that CD21^{lo} transitional B cells are precursors of CD21^{hi} transitional B cells. To examine this in more detail, we analyzed the kinetics of B-cell reconstitution in a cohort of patients undergoing HSCT. Figure 5A-B depicts the data from one patient, whereas the data for all patients are compiled in Figure 5C. In the initial stages after HSCT (0-10 weeks), only a low frequency of PB CD20⁺ cells was detected ($<1\%$ of all lymphocytes). However, the majority of them ($>75\%$) were CD10⁺ transitional B cells. At subsequent times, the frequency of CD20⁺ lymphocytes increased. This was accompanied by a decrease and increase, respectively, in the frequency of transitional and naive B cells (Figure 5). Interestingly, the composition of the transitional B-cell population also changed after HSCT such that, at the earliest times examined, more than 95% of them were CD21^{lo} whereas over time the CD21^{hi} subset emerged and increased in frequency (Figure 5B-C). Overall, the B-cell reconstitution data demonstrate that the first B-cell population to be exported from the BM to the periphery is the CD21^{lo} transitional subset, which subsequently gives rise to CD21^{hi} transitional B cells, the precursor to the naive B-cell pool.

Discussion

Much is known of the biology of murine transitional B cells, yet our understanding of human transitional B cells is only just beginning to be unraveled. Here, we identified and characterized

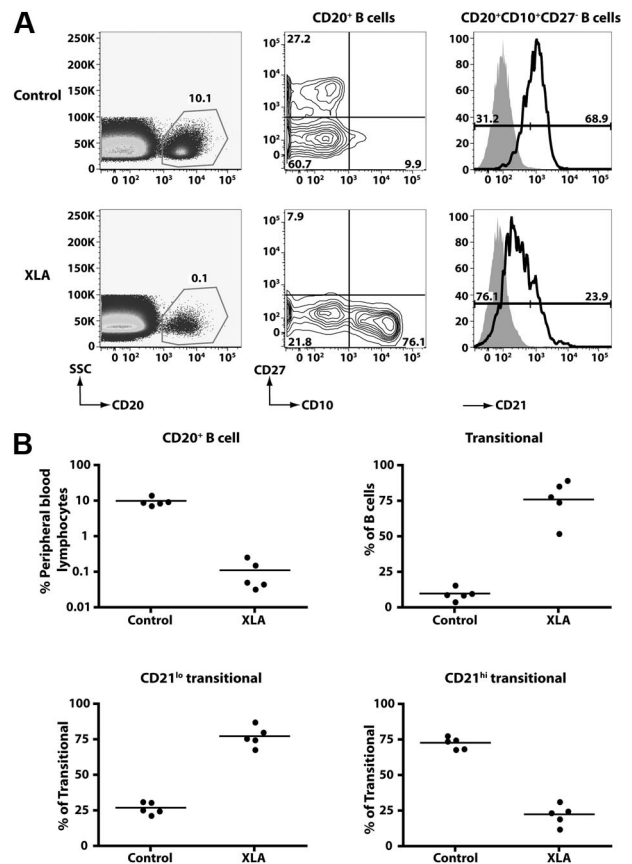


Figure 4. CD21^{lo} transitional B cells are the predominant B-cell population in the blood of B-cell-deficient XLA patients. (A) PBMCs from a healthy donor and an XLA patient were stained with anti-CD20, -CD27, -CD10, and -CD21 mAbs. (Left panel) The frequency of CD20⁺ B cells; these cells were divided into transitional, naive, and memory B-cell subsets based on differential expression of CD10 and CD27 (middle panel). The transitional B cells were further divided into CD21^{lo} and CD21^{hi} subsets. The histograms presented for CD21 expression (right-hand panel) were obtained by gating on the CD20⁺CD10⁺CD27⁻ B cells (which appear in the lower right-hand quadrant of the contour plot depicted as the middle panel). The overlay solid gray histogram represents the fluorescence of lymphocytes incubated with an isotype control mAb. Flow-cytometric analysis of transitional B-cell subsets detected in donors and XLA patients was performed on the same day with identical instrument settings. The values in each plot are the mean percentages of each of the indicated B-cell subsets from all studied healthy donors and patients. (B) Summary of the frequency of total B cells, transitional B cells, and transitional B-cell subsets in healthy donors (HD) and XLA ($n = 5$).

2 populations of human transitional B cells: CD21^{lo} and CD21^{hi}. Our data revealed that these 2 populations have distinct phenotypes, with higher expression of CD23, CD44, IgD, and BAFF-R by the CD21^{hi} subset (Figure 1). Notably, the greater expression of CD21, CD23, and IgD on the CD21^{hi} versus CD21^{lo} transitional subsets correlates with the phenotypes of murine transitional B cells, with expression of these molecules also increasing along a T1 \rightarrow T2 \rightarrow T3 gradient^{7,8}

The phenotypic data, together with greater proliferation and Ig secretion by the CD21^{hi} transitional B cells (Tables 2-3), suggest that CD21^{lo} and CD21^{hi}CD10⁺ B cells correspond to distinct stages of B-cell development, with the CD21^{lo} subset representing a less mature population. More compelling support for this conclusion came from assessment of the composition of the B-cell compartment in patients with XLA or those recovering from HSCT, as well as microarray analyses. First, although *BTK* mutations cause a severe block in B-cell development²¹ (Figure 4), the few circulating B cells detectable in XLA patients were predominantly CD21^{lo} transitional cells, suggesting that loss of *Btk* prevents CD21^{lo}

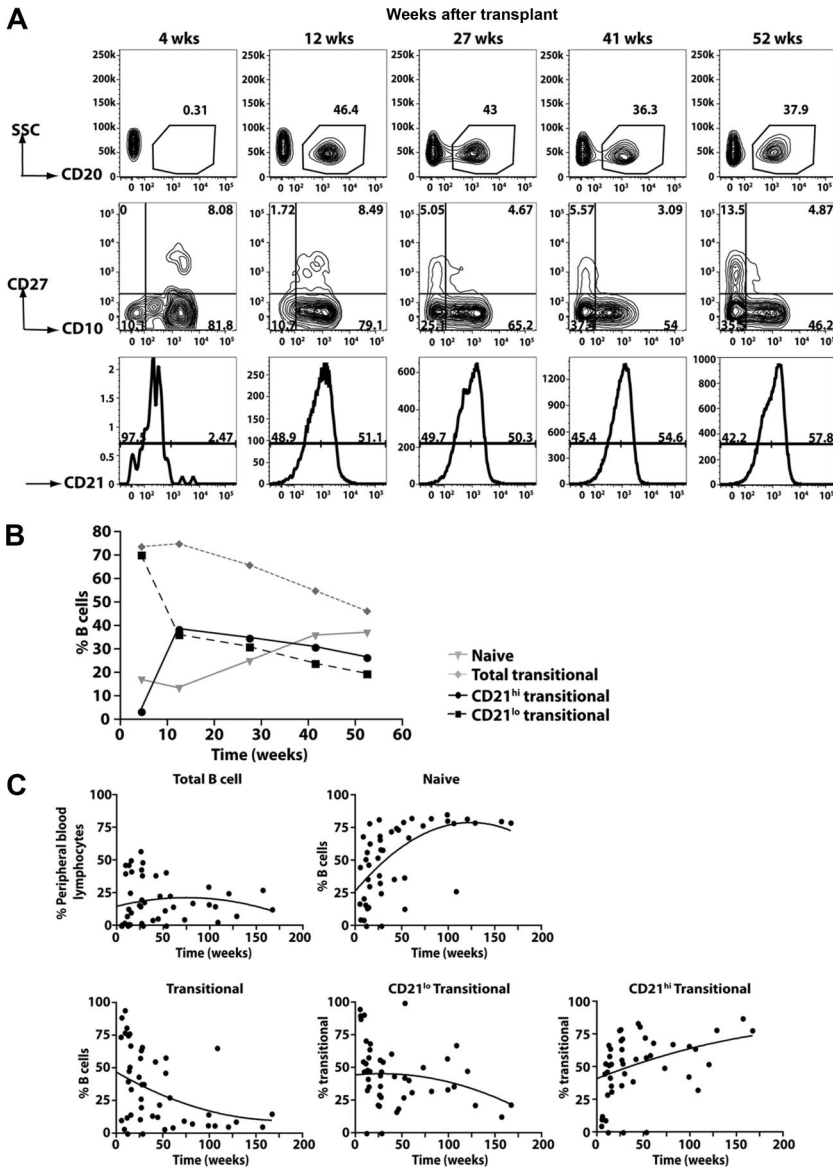


Figure 5. Kinetics of B-cell reconstitution after HSCT reveals the early appearance of CD21^{lo} transitional B cells.

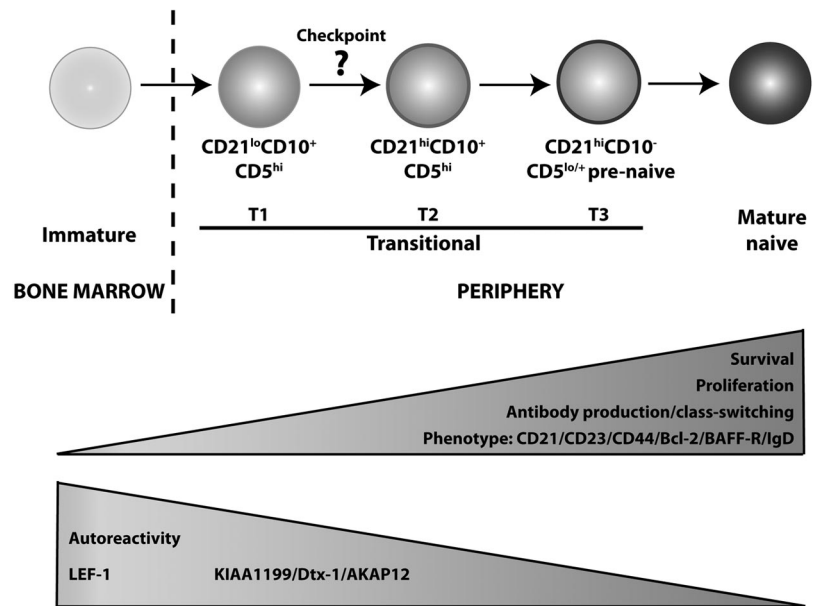
(A) PBMCs were collected from patients at different times after HSCT and then stained with anti-CD20, -CD27, -CD10, and CD21 to determine the B-cell reconstitution profile. CD20⁺ B cells were gated (top panel) and divided into transitional, naive, and memory B-cell subsets based on differential expression of CD10 and CD27 (middle panel). The transitional B cells were further divided into CD21^{lo} and CD21^{hi} populations (bottom panel). The numbers indicate the percentage of total B cells (top panel) and the corresponding subsets (middle and bottom panels). (B) Summary of the reconstitution of naive and transitional B-cell subsets after HSCT in the patient presented (A) as a percentage of all B cells. (C) Combined B-cell reconstitution profile in 12 post-HSCT patients.

transitional B cells from maturing into CD21^{hi} transitional, and subsequently naive, B cells (Figure 4). Second, and consistent with the observations in XLA patients, CD21^{lo} transitional B cells were the first B-cell subset to populate the periphery after HSCT. Over time, these cells declined in number, and this coincided with the appearance of CD21^{hi} transitional cells, thereby suggesting that CD21^{lo} transitional B cells represent the initial population of BM emigrant B cells that subsequently develops into the CD21^{hi} transitional subset (Figure 5). Third, gene profiling revealed that *LEF-1* was expressed at the highest level in CD21^{lo} transitional B cells, with expression being reduced in CD21^{hi} transitional B cells and extinguished in mature naive and memory cells (Figure 2). Hystad et al recently reported that *LEF-1* was absent in human early B cells (CD34⁺CD10⁺CD19⁻), induced in pro-B cells (CD34⁺CD10⁺CD19⁺), further up-regulated in pre-B cells (CD34⁺CD10⁺CD19⁻), and reduced, but still expressed, in immature B cells (CD34⁻CD10⁺CD19⁺).³⁷ The declining level of *LEF-1* expression between human BM pre-B cells and immature B cells,³⁷ and CD21^{lo} and CD21^{hi} subsets of transitional B cells, and further down-regulation between the CD21^{hi} transitional and naive B cells suggest that B-cell development progresses in the

following linear manner: immature → CD21^{lo} transitional → CD21^{hi} transitional → naive (Figure 6). The finding that CD21^{lo} transitional cells were enriched for autoreactive Ab is also consistent with the notion that CD21^{lo} transitional B cells are the earlier BM emigrants and that they are still undergoing negative selection.

The results from our study provide novel and significant insights into human B-cell development in the periphery and clarify our understanding of the requirements for this process. First, we identified several genes (eg, *LEF1*, *KIAA1199*) that are highly expressed in CD21^{lo} transitional B cells and down-regulated at subsequent stages of B-cell development (Figure 2). The exact roles of these genes in human B-cell development are unknown. However, *LEF-1*-deficient mice have a reduction in the total number of B cells in the fetal liver and perinatal BM,³⁹ thus highlighting the importance of this gene in controlling B-cell development. In contrast, overexpression of *KIAA1199*⁴⁰ in cancer cells was associated with increased cell death and/or growth suppression. Based on these observations, it is possible that the heightened expression of *KIAA1199* in transitional B cells contributes to their reduced survival and proliferation in vitro relative to naive B cells. Further examination of these genes in immune cells

Figure 6. Proposed model of human B-cell development. We propose that B cells emigrating from the BM to the periphery are CD21^{lo} transitional/T1 B cells, which subsequently differentiate into CD21^{hi} transitional/T2 B cells. This maturation step requires Btk, indicating a role for BCR engagement, and is accompanied with improved survival and the potential to proliferate and differentiate into Ig-secreting cells. There is also a reduction in autoreactivity, suggesting that a checkpoint for self-tolerance exists at the step between these 2 stages of transitional B-cell development. Phenotypically, the CD21^{hi} transitional B cells increase expression of CD21, CD23, CD44, Bcl-2, BAFF-R, and IgD. Meanwhile, expression of *LEF-1*, *KIAA1199*, *Dtx-1*, and *AKAP12* is reduced as the cells enter the mature B-cell repertoire. CD21^{hi} transitional/T2 B cells then give rise to T3/prenaive CD5⁺ B cells,³⁸ which are characterized by loss of expression of CD10, high expression of CD21, but impaired extrusion of R123. The autoreactive specificity of this population remains to be determined.



should reveal more about their role during human B-cell development.

Our finding that *BTK* mutations severely block B-cell development at the CD21^{lo} transitional stage (Figure 4) questions a previous finding that BTK is required to establish B-cell tolerance in humans.⁴¹ Ng et al, noting the predominance of transitional (ie, CD10⁺) B cells in XLA PB,⁴¹ demonstrated that these cells had a greater frequency of autoreactivity (~55%-60%) than CD10⁺ B cells from normal donors (37%).⁴¹ They concluded that altered signaling through the BCR resulting from mutations in *BTK* allowed the escape of an increased proportion of autoreactive B cells; thus, intact BTK function was necessary for the maintenance of B-cell tolerance.⁴¹ However, an alternative interpretation is that the increase in autoreactivity noted in XLA B cells⁴¹ simply reflects the fact that approximately 80% of transitional B cells in these patients belong to the CD21^{lo} subset (Figure 4), and a greater frequency of these cells are autoreactive Abs compared with the CD21^{hi} subset (Figure 2C), which composes most of the CD10⁺ population in healthy donors (Figure 1D). Thus, it is possible that BTK does not play a role in establishing tolerance; rather, it is necessary for the maturation of CD21^{lo} transitional B cells into CD21^{hi} transitional B cells.

Although the CD21^{lo} and CD21^{hi} transitional B-cell subsets exhibited numerous differences, they did share several phenotypic and functional attributes. For instance, based on replication history, these 2 B-cell subsets were both nonproliferating cells in vivo (Figure 1F). Thus, the development of CD21^{lo} transitional cells into CD21^{hi} transitional cells is unlikely to involve proliferation. Interestingly, naive B cells had undergone several divisions in vivo (Figure 1F),^{30,31} implying that differentiation of transitional B cells into naive cells may involve a proliferative phase. Notably, BAFF had very little effect on the survival of either transitional B-cell subset despite detectable expression of BAFF-R (Figures 1,3). We also found that BAFF did not modulate expression of CD21 or CD23 on human transitional B cells in vitro (supplemental Figure 3). Because BAFF is critical for the in vivo survival^{13,42} of and up-regulation of expression of both CD21 and CD23⁴³ on murine B cells, it is possible that B-cell development in humans is less dependent on BAFF than that in mice. This is supported by the recent description of 2 related persons with mutations in

TNFRSF13C (encoding BAFF-R) who, in contrast to *Baffr*-deficient mice,^{13,42} still developed naive and memory B cells, albeit at reduced frequencies compared with normal healthy controls.⁴⁴

Several studies have partitioned human transitional B cells into subsets according to phenotype and/or function. Anolik et al defined human "T1" and "T2" transitional cells based on expression levels of CD24 and CD38, with T1 B cells being CD38⁺⁺⁺CD24⁺⁺⁺ and T2 B cells CD38⁺⁺CD24⁺⁺.²⁶ These transitional subsets were detected in the PB of patients recovering from B-cell depletion therapy.²⁶ More recently, this group identified a third population of human transitional B cells (termed T3) that exhibit a phenotype of naive B cells (ie, CD10⁻CD24^{int}CD38^{int}IgD⁺CD27) but can be resolved from naive cells by their inefficiency in extruding R123.⁴⁵ Consistent with our data from HSCT patients, the appearance of the T1 subset preceded that of the T2 and T3 populations in the setting of B-cell reconstitution after B-cell depletion.^{26,45} Interestingly, another study described a population of human CD5⁺ prenaive B cells that can be distinguished from transitional and naive B cells by their intermediate levels of expression of CD9, CD10, and CD38, and efflux of R123.³⁸ Based on surface phenotype, their predominance in CB, and ability to mature into naive B cells in vitro,^{38,45} it is probable that T3 cells and CD5⁺ prenaive B cells represent the same population of developing B cells. Because our study relied on CD10 expression to identify transitional B cells, neither the CD21^{lo} nor CD21^{hi} subset corresponds to T3/CD5⁺ (CD10^{-/lo}) prenaive B cells. Our analysis demonstrated that CD21^{lo} and CD21^{hi} transitional B cells in PB of normal donors express comparable levels of CD24 and CD38, raising the possibility that they do not correspond to CD38⁺⁺⁺CD24⁺⁺⁺ T1 and CD38⁺⁺CD24⁺⁺ T2 transitional cells, respectively. However, when we assessed CD24 and CD38 on transitional B-cell subsets in post-HSCT patients, we, like Anolik et al,²⁶ observed that, at the earliest times of analysis, CD21^{lo} transitional B cells expressed higher levels of both markers compared with CD21^{hi} transitional B cells (supplemental Figure 4). Despite this initial difference, expression of CD24 and CD38 on the transitional B-cell subsets eventually approximated one another (supplemental Figure 4), thus suggesting that expression levels of CD24 and CD38 on transitional B-cell subsets reflect the time since their generation and export from the BM, rather than a delineation between transitional

subsets. Based on this, it is probable that the CD21^{lo} and CD21^{hi} transitional B-cell subsets are the CD38⁺⁺⁺CD24⁺⁺⁺ T1 and CD38⁺⁺CD24⁺⁺ T2 cells.²⁶ This is further supported by the findings that expression of CD21 and CD23 was increased on T2/CD21^{hi} compared with T1/CD21^{lo} transitional B cells and that neither population was responsive to BAFF in in vitro survival or differentiation assays.^{26,45}

Combining the data from our current study and those recently published, it is possible to propose a map of human peripheral B-cell development whereby immature B cells exit the BM as CD21^{lo} transitional/T1 cells that develop into CD21^{hi} transitional/T2 cells in a BTK-dependent manner (Figure 6). Interestingly, the reduction in autoreactivity between the CD21^{lo} and CD21^{hi} transitional stages suggests that a checkpoint for self-tolerance exists at this step. This is analogous to the checkpoints between early immature and immature B cells, and transitional and naive B cells where autoreactive B cells are deleted from the repertoire,⁵ and is consistent with the increased frequency of T1/CD21^{lo} transitional B cells in some patients with systemic lupus erythematosus.^{24,46} CD21^{hi} transitional B cells then develop into T3/prenaive B cells, which undergo a final maturation process to become naive B cells (Figure 6). It will be important to investigate the autoreactivity and proliferative history of the T3/prenaive B-cell population to determine whether replication is required for the development of these cells into the naive compartment and if deletion of autoreactive cells occurs beyond the CD10⁺ stage of B-cell development. Overall, this framework will allow refined molecular investigation of human B-cell development in cases of

immunologic dyscrasias characterized by perturbed B-cell behavior, such as immunodeficiency, autoimmunity, and malignancy.

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Authorship

Contribution: S.S. designed the research, performed experiments, analyzed and interpreted results, and wrote the manuscript; B.S.-N. and R.N. provided CB samples; D.A.F., J.G., M.W., A.W., and P.J.S. provided patient samples; S.G.T. conceptualized and designed the research and wrote the manuscript; and all authors commented on the manuscript.

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Correspondence: Stuart G. Tangye, Garvan Institute of Medical Research, 384 Victoria St, Darlinghurst 2010, NSW, Australia; e-mail: s.tangye@garvan.org.au.

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