Brief Report

Nonpermissive bone marrow environment impairs early B-cell development in common variable immunodeficiency

Arianna Troilo,[1] Claudia Wehr,[2] Iga Janowska,[1] Nils Venhoff,[1] Jens Thiel,[1] Justyna Rawluk,[2] Natalie Frede,[1] Julian Staniek,[1,3] Raquel Lorenzetti,[1] Marei-Theresa Schleyer,[1] Georg W. Herget,[4] Lukas Konstantinidis,[4] Miriam Erlacher,[5] Michele Proietti,[6] Nadezhda Camacho-Ordonez,[6] Reinhard Edmund Voll,[1] Bodo Grimbacher,[6-9] Klaus Warnatz,[1,6] Ulrich Salzer,[1] and Marta Rizzi[1,10]

[1]Department of Rheumatology and Clinical Immunology, [2]Department of Medicine I, [3]Faculty of Biology, [4]Department of Orthopedics and Trauma Surgery, [5]Faculty of Medicine, Division of Pediatric Hematology and Oncology Medical Center, Department of Pediatrics and Adolescent Medicine, and [6]Center for Chronic Immunodeficiency (CCI), Institute for Immunodeficiency, Medical Center, Faculty of Medicine, Albert-Ludwigs University of Freiburg, Freiburg, Germany; [7]DZIF–German Center for Infection Research, Satellite Center Freiburg, Germany; [8]Centre for Integrative Biological Signalling Studies, Albert-Ludwigs University of Freiburg, Freiburg, Germany; [9]RESIST–Cluster of Excellence 2155 to Hannover Medical School, Satellite Center Freiburg, Germany; and [10]CCI, Faculty of Medicine, Albert-Ludwigs University of Freiburg, Freiburg, Germany

KEY POINTS

- Study of B-cell developmental dynamic in vitro distinguishes intrinsic B-cell defects from bone marrow microenvironment defects in CVID.

- Impairment of the bone marrow niche can contribute to B-cell defects and CVID pathogenesis.

Common variable immunodeficiency (CVID) is a disease characterized by increased susceptibility to infections, hypogammaglobulinemia, and immune dysregulation. Although CVID is thought to be a disorder of the peripheral B-cell compartment, in 25% of patients, early B-cell development in the bone marrow is impaired. Because poor B-cell reconstitution after hematopoietic stem cell transplantation has been observed, we hypothesized that in some patients the bone marrow environment is not permissive to B-cell development. Studying the differentiation dynamics of bone marrow-derived CD34+ cells into immature B cells in vitro allowed us to distinguish patients with B-cell intrinsic defects and patients with a nonpermissive bone marrow environment. In the former, immature B cells did not develop and in the latter CD34+ cells differentiated into immature cells in vitro, but less efficiently in vivo. In a further group of patients, the uncommitted precursors were unable to support the constant development of B cells in vitro, indicating a possible low frequency or exhaustion of the precursor population. Hematopoietic stem cell transplantation would result in normal B-cell repopulation in case of intrinsic B-cell defect, but in defective B-cell repopulation in a nonpermissive environment. Our study points to the importance of the bone marrow niche in the pathogenesis of CVID. (*Blood*. 2020;135(17):1452-1457)

Introduction

Common variable immunodeficiency disorders (CVID) are characterized by hypogammaglobulinemia and susceptibility to infections,[1] and can be associated with immune dysregulation.[2] Beside molecular diagnosis,[3] immunological and clinical classifications as well as functional characterization of the immunopathology are important to guide management.[4-6] In some CVID, a decrease in B cells emerging from the bone marrow (BM) (ie, transitional B cells) suggests a defect in BM output.[7,8] Indeed, impaired early B-cell development in the BM has been reported in 25% of CVID,[7,9,10] and after hematopoietic stem cell (HSC) transplantation one-half of surviving CVID patients presented incomplete B-cell reconstitution, independently of other confounding factors like mixed donor chimerism or graft-versus-host disease.[11] Thus, we hypothesized that some CVID patients have a nonpermissive BM environment for B-lymphopoiesis. B-cell development is a dynamic process (Figure 1A) regulated by a complex network of intrinsic signals and transcription factors, such as E2A, EBF-1, and Ikaros important for lineage specification, and
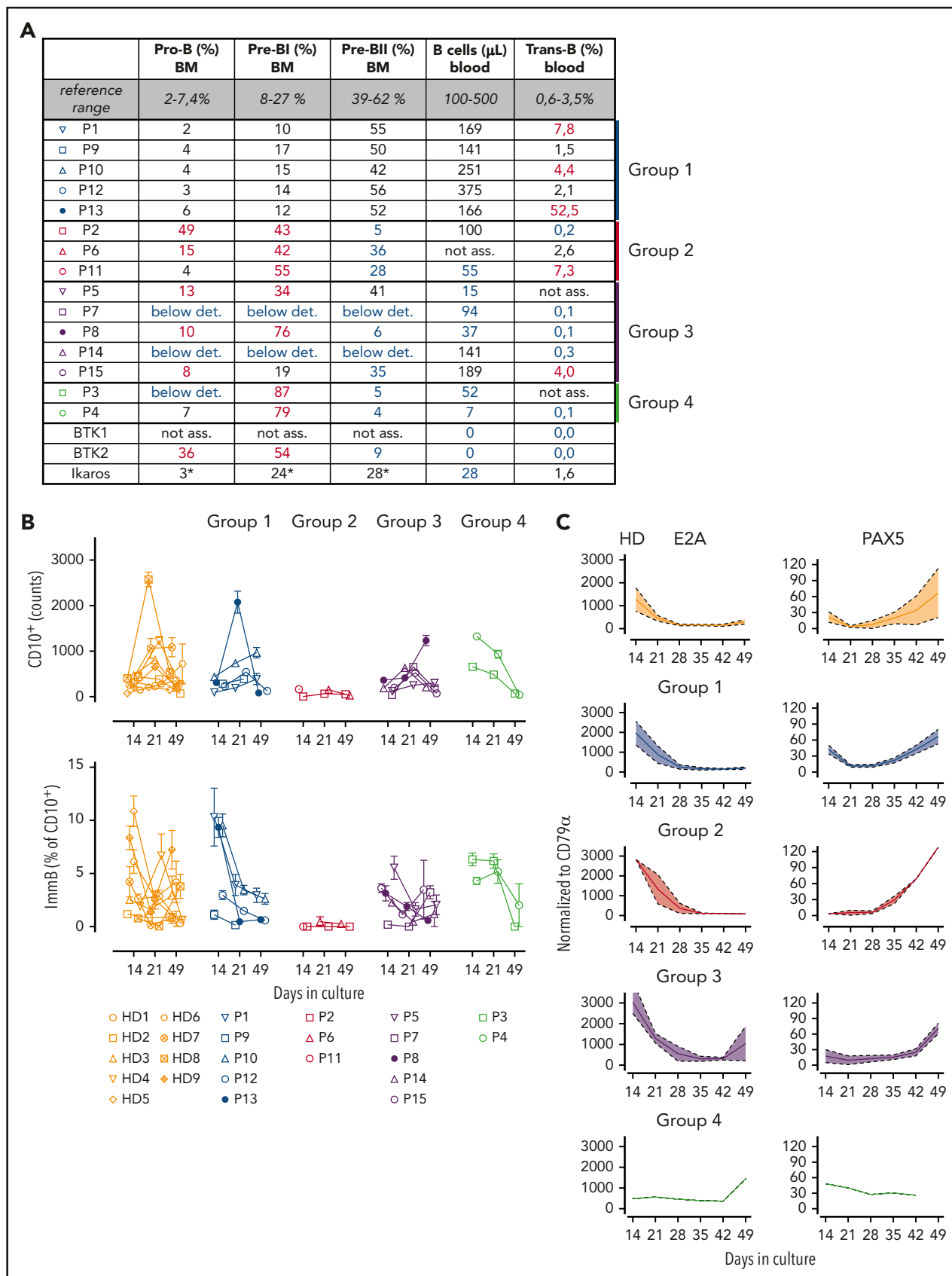
PAX5 for B-cell committment.[12] Cytokines and growth factors released by the niche can support[13] or actively inhibit[14,15] B-cell development. On the other side, if essential intrinsic signals driving B-lymphopoiesis fail, like in Bruton tyrosine kinase (BTK) deficiency, B-cell development is blocked.[16] BM flow cytometry analysis reliably identifies a skewed distribution of B-cell precursors, but is unable to establish its origin. To study the dynamic of early B-cell maturation starting from CD34+ cells of CVID patients independently of the BM environment and to assess the intrinsic potential of the HSCs to become B cells, we used a feeder-free system[17] developing immature B cells in vitro.

Study design

The study was approved by the Ethic Committee of the University Medical Center Freiburg (188/11). Each participant provided informed consent. We included 15 CVID patients, 2 BTK-deficient, 1 IKFZ1 (Ikaros)-deficient patients, and 9 healthy donors (HDs) (supplemental Table 1, available on the *Blood* Web
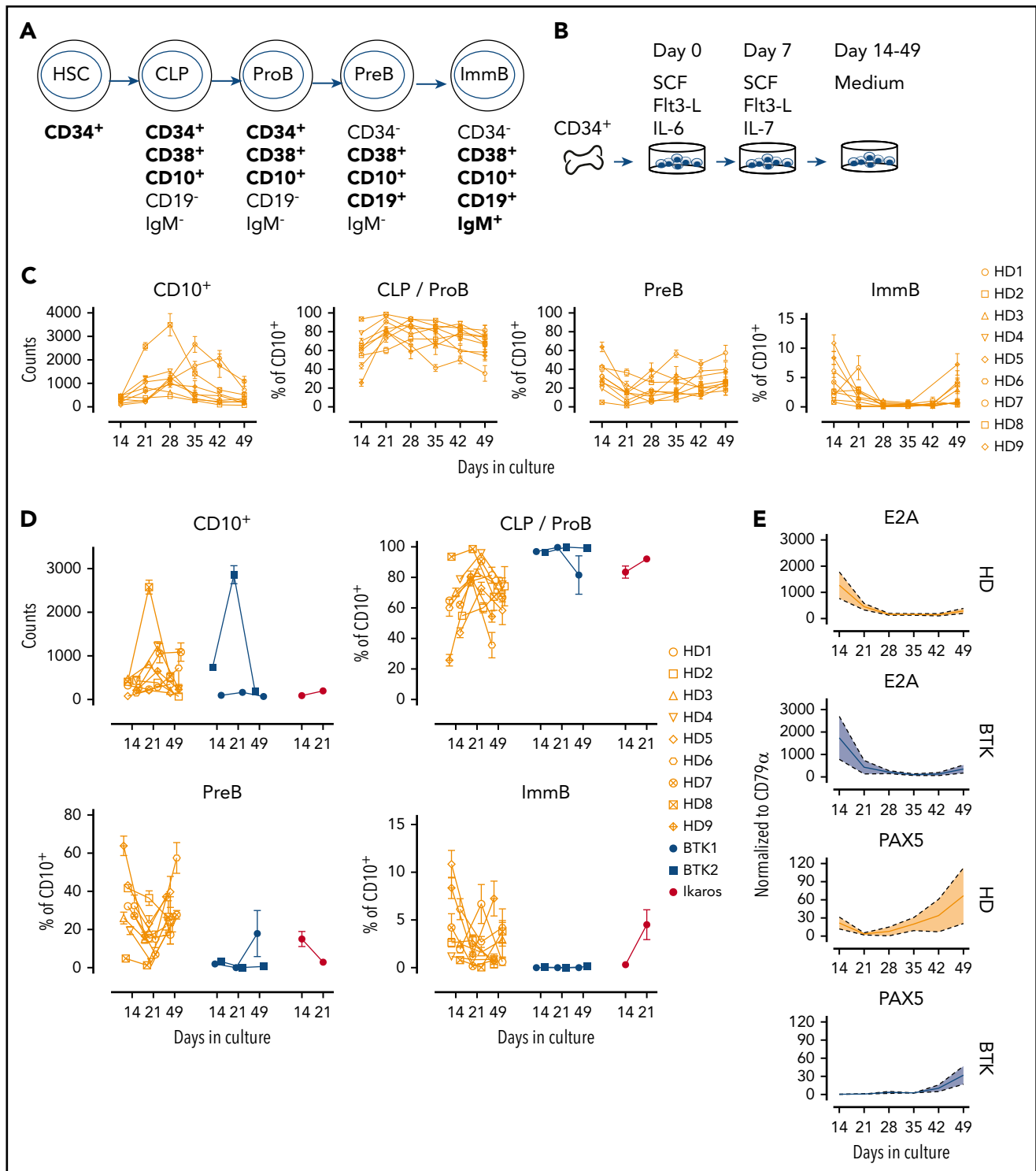
Figure 1. Developmental dynamic of BM-derived CD34+ in B-cell differentiating condition. (A) Early B-lymphocyte development with stage-characteristic surface markers. In bold are the markers used to identify distinct populations in vitro. ImmB: immature IgM+ B cells; PreB: pre-B-cells; ProB: pro-B-cells. (B) Scheme of the experimental setup. Magnetically isolated CD34+ cells from BM aspirates were expanded in the presence of SCF, Flt3-L, and IL-6, then in presence of SCF, Flt3-L, and IL-7. From day 14 to 49, cells were cultivated in cytokine-free medium and developing common lymphoid progenitors (CLP), as well as pro-, pre-, and immature B cells were analyzed weekly by flow cytometry. (C) Distribution of B-cell subpopulations over time in culture: Live CD10+ cells counts and within the CD10+ population percentages (%) of CLP and pro-B-cells (CLP/ProB), of pre-B-cells (PreB) and of immature B-cells (ImmB) between day 14 and 49 of culture in healthy donors (HDs). Each symbol shows a different HD represented as mean and standard error of mean of 4 to 10 technical replicates at each timepoint. (D) Outcome of in vitro development of BTK-deficient (BTK1, BTK2) and IKZF1-deficient (Ikaros) CD34+ cells. Live CD10+ cell counts, and within the CD10+ population percentages (%) of CLP and pro-B-cells (CLP/ProB), of pre-B-cells (PreB), and of immature B-cells (ImmB) at days 14, 21, and 49. Four to 10 replicates were analyzed at each timepoint for each patient and HD. Each symbol represents a patient; mean and standard error of mean are depicted. (E) Expression of transcription factors driving B-cell specification (E2A) and commitment (PAX5) in relation to CD79a expression, evaluated by quantitative polymerase chain reaction. Mean (line) and standard error of mean (shadow) of 2 BTK patients (blue) and 7 HDs (yellow).

Figure 2. Different developmental dynamics of BM CD34+ cells from CVID patients distinguish intrinsic defects from nonpermissive BM environment. (A) The table indicates the distribution of early B-cell stages in BM aspirates, analyzed by flow cytometry in the cohort of CVID patients and in patients with defined mutations in *BTK* (BTK1, BTK2) and *IKZF1* gene (Ikaros). Pro-B-cells are defined as CD22+ CD79a+ CD34+ CD22+, pre-BI cells as CD19+, cytoplasmic IgM−, cytoplasmic CD179a+, pre-BII cells as CD19+,

TROILO et al

site). Genetic analysis was performed in 10 CVID patients and 2 carried heterozygous *NFKB1* mutations (supplemental Table 2). BM isolated CD34$^+$ cells were cultivated in vitro (Figure 1B) as previously described.[17] Details on patient cohort and methods are provided in supplemental Material and Methods.

Results and discussion

After HSC transplantation, B cells appear in circulation after 40 to 60 days.[18] Similarly, in vitro development of cord blood CD34 cells resulted in a single wave of development first pro-B then pre-B and finally after 42 to 49 days of immunoglobulin M positive (IgM$^+$) immature B cells (supplemental Figure 1A). In contrast, healthy BM CD34$^+$ cell culture resulted in a rapid first wave of immature IgM$^+$ cell development at days 14 to 21 (Figure 1C; supplemental Figure 1B-C). This derived from already committed progenitors and pro-B-cells that also express CD34 (Figure 1A) and are isolated together with the HSCs. The second wave of immature IgM$^+$ cells at days 42 through 49 (Figure 1C; supplemental Figure 1B-E) derived from uncommitted progenitors present in the CD34$^+$ pool, with a dynamic similar to more undifferentiated cord blood-derived CD34$^+$ cells. To validate our system, we used BTK-deficient BM CD34$^+$ cells. These developed into CD10$^+$ common lymphoid progenitors (CLP) and pro-B-cells, but showed reduced proportion of pre-B-cells and failed to reach the immature B-cell stage (Figure 1D; supplemental Figure 2A). The differential amount of CLP and pre-B-cells developing in vitro may be the consequence of the severity of the 2 distinct mutations in *BTK* (supplemental Table 2). The specification into the B-cell lineage in BTK-deficient cells was preserved with normal induction of the transcription factor E2A (Figure 1E), but the B-cell lineage commitment was inefficient with delayed and reduced transcription of EBF-1 (supplemental Figure 2B) and PAX5 (Figure 1E). In correspondence with immunoglobulin gene rearrangements, RAG2 and TdT messenger RNA were detected (supplemental Figure 2B). As a second model, we used a patient with *IKZF1* germline mutation. These patients have been shown to have low frequencies of CLPs and a normal relative distribution of BM B-cell precursors.[19,20] IKZF1-deficient BM CD34$^+$ cells inefficiently developed into CD10$^+$ cells in vitro, in accordance with the very low amount of CLPs in the BM aspirate of our patient (Figure 2A). Within this scarce CLP compartment, the dynamic of development was conserved, showing IgM$^+$ immature B cells at day 21 of culture (Figure 1D; supplemental Figure 2A) recapitulating the phenotype of BM B cells in *IZKF1* deficiency. Hence, our in vitro model is suitable to study the dynamic of defects in early B-cell development.

We analyzed BM aspirates from 15 CVID patients and found that the distribution of B-cell precursors was normal in 5/15 (group 1),

and altered in 10/15 patients, with an accumulation of pro- and pre-BI cells (group 2-4; Figure 2A). The latter correlated with low blood B-cell numbers (mean: 77/μL in group 2-4 vs 220/μL in group 1; *P* = 0.0045), and with low transitional B cells, underlying low BM output. In group 1, normal B-cell precursor frequencies ex vivo corresponded to a normal pattern of the dynamic of in vitro B-cell development (Figure 2B; supplemental Figures 3 and 4A) and to a timely induction of fate determining transcription factors such as E2A, EBF-1, and PAX5 (Figure 2C; supplemental Figure 3B). In group 2, CD34$^+$ cells (Figure 2B, red) did not develop into immature B cells in vitro. PAX-5 drives CD19 expression, and its detection supported the presence of pre-B-cells in the culture (Figure 2C). Factors related to immunoglobulin gene recombination (TdT, FOXO1) were only expressed early on in culture (supplemental Figure 4B). Genetic analysis performed in P2 did not result in molecular diagnosis (supplemental Table 2), and genetic information on P6 and P11 were not available. Nevertheless, this intrinsic inability to develop into B cells in vitro resembled the phenotype observed in agammaglobulinemia with defects in pre-B-cell receptor signaling,[21] in fate-determining transcription factors, or in zinc homeostasis.[22] Also, epigenetic defects[23] affecting transcription factors expression, cell growth, or development[24] may underly this phenotype, and further studies will be needed to address this topic.

In group 3, which had altered BM B-cell distribution ex vivo, CD34$^+$ cells (Figure 2B; supplemental Figures 3, 4A, and 5, purple) reached the immature B-cell stage in vitro, with developmental dynamics similar to HDs. Hence, the lymphoid precursors were able to become B cells in a permissive environment, suggesting that signals deriving from the niche may actively have impaired B-cell development. Supporting this hypothesis, B-cell depletion by rituximab in P7 and P14 resulted in permanent B-cell depletion (supplemental Figure 6A). Additionally, stroma cells isolated from P5 BM (supplemental Figure 6B) were less efficient in supporting immature B-cell development from HD isolated pro-B-cells (supplemental Figure 6C), and the addition of cytokines did not compensate this defect (supplemental Figure 6D). Indeed, several factors produced within the BM niche can negatively influence early B-cell development such as Notch[14] and transforming growth factor-β.[25] T-cell infiltrates in the BM can disturb the niche structure and affect progenitors,[26] and immunosuppressive therapy can also negatively influence B-cell development, but neither of them correlated with our findings (supplemental Table 1). Therefore, further studies will be needed to identify specific factors.

In group 4, CD34$^+$ cells (Figure 2B; supplemental Figures 3, 4, and 5A-D, green) reached the immature B-cell stage at day 14,

Figure 2 (continued) cytoplasmic IgM$^-$, IgM$^-$. Also, peripheral B-cell counts per microliter and the percentages (%) of transitional B cells in the CD19 B-cell population are indicated. In gray shade is the reference range, high values are indicated in red, and low values in blue. Pro-B: pro-B-cells; pre-BI and pre-BII: pre-BI and pre-BII cells. *Lymphoid precursors 0.7% (reduced, normal range 2% to 22%). Below det.: below detection level; Not ass.: not assessed. (B) Distribution of developing B-cell subpopulations over time in culture starting from CVID CD34$^+$ cells. Live CD10$^+$ cell counts (top) and proportion of immature B cells (ImmB, %, bottom) within the CD10$^+$ gate at days 14, 21, and 49 in HD and CVID patients. CD34$^+$ cells from HD (yellow), from CVID patients with normal BM analysis ex vivo (group 1, blue), from CVID patients with altered early B-cell development ex vivo (group 2, red: P2, P6, and P11; group 3, purple: P5, P7, P8, P14, and P15; group 4, green: P3 and P4). Each experiment was performed with 3 to 10 replicates. Each symbol represents 1 patient. Mean and standard error of mean are represented. Filled symbols represent NFkB1-deficient (P8 in purple and P13 in blue) patients. (C) Quantitative polymerase chain reaction analysis of expression levels relative to CD79a of transcription factors driving B-cell specification (E2A) and commitment (PAX5) in cultivated B cells. Mean (line) and standard error of mean (shade) are shown and evaluated on 7 HD, 3 CVID patients in group 1 (blue), 2 patients from group 2 (red), 3 patients from group 3 (purple), and 1 patient from group 4 (green).

but failed to generate new progenitors and pro-B-cells at days 21 to 35 and consequently the second wave of immature B cells (supplemental Figure 5E). The progressive decrease in PAX-5 expression and low expression of E2A and EBF-1 confirmed the short duration of the culture (Figure 2C; supplemental Figure 4B). Hence, group 4 presented a combined defect. On 1 side, the niche seemed less supportive of B-cell development, as also indicated by the defective B-cell reconstitution of P3 upon allogenic HSC transplantation (supplemental Figure 6A). On the other side, the short duration of culture may indicate a possible exhaustion or a reduced frequency of the HSC pool in these patients.

Two CVID patients carried heterozygous mutations in *NFKB1* (P8 and P13). Although P13 had normal ex vivo B-cell subpopulations in BM aspirate, P8 showed an impaired cell distribution among B-cell subpopulations (Figure 2A, filled symbols). Interestingly, both patients' CD34+ cells developed normally in vitro, indicating that NF-kB1 signaling is dispensable for B-cell development in humans, in line with normal BM development observed in the *nfkb1*-deficient mouse.[27] Combining phenotyping and in vitro modeling of early B-cell development, we were able to distinguish a B-cell-intrinsic defect from a defect of the BM microenvironment. To our knowledge, this is the first study of in vitro analysis of early B-cell development in CVID. Besides B-cell intrinsic mechanisms our results point to the importance of the BM niche in the pathogenesis of B-cell defects. The analysis described here may have future therapeutic implications as the latter group may exhibit a defect in B-cell repopulation after B-cell depletion or HSC transplantation.

Authorship

Contribution: M.R., C.W., U.S., and A.T. designed research; A.T., I.J., N.F., J.S., M.-T.S., and R.L. performed research; N.V., J.T., J.R., G.W.H., L.K., M.E., B.G., and K.W. contributed patients material and data; A.T. and M.R. analyzed data; M.P., N.C.-O., and B.G. analyzed genetic data; and M.R., A.T., C.W., U.S., and K.W. wrote the paper; and all authors critically read and reviewed the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: N.V., 0000-0001-7729-2759; G.W.H., 0000-0002-6225-0852; R.E.V., 0000-0002-5542-9133; B.G., 0000-0002-6897-6806; U.S., 0000-0002-1769-1157.

Correspondence: Marta Rizzi, Department of Rheumatology and Clinical Immunology, Medical Center – University of Freiburg, Hugstetter Strasse 55, 79106 Freiburg; e-mail: marta.rizzi@uniklinik-freiburg.de.

REFERENCES

1. Bonilla FA, Barlan I, Chapel H, et al. International Consensus Document (ICON): common variable immunodeficiency disorders. *J Allergy Clin Immunol Pract.* 2016;4(1):38-59.

2. Resnick ES, Moshier EL, Godbold JH, Cunningham-Rundles C. Morbidity and mortality in common variable immune deficiency over 4 decades. *Blood.* 2012;119(7):1650-1657.

3. Kienzler AK, Hargreaves CE, Patel SY. The role of genomics in common variable immunodeficiency disorders. *Clin Exp Immunol.* 2017;188(3):326-332.

4. Chapel H, Lucas M, Lee M, et al. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. *Blood.* 2008;112(2):277-286.

5. Wehr C, Kivioja T, Schmitt C, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. *Blood.* 2008;111(1):77-85.

6. Giovannetti A, Pierdominici M, Mazzetta F, et al. Unravelling the complexity of T cell abnormalities in common variable immunodeficiency. *J Immunol.* 2007;178(6):3932-3943.

7. Ochtrop ML, Goldacker S, May AM, et al. T and B lymphocyte abnormalities in bone marrow biopsies of common variable immunodeficiency. *Blood.* 2011;118(2):309-318.

8. Driessen GJ, van Zelm MC, van Hagen PM, et al. B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency. *Blood.* 2011;118(26):6814-6823.

9. Lougaris V, Baronio M, Masneri S, et al. Correlation of bone marrow abnormalities, peripheral lymphocyte subsets and clinical features in uncomplicated common variable immunodeficiency (CVID) patients. *Clin Immunol.* 2016;163:10-13.

10. Anzilotti C, Kienzler AK, Lopez-Granados E, et al. Key stages of bone marrow B-cell maturation are defective in patients with common variable immunodeficiency disorders. *J Allergy Clin Immunol.* 2015;136(2):487-490.

11. Wehr C, Gennery AR, Lindemans C, et al. Multicenter experience in hematopoietic stem cell transplantation for serious complications of common variable immunodeficiency. *J Allergy Clin Immunol.* 2015;135(4):988-997.

12. Somasundaram R, Prasad MA, Ungerbäck J, Sigvardsson M. Transcription factor networks in B-cell differentiation link development to acute lymphoid leukemia. *Blood.* 2015;126(2):144-152.

13. Fistonich C, Zehentmeier S, Bednarski JJ, et al. Cell circuits between B cell progenitors and IL-7+ mesenchymal progenitor cells control B cell development. *J Exp Med.* 2018;215(10):2586-2599.

14. Pui JC, Allman D, Xu L, et al. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity.* 1999;11(3):299-308.

15. Ichii M, Oritani K, Yokota T, et al. Regulation of human B lymphopoiesis by the transforming growth factor-beta superfamily in a newly established coculture system using human mesenchymal stem cells as a supportive microenvironment. *Exp Hematol.* 2008;36(5):587-597.

16. Vogler LB, Pearl ER, Gathings WE, Lawton AR, Cooper MD. Letter: B lymphocyte precursors in bone-marrow in immunoglobulin deficiency diseases. *Lancet.* 1976;2(7981):376.

17. Kraus H, Kaiser S, Aumann K, et al. A feeder-free differentiation system identifies autonomously proliferating B cell precursors in human bone marrow. *J Immunol.* 2014;192(3):1044-1054.

18. Marie-Cardine A, Divay F, Dutot I, et al. Transitional B cells in humans: characterization and insight from B lymphocyte reconstitution after hematopoietic stem cell transplantation. *Clin Immunol*. 2008;127(1): 14-25.

19. Schwickert TA, Tagoh H, Gültekin S, et al. Stage-specific control of early B cell development by the transcription factor Ikaros. *Nat Immunol*. 2014;15(3):283-293.

20. Hoshino A, Okada S, Yoshida K, et al. Abnormal hematopoiesis and autoimmunity in human subjects with germline IKZF1 mutations. *J Allergy Clin Immunol*. 2017;140(1): 223-231.

21. Espeli M, Rossi B, Mancini SJ, Roche P, Gauthier L, Schiff C. Initiation of pre-B cell receptor signaling: common and distinctive features in human and mouse. *Semin Immunol*. 2006;18(1):56-66.

22. Anzilotti C, Swan DJ, Boisson B, et al. An essential role for the Zn^{2+} transporter ZIP7 in B cell development. *Nat Immunol*. 2019;20(3): 350-361.

23. Rae W. Indications to epigenetic dysfunction in the pathogenesis of common variable immunodeficiency. *Arch Immunol Ther Exp (Warsz)*. 2017;65(2):101-110.

24. Robertson KD. DNA methylation and human disease. *Nat Rev Genet*. 2005;6(8):597-610.

25. Tamayo E, Alvarez P, Merino R. TGFβ superfamily members as regulators of B cell development and function-implications for autoimmunity. *Int J Mol Sci*. 2018;19(12): E3928.

26. Nakamori Y, Liu B, Ohishi K, et al. Human bone marrow stromal cells simultaneously support B and T/NK lineage development from human haematopoietic progenitors: a principal role for flt3 ligand in lymphopoiesis. *Br J Haematol*. 2012;157(6):674-686.

27. Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. *Cell*. 1995;80(2):321-330.