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A Feeder-Free Differentiation System Identifies Autonomously Proliferating B Cell Precursors in Human Bone Marrow

Helene Kraus,*† Sandra Kaiser,* Konrad Aumann,§ Peter Bönelt,* Ulrich Salzer,* Dietmar Vestweber,§ Miriam Erlacher,¶ Mirjam Kunze,‖ Meike Burger,¶ Kathrin Pieper,¶ Heiko Sic,* Antonius Rolink,** Hermann Eibel,* and Marta Rizzi*

The peripheral B cell compartment is maintained by homeostatic proliferation and through replenishment by bone marrow precursors. Because hematopoietic stem cells cycle at a slow rate, replenishment must involve replication of precursor B cells. To study proliferation of early human B cell progenitors, we established a feeder cell–free in vitro system allowing the development of B cells from CD34+ hematopoietic stem cells up to the stage of immature IgM+ B cells. We found that pro-B and pre-B cells generated in vitro can proliferate autonomously and persist up to 7 wk in culture in the absence of signals induced by exogenously added cytokines. Nevertheless, addition of IL-7 enhanced pre-B cell expansion and inhibited maturation into IgM+ B cells. The B cell precursor subsets replicating in vitro were highly similar to the bone marrow B cell precursors cycling in vivo. The autonomous proliferation of B cell precursor subsets in vitro and their long-term persistence implies that proliferation during pro-B and pre-B cell stages plays an important role in the homeostasis of the peripheral B cell compartment. Our in vitro culture can be used to study defects in B cell development or in reconstitution of the B cell pool after depletion and chemotherapy. The Journal of Immunology, 2014, 192: 1044–1054.

Throughout life, B lymphocytes develop in the bone marrow (BM) from hematopoietic and lymphocyte precursor cells. Starting from hematopoietic stem cells (HSCs), progressive rearrangement of IgH and IgL chain gene loci (1), stage-specific proliferation (2–4), and expression of transcription factors (5, 6) precisely regulate early stages of B cell development. In humans, circulating B cells have an average half-life of 18 d (7). They are maintained by continuous development from BM precursors and by peripheral homeostatic proliferation accounting for 1 cell cycle/36 d (7).

Although in mice HSCs are constantly cycling at a very slow rate (8), a fraction of murine pro-B and pre-B cells is rapidly proliferating (9). Depletion of dividing cells by 5-fluorouracil treatment allows the proliferation of common lymphocyte progenitors (CLP) and of pro-B cells to replenish the peripheral compartment (10). In contrast, most of the immature B cells are resting cells (9). Thus, in mice, the B cell compartment is maintained by proliferating HSCs, CLPs, pro-B cells, and pre-B cells. Although in humans, cell division of a small fraction of IgM+ immature B cells has been reported (11), the contribution of proliferating pro-B and pre-B cells to maintain the peripheral B cell compartment is unknown.

When comparing B cell development between mice and humans, significant differences become evident in the pattern of cell-surface markers (4, 12, 13), cytokine requirements (14–18), and in the role of pre-BCR and BCR-associated signaling molecules (19). Thus, it is difficult to extrapolate results from mouse models directly to B cell development in humans. However, BM transplantation (20) and immunosuppressive therapies in cancer or autoimmunity (21, 22) affect proliferation and development of B cells, thus changing B cell homeostasis, immune responses, and the susceptibility to infections.

Because B cell precursors in human BM are experimentally difficult to access, several in vitro model systems addressing the biology of B lymphopoiesis have been developed. Starting from CD34+ cells, development of human B cells has been achieved in vitro by cocultivation with feeder cells (23–26) or by adding supernatants of mesenchymal stem cell cultures (27). Although these in vitro systems proved to be effective, the presence of ill-characterized feeder cells and feeder cell-products bears an inherent disadvantage in studying the signaling responses and proliferation requirements of human precursor B cells developing in vitro.

In this article, we present a novel feeder cell–free in vitro model of human B cell development starting from CD34+ cells isolated from cord blood (CB) and from BM. As shown by the expression of stage-specific markers, transcription factors, and IgH chain recombination events, our model recapitulates the characteristic
steps of BM-dependent B cell development up to the stage of IgM+ immature B cells. After a relative expansion of CLP/pro-B cells, precursor B cells proliferated up to 7 wk in culture independent of IL-7 and other exogenously added cytokines, thus demonstrating longevity and autonomous proliferation of human precursor B cells. Therefore, different from mice, the B cell compartment in humans is replenished independently from IL-7 signaling by the proliferation of HSCs, pre-B cells, and pro-B cells. Comparing CB- and BM-derived cells, our findings emphasize the role of the pro-B and pre-B cells in reconstituting the B cell compartment after depletion, for example, after chemotherapy or transplantation. Moreover, our feeder cell–free in vitro system is a feasible and robust tool to study factors, therapeutic protocols, compounds, and mutations that affect the development of human B cells.

Materials and Methods
Human BM and CB
Human umbilical CB was obtained after cesarian delivery upon signed informed consent under approval of the University Freiburg Ethics Committee (174/13). BM was obtained from material remaining from diagnostic aspirations upon signed informed consent under the ethics approval (281/12); age of BM donors ranged between 22 and 38 y (mean ± SD: 32.6 ± 5.6 y).

CD34+ isolation
CD34+ cells were isolated from CB or BM using the CD34 microbead kit (Miltenyi Biotec). Purity of isolated cells was monitored by flow cytometry.

In vitro culture
CD34+ cells were cultivated in vitro at 10^5 cells/ml in Iscove’s medium supplemented with insulin, transferrin, fatty acids, nonessential amino acids, glutamine, and reduced glutathione with the addition of human IL-6, stem cell factor (SCF), Flt3-L (each at 25 ng/ml; Immunotools), and 10% FCS (catalog no. DE14-801F; lot no. 9SB029; Lonza). After 7 d, non-adherent cells were harvested from original wells and transferred to ICAM-1–Fc–coated plates at 1 × 10^5 cells/ml and cultivated in the presence of human IL-7 (20 ng/ml), SCF, and Flt3-L (each 25 ng/ml). From day 14 on, cytokine-free medium was changed two times per week (Supplemental Fig. 1A).

Quantitative PCR
Total RNA was extracted using TRizol (Invitrogen) and quantified with the NANO DROP 2000c (Thermo Scientific). cDNA was synthesized from 200 ng total RNA using SuperScript III reverse transcriptase (Invitrogen) and random hexamer primers (Amersham Pharmacia Biotech). Quantitative PCR (qPCR) was performed using the TaqMan gene expression master mix (Applied Biosystems) and a StepOnePlus Real Time PCR system (Applied Biosystem) with the primer/probe real-time PCR assays for: CD10 (HS00153510), CD79A (HS00998119), E2A (HS01029649), BFB1 (HS01092694), RAG2 (HS00791777) (all Applied Biosystems) and PAX5 (28). PCR fragments were amplified for 2 min at 50˚C, 10 min at 95˚C followed by 45 cycles consisting of 15 s at 95˚C and 1 min at 58˚C. Relative expression was calculated using the 2^(-ΔΔCt) method with cDNA concentrations standardized to the reference gene RPLPO (Applied Biosystem).

Flow cytometry
Phenotype of cells was determined by flow cytometry using anti-CD34 FITC, anti-CD10 FITC, anti-CD45 FITC, anti-BAFFR PE, anti-CD38 PE/Cy7, anti-CD38 PerCP/Cy5.5, anti-CD19 allophycocyanin-H7, anti-CD4 PerCP/Cy5.5, and anti-CD8 V500, anti-CD33 allophycocyanin-H7 (all BD), anti-CD33 PerCP/Cy5.5, anti-Idg PE/Cy7, anti-CD34 PE/Cy7, anti-IgM allophycocyanin-Cy7, anti-CD19 allophycocyanin-Cy7, anti-CD20 PE-Cy7, anti-CD38 PE-Cy7 (all BD), anti-CD10 PE (Beckman Coulter), anti-CD179a PE (Beckman Coulter), anti-CD179b PE (Beckman Coulter), anti-CD14 PE (BD Biosciences), anti-CD3 PE (Miltenyi Biotec), and anti-CD5 allophycocyanin (Invitrogen). Dead cells were excluded by DAPI staining. Intracellular staining was performed using the IntraPrep kit (Beckman Coulter), and dead cells were excluded using the Live/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen). Proliferation was tested by intracellular staining of Ki67 (Dako). BrdU (10 μM) incorporation by cells cultivated over 16 h was analyzed fixing the cells first in 95% ethanol followed by 1% paraformaldehyde and 0.01% Tween 20. After incubation with 50 U/ml DNase I, cells were stained with anti-BrdU PE (BioLegend). FACS-sorted CD22+CD10+IgM+ CD34+ cells from human BM were labeled with CFSE (Molecular Probes) and cultivated for 6 d. Data were acquired using a FACSCanto II (BD) and analyzed with FlowJo (TreeStar).

Reconstitution of Rag2−/- IL2r−/- mice
Human CD34+ cells were cultivated for 7 d in the presence of IL-6, Flt3-L, and SCF, and injected in the facial vein of irradiated (4 Gy) 1– to 3-d-old newborn Rag2−/- IL2r−/- mice. Femurs were either flushed with PBS for flow cytometry or fixed in 4% formalin for immunohistochemical analysis. Single-cell suspensions of thyln were analyzed by flow cytometry. All animal experiments were approved by the regional council under 35-9185.81/G-10/26.

Immunohistochemistry
Immunohistochemistry of paraffin sections from mouse BM of decalcified mice was performed as described previously (29), using mouse mAbs or rabbit polyclonal Abs against CD34, CD10, CD19, IgM, IgG, IgA, Ki67, myeloperoxidase, and lactoferrin (all from Dako). Slides were mounted in DPX medium, and images were taken using a Zeiss Axioscope equipped with Zeiss AxioCam and Axiovert software. Figures were made with Adobe Photoshop CS5 and white point adjustment to account for differences in exposure time and color temperature.

DJ and VDJ recombination assay
For the analysis of H chain recombination, DNA was extracted from 60,000 to 100,000 cells by direct lysis method (30). DJ and VDJ recombination was analyzed by multiplex PCR and BIOMED-2 primers (31) with Hot-Start Taq polymerase (Invitrogen) for 7 min at 95˚C followed by 40 cycles of 30 s at 94˚C and 45 s at 59˚C, 1 min at 72˚C, and a single step at 72˚C for 10 min. For DJ-recombination: 1 JH primer and 6 DH primers (DH1-6) were used in a single PCR. For VDJ-recombination: 1 JH and 7 VH (VH1-7)-specific primers were used in a single PCR. GAPDH was amplified as internal standard using Platinum Taq polymerase (Invitrogen) at 3 min at 94˚C, 35 cycles consisting of 30 s at 94˚C, 30 s at 62˚C, 1 min at 72˚C, and a single step at 72˚C for 10 min.

Detection of stroma cells
CD34+ cells were cultivated in conditions favoring B cell development in vitro up to 49 d as described earlier. To assess the presence of stroma cells, cells growing in suspension were collected. Consequently, the wells were washed with PBS and adherent cells detached by incubation with trypsin. After addition of medium, cells were collected and added to previous ones, washed, and stained with anti-CD34, -CD45, -CD14, and –Stro-1 (32).

IL-7 ELISA
Supernatant of culture was collected weekly and stored at −20˚C. Human IL-7 concentration in the supernatant of culture was determined by ELISA (Human IL-7 ELISA Set; Diaclone), following manufacturer’s instructions.

Results
CB-derived CD34+ cells develop into immature B cells in vitro
To study early phases of human B cell development in vitro independent from feeder cells, we established a three-stage cultivation system (Supplemental Fig. 1A). First, CD34+ cells isolated from CB were expanded for 7 d in the presence of SCF, Flt3-L, and IL-6. The second stage lasted an additional 7 d in which CD34+ cells were grown in medium containing IL-7 instead of IL-6 to promote the development of lymphocyte progenitors (33, 34). CD34+ HSC and B cells expressed the ICAM-1 ligand LFA-1 (Supplemental Fig. 1B–D). Because mesenchymal stroma cells express ICAM-1 and LFA-1, they have been shown to support B cell development in vitro (24), we cultured the CD34+ cells from day 7 on ICAM-1–Fc–coated plates. Comparing cultures with or without ICAM-1, we found consistently higher absolute numbers of
B cells developing in ICAM-1-Fc–coated plates, although the development was ICAM-1 independent (Supplemental Fig. 1E). In the third stage, which lasted up to 49 d, the transition of proliferating into differentiating cells was promoted by replacing cytokine substituted with cytokine-free medium.

Developing B cells express CD10 from CLPs on up to the stage of IgM+ immature B cells (37) (Fig. 1A). In vitro, the development of CD10+ cells progressed up to day 49 (Fig. 1B). Like ex vivo cells, CD10+ cells also expressed CD38 (Fig. 1B, 1C) correlating with increasing CD10 mRNA levels (Fig. 1D). Because CD33+CD10− cells were large in size (Supplemental Fig. 2A, 2B) and expressed CD45 and CD14 (data not shown), they were identified as monocytes.

CD79a (Igα) and CD79b (Igβ) are part of the pre-BCR and BCR complex. Preceding CD19, CD79a expression begins at the pro-B cell stage (38). In vitro, CD79a was detected from day 14 on up to day 49 (Fig. 1E, 1G), matching the CD10+ expression pattern of human BM B cells (Fig. 1F). Interestingly, CD79a expression was higher in CB than in BM, indicating either age-specific differences or that CD79a expression changes in B cells exiting from BM (Fig. 1F). Immature B cells expressing surface IgM appeared in vitro at day 35 (Fig. 1H). The percentage of IgM+...
cells increased until day 49, reaching levels found in human BM (Fig. 1H, 1I).

The pre-BCR is formed by IgH and surrogate L chains composed of CD179a (VpreB1) and CD179b (lambda5). The pre-BCR plays an essential role as sensor for functional IgH chain rearrangement, allelic exclusion, regulation of \textit{RAG} expression, and the initiation of IgL chain gene rearrangement (2). Cytoplasmic CD179a expression is detectable in late pro-B cells and in pre-B cells (19). In vitro, CD179a+ cells developed into surface IgM+ immature B cells (Fig. 1J). Similar ratios between CD179a+ IgM+ pro-/pre-B and IgM+ CD179a- immature B cells were found in human BM, whereas pro-B and pre-B cells were not detected in CB (Fig. 1K). When BM B cells reach the IgM+ stage, they start to express low levels of surface IgD, leave the BM, and complete maturation in the spleen (39). In vitro, IgM+ IgDlow B cells were detected at day 49 (Fig. 1L) at a percentage similar to human BM (Fig. 1M). In conclusion, this feeder cell–free in vitro system reproduces development from CLP to immature B cells.

**In vitro B cell development from BM CD34+ cells**

In contrast with B cells generated in vitro from CB-derived CD34+ cells, human B cell precursors develop less efficiently from BM-derived CD34+ cells (27, 33, 40). Nevertheless, we observed steady increase of CD10+ cells generated in vitro from CD34+ precursors of adult BM (Fig. 2A), which was mirrored by the increase in CD10 mRNA levels (Fig. 2B). Like CB, BM CD34+ cells differentiated into CLP, pro-B, pre-B, and immature B cells. The subset of CD19+IgM− and of CD19+IgM+ cells formed between days 7 and 14 (Fig. 2C, 2E) most likely originated from CD34+ CLP and pro-B cells copurifying with CD34+ HSCs. The CD19+IgM− and CD19+IgM+ cells developing at later time points were most likely originating from CD34+ HSCs. This notion is supported by the steady increase in CLP/pro-B cells forming the major proportion of BM-derived precursor cells from day 28 on (Fig. 2C–F).

Catalyzed by RAG activity, precursor B cells in the BM rearrange IgH and IgL gene segments (41). CLPs start recombining IgH chain D\textsubscript{H} and J\textsubscript{H} gene segments followed by V\textsubscript{H} to DJ\textsubscript{H} rearrangement in pre-B cells. Then, IgL chain rearrangement in small pre-BI cells (1) allows surface IgM expression in immature B cells. \textit{RAG2} expression and IgH chain D\textsubscript{H} to J\textsubscript{H} rearrangement in both CB- and BM-derived B cell precursors (Fig. 3A) demonstrated the continuous generation of pro-B cells from lymphocyte precursors between days 7 and 49. In vitro development of pre-BI cells from pro-B cells between days 28 and 42 was documented by ongoing VDJ recombination (Fig. 3B–D). Sequencing of the VDJ junctions of rearranged IgH chains revealed a diverse repertoire and excluded expansion of single clones in vitro (data not shown).

**Long-term persistence of CLP/pro-B cells differentiating from BM-derived CD34+ cells**

CD34+ cells from seven different CB donors displayed a very similar pattern of B cell development. At day 28, on average, >20% of cells were CD10+ CLP, giving rise to >30% of B cell

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**FIGURE 2.** Early human B cell development in vitro starting from BM-derived CD34+ cells. (A) Starting from BM CD34+ cells, expression of CD10 and CD38 in live cell gate was monitored over time by flow cytometry. (B) Expression of CD10 mRNA was determined by qPCR. Shown are mean and SEM of two independent experiments. (C) Expression of surface CD19 and cytoplasmic (cy) CD79a over time in CD10+ live cells. (D) qPCR analysis of CD79A expression at indicated time points from two independent experiments. Mean and SEM are shown. (E) IgM and CD19 or (F) cyCD179a and CD19 expression over time in CD10+ live cells. (A, C, E, F) Data are representative of three to seven independent experiments.
precursors at day 35 (Fig. 4A). Between days 14 and 21, >50% of CD10+ cells were CD19+ IgM+ CLP and pro-B cells (Fig. 4B), whereas from day 28 on, the majority of CD10+ cells were CD19+ pre-BI and pre-BII cells (Fig. 4C). IgM+ CD19+ immature B cells were detected from day 35 on and accumulated up to 10% until day 49 (Fig. 1H).

In vitro differentiating CD34+ cells from seven different BM samples developed into CD10+ cells reaching on average 7% of the cells in culture at day 49 (Fig. 4D). Paradoxically, at day 14, >70% of CD10+ cells were CD19+ CD19+ IgM+ pre-BI and pre-BII cells. The percentage of these subsets declined to 35% at day 35 (Fig. 4F), whereas the fraction of CD10+ CD19− pro-B cells reached 50–80% between days 28 and 42 (Fig. 4E). Thus, CLP/pro-B cells were the major population found in long-term BM-derived culture. These cells most likely supported development of CD19+ cells throughout this period.

B cell lineage commitment is initiated by the transcription factors E12/E47 (E2A) and EBF1 (5). E2A and EBF1 induce expression of CD79A, CD79B, CD179A, CD179B, RAG1, RAG2, and PAX5 (42). PAX5 activity completes B lineage commitment (43) and promotes the expression of genes encoding the pre-BCR/BCR-associated proteins CD19 and BLNK (5). Similar levels of

**FIGURE 3.** DJ and VDJ recombination in newly developed B cells in vitro. CD34+ cells isolated from CB or BM were developed in vitro into B cells as described. (A) Expression of RAG2 mRNA relative to RPLPO in CB (left) and BM (right)-derived cells. Data represent mean and SEM of three and two experiments, respectively. (B–D) DNA was isolated at indicated time points. The DJ recombination (upper panels) was assessed by using six Dn primers and one Jn primer. The VDI recombination (middle panels) was detected by using seven Vn primers and one Jn primer (31). GAPDH control (lower panels). Lane M: DNA ladder; lanes 1 and 2: culture from CB-derived CD34+ cells; lane 3: culture from BM-derived CD34+ cells; lane 4: EBV immortalized B cell line as control.

**FIGURE 4.** Differences in developmental dynamics in CB- and BM-derived cultures. (A and D) Appearance of CD10+ cells was monitored by flow cytometry over time. Data show the percentage of CD10+CD38−CD33− out of live cells. In the CD10+ B and precursor cell gate, the percentages of (B, E) CD19+ IgM+ CLP and pro-B cells and (C, F) CD19+ IgM+ pre-BI and pre-BII cells were identified. Data represent duplicate or triplicate experiments of seven different (A–C) CB or (D–F) BM donors. Each dot represents a single well, and bars the mean ± SEM. (G–I) Expression levels of transcription factors driving B cell differentiation were determined by qPCR. Shown are mean and SEM of independent experiments using three CB and two BM donors.
E2A transcripts were found in CB- and BM-derived samples over the entire period of 49 d (Fig. 4G). In both CB and BM, EBF1 expression increased between days 14 and 28 (Fig. 4H), followed by upregulation of PAX5 from day 28 on (Fig. 4I). However, in BM-derived samples, EBF1 and PAX5 expression were ∼10 times lower than in CB samples, reflecting the lower efficiency in generating B cells.

Thus, CD34+ HSC and CLP/pro B cells from human BM develop in vitro into pre-B and immature B cells. Notably, pro-B and pre-B cells continued to proliferate for 35 d in the absence of exogenously added cytokines.

**Cytokine-independent proliferation of human precursor B cells in vitro**

Murine pro-B and pre-B cells proliferate in response to IL-7 (44). Pre-BCR signals induce at first proliferation of large pre-B cells followed by allelic exclusion and the differentiation into small pre-B cells (2). Human pre-B cells develop independently of IL-7R signaling (45). To study proliferation during the different stages of development in vitro, we analyzed BrdU incorporation and expression of the cell division marker Ki67. As expected, within the first 14 d, 40–70% of the cells grown in medium supplemented with SCF, Flt3-L, and IL-6 or IL-7 incorporated BrdU.
(Fig. 5A). After cytokine withdrawal, the fraction of proliferating cells decreased steadily; but surprisingly, after 4 wk of cultivation in cytokine-free medium, 7–9% of the cells were still dividing as they incorporated BrdU. To compare the B cell precursor subsets proliferating in vitro directly to BM B cell subsets proliferating in vivo, we analyzed expression of the proliferation marker Ki67. During in vitro differentiation of BM-derived precursor B cells, between 7.2% (day 28) and 18.8% (day 49) of CD10+CD19− and 2.5–4.3% of CD10+CD19+ cells expressed Ki67 (Fig. 5B). In fresh BM, 2.4% of CD10+CD19− and 12.3% of CD10+CD19+ cells were Ki67+ (Fig. 5C). In both ex vivo and in vitro samples, most, if not all, Ki67+ cells were pre-B or pro-B cells, because they did not express surface IgM (Fig. 5B, 5C). For CB-derived B cell precursors, the majority of Ki67+ cells were CD10+CD19− IgM− pre-B cells (Fig. 5D, 5E). During in vitro differentiation, CB- and BM-derived cultures did not only differ by the proportions of proliferating pro-B and pre-B cells (Fig. 5E), but also by absolute numbers (Fig. 5F). In CB samples, CD10+ cells appeared between 14 and 21 d, reaching their maximum after 35 d. Although the CD34+ progenitors from BM differentiated less efficiently into precursor B cells, the largest number of CD10+ cells was also reached after 35 d of in vitro cultivation (Fig. 5F). In both settings, however, pro-B and pre-B cells were found to proliferate even 5 wk after cytokine withdrawal, suggesting that cytokine-independent proliferation allows the long-term persistence of human pro-B and pre-B cells.

In vitro, in addition to the B cell precursors, also monocytes develop from CD34+ progenitor cells. To exclude that factors produced by monocytes promoted the proliferation of precursor B cells in the absence of exogenously added cytokines, we sorted pro-B and pre-B cells from human BM, labeled the cells with CFSE, and cultivated them for 7 d in cytokine-free medium or in the presence of IL-7 and SCF. In absence of stimuli or feeder cells, ex vivo purified CLP/pro-B cells performed four to five cell divisions (Fig. 6A). The addition of IL-7 and SCF increased the proliferation rate of CD10−CD19+ CLP/pro-B cells (Fig. 6B) but suppressed their differentiation into immature B cells (Fig. 6C, 6D). Although we could not discriminate whether the fraction of proliferating pre-B cells had developed from pro-B cells, these results clearly demonstrate that precursor B cells can proliferate autonomously in the absence of cytokines like IL-7 and SCF.

Another possible source of IL-7 in culture might be the production by developing stroma cells that might have been contained in the starting CD34+ population. In line with the results obtained from ex vivo sorted CLP/pro-B cells (Fig. 6), in absence of the initial IL-7R stimulation, immature IgM+ B cells appeared earlier, whereas IL-7 treatment between days 7 and 14 increased the amount of precursors and pre-B cells (Fig. 7A–D). In addition, proliferation of cultured B cells was detectable both in the presence and the absence of IL-7 (Fig. 7E, 7F). Thus, IL-7R signaling was dispensable for human B cell development and proliferation, but increased the proportion of early B cell precursors.

**CD34+ cells developing in vitro into B cells give rise to B cells in vivo and are multipotent**

Finally, we demonstrated that B cells developing in our feeder cell–free in vitro system are highly similar to cells developing in vivo. In vitro, we analyzed the presence of stroma cells by detaching the adherent cells. Both fractions were then combined and analyzed by flow cytometry. As shown in Supplemental Fig. 3A–D, the number of CD34+CD45−Stro-1+ adherent cells was below detection levels. Moreover, because IL-7 was detected in the culture supernatants only when it was added as a supplement between days 7 and 14 (Supplemental Fig. 3E), it is unlikely that cells present in the in vitro culture produced significant amounts of IL-7.

To investigate the contribution to B cell differentiation of IL-7R triggering between days 7 and 14 of culture, we differentiated CD34+ cells into B cells in absence of IL-7. In line with the results obtained from ex vivo sorted CLP/pro-B cells (Fig. 6), in absence of the initial IL-7R stimulation, immature IgM+ B cells appeared earlier, whereas IL-7 treatment between days 7 and 14 increased the amount of precursors and pre-B cells (Fig. 7A–D). In addition, proliferation of cultured B cells was detectable both in the presence and the absence of IL-7 (Fig. 7E, 7F). Thus, IL-7R signaling was dispensable for human B cell development and proliferation, but increased the proportion of early B cell precursors.

**FIGURE 6.** Ex vivo sorted CLP/pro-B and pre-B cells proliferate in culture and give rise to IgM+ cells. FACS sorted CD22−CD10−IgM−CD33− precursors and B cells from human BM were labeled with CFSE and plated at 105 cells/ml in (A, C) medium only or (B, D) in presence of SCF and IL-7 for 6 d. (A and B) CFSE dilution in the three indicated gates. (C and D) Data show proportion of CD19 and IgM expressing cells in live gate. Representative of two independent experiments.

**FIGURE 7.** B cell development is independent from IL-7R signaling. CB-derived CD34+ cells were cultured for 49 d in presence or absence of IL-7 between days 7 and 14. (A) Absolute numbers of precursor and B cells, and (B–D) the proportion of CLP/pro-B, pre-BL, and pre-BII cells and immature B cell is shown. Data show mean and SEM of two independent experiments done in duplicates or in triplicates. (E and F) Ki67 expression in B cells in the CD10+CD38− live cell gate. Experiment was performed in triplicates, using cells from the same CB donor. (F) Mean and SEM of proliferating CD19+ cells in culture at indicated time points are shown. Statistical analysis was performed using the unpaired *t* test, two-tailed; *p = 0.038, **p = 0.0047–0.0098, ***p < 0.0001. ns, not significant.
in vivo by comparing in vitro developing B cells to B lymphocytes developing in humanized mice (46). To this end, the same batches of expanded CD34+ cells that were used for the in vitro differentiation were transplanted in newborn Rag2^{−/−} IL2rg^{−/−} mice. Like in human BM and very similar to the in vitro differentiation system, we found CLPs, pro-B, pre-BI, pre-BII, and immature B cells in the BM. All B cell subsets had developed in 15- to 18-wk-old mice. However, differences from in vitro long-term B lymphopoiesis were found even 24–36 wk after reconstitution (Fig. 8 and data not shown). Immunohistochemical analyses of BM paraffin sections revealed that CD34+ precursor cells were closest to the endosteum (Fig. 9A), whereas CD10+ cells including CLPs and B cell precursor stages, as well as proliferating Ki67+ cells, were distributed between the endosteum and the center of the BM. Immature B cells expressing CD19, IgM, Igκ, and Igλ L chain (Fig. 9A) were located closer to the BM sinuses. This pattern of precursor cells found in humanized mice was similar to ex vivo human BM biopsies (Fig. 9B).

Similar to CB, transplantation of human BM-derived CD34+ cells into newborn Rag2^{−/−} IL2rg^{−/−} mice led to the reconstitution of all B cell stages in 8- to 14-wk-old mice (Fig. 10A, 10B), again resembling all stages found in adult human BM (Fig. 10C). However, compared with CB-derived cells, B lymphopoiesis strongly declined with time, and higher numbers (4 × 10^6) of adult CD34+ cells were needed to reach comparable levels of engraftment (Fig. 10D, 10E). This indicated a lower frequency of precursor cells in the adult CD34+ pool.

The multipotency of transplanted CD34+ cells was proved, as they did not only develop into cells of the B cell lineage but also into immature and mature T cells (Supplemental Fig. 4A–F), and into myeloid lineage cells (Supplemental Fig. 4G). Human mesenchymal stem cells or stroma cells were not detectable by flow cytometry (Supplemental Fig. 3A–D).

**FIGURE 8.** Human CB-derived CD34+ cells reconstitute early B cell development in BM of Rag2^{−/−} IL2rg^{−/−} mice. Magnetically separated CD34+ cells from human CB were cultured for up to 1 wk with SCF, Flt3-L, and IL-6, and transplanted into sublethally irradiated Rag2^{−/−} IL2rg^{−/−} newborn mice. BM was analyzed 15–24 wk after transplantation. The different B cell populations were identified by flow cytometry. (A) In the CD34+CD38+ live gate CLP and pre-BI cells were defined as CD10+CD19 and pre-BI as CD10+CD19+. The CD38+CD34- live population contained mainly CD19+ cells. In this gate IgM- are pre-BII cells and IgM+ are immature B cells. This gating strategy was used to generate the statistic shown in (B). (B) Percentage of CLP and early B cell subsets found in humanized mice at different time post transplantation. Mean and SEM are shown. (C) Summary of analyzed mice, with details about transplanted cell numbers, number and age of analyzed mice and reconstitution efficiency. (D) Phenotype of B cells found in the BM of humanized mice by flow cytometry. Plots show cytoplasmic (cy) CD179a, IgM, CD19, and cyCD79a expressing cells in CD10+CD33+ living cells.
Discussion

In humans, early, BM-dependent stages of B cell development are experimentally almost inaccessible. Therefore, the proliferative capacity of pro-B and pre-B cells has remained elusive. Comparing the proliferation of pro- and pre-B cells generated in a feeder cell–free in vitro differentiation system, the division of purified pro-B and pre-B cells, the expression of Ki67 by B cell subsets, and the development of B cells in humanized mice, we demonstrated that human pro-B and pre-B cells proliferate autonomously in the absence of cytokines. Thus, human precursor B cells differ from corresponding murine subsets, as the latter stop proliferating in the absence of IL-7R signaling and undergo cell death (14, 47). Development of human B cells independent of IL-7R signaling has been evident since the discovery that B cells develop normally in immunodeficient patients carrying mutations in the IL-7R \( \beta \) (16) or the IL-7R \( \alpha \) chain (18). Previous in vitro studies reported inefficient development of human pro-B cells (34), reduced proliferation of B cell precursors, as well as lowered CD19 expression (33, 48) in absence of IL-7. In line with this, we found that the addition of IL-7 supports the expansion of B cells in early stages, whereas the development and proliferation of early B cells is completely independent of IL-7R signaling. Other cytokines like thymic stromal lymphopoietin (3) have been reported to induce human precursor B cell proliferation. However, mutations affecting components downstream of the pre-BCR signaling pathway like BTK (49) and BLNK (50) suggest that pre-BCR function is an essential element required for proliferation and survival of human precursor B cells. Our observation that purified human pro-B and pre-B cells replicate autonomously in vitro in the absence of any exogenously added cytokines strongly support this notion. Thus, the human pre-BCR integrates most likely the functions that are transmitted in mice separately through the IL-7R and the pre-BCR. In mice, pre-BCR signals are required to initiate proliferation but not to sustain the expansion of precursor B cell subsets because this is the task of the IL-7R (2). Chromosomal aberrations in genes regulating the expression of pre-BCR–associated molecules and of downstream signaling components like PAX5, E2A, and BTK have been reported to affect precursor B cell development in humans (19).
c-ABL, and IKAROS are frequently found in pre-B acute lymphoblastic leukemia (51), thus emphasizing the prominent role of the pre-B/BCR in regulating growth and differentiation of human precursor B cells. Because the pre-BCR is composed from the rearranged IgH chain, the surrogate L chain, and the signal transducing components Igα and Igβ, the receptor is competent to signal only after IgH chain rearrangement has been completed in pre-B1 cells. Thus, pro-B cell proliferation must be regulated by other signals. TLR activation, for instance, may play an important role because it has been shown that the function of gp96, a major chaperone of multiple TLRs passing the endoplasmatic reticulum, is essential for pro-B cell replication and for the transition from pro-B to pre-B cells (52).

In vitro, the replicating pro-B and pre-B cells constitute the majority of proliferating cells. This implies an important role for both precursor B cell subsets in the maintenance of the B cell compartment as changes in the proliferative capacity of CLP/pro-B and pre-B cells may affect B cell reconstitution in patients treated with B cell depletion (21) or chemotherapy (22).

Several aspects of our in vitro system differ from previously reported schemes. Instead of using feeder cells, we transferred the expanded HSCs to plates coated with ICAM-1–Fc, because CD34+ HSCs are normally in close contact with BM stroma cells. ICAM-1 strongly binds to integrins CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1) expressed by human CD34+ cells (53). It was demonstrated that survival of HSCs cultured in vitro on osteo-blasts depends on ICAM-1, and the addition of ICAM-1 has been found to replace direct cell–cell contacts (54). LFA-1 is also expressed by B cells promoting cell adhesion, thereby facilitating immunological synapse formation and B cell activation (55), mAbs that block adhesion of B cell progenitors to BM stroma cells in vitro prevent B cell differentiation in vivo (56). In line with this, we observed that ICAM-1–Fc consistently increased the absolute amount of B cells, although it was dispensable for early B cell development.

In this precisely defined system, the stage-specific expression of characteristic markers and of lineage-determining transcription factors recapitulates all phases of B cell development found in adult human BM. Like in vivo, E2A is expressed from very early on (50), followed by EBF1 expression starting when CD34+ differentiate into CLPs and early pro-B cells. E2A and EBF1 induce PAX5 expression, the in vitro differentiating factor that recapitulates all phases of B cell development found in adult human BM. TheJournal ofImmunology1053

Commitment of CD34+ cells to the B cell lineage. Because E2A is expressed from very early on (50), followed by EBF1 expression starting when CD34+ differentiate into CLPs and early pro-B cells. E2A and EBF1 induce PAX5 expression, the in vitro differentiating factor that recapitulates all phases of B cell development found in adult human BM. TheJournal ofImmunology1053

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