Supplemental material and methods

Troilo et al.

Patients

BM aspirates from patients were obtained from leftover material from routine diagnostic testing. The analysis of patients BM was approved by the University Freiburg Ethics Committee (188/11) and the collection control BM was approved by the University Freiburg Ethics Committee (507/16). Control BM derived from otherwise healthy subjects undergoing orthopedic surgery, or BM aspirate performed for exclusion of malignancy. All patients and controls provided informed consent. The mean age of patients at BM sampling was 39.9 years (range 22-71) and 36.1 years (range 27-65) for healthy donors. The clinical characteristics of the patients are listed in Supplemental Table S1. The genetic analysis is described in Table S2. Umbilical cord blood (CB) was obtained after caesarean delivery upon signed informed consent under approval of the University Freiburg Ethics Committee (353/07_120590).

$CD34^+$ isolation

$CD34^+$ cells were isolated from BM aspirate or from CB by positive selection using the CD34 MicroBead Kit (Miltenyi Biotec), following manufacturer instructions. Purity of isolated cells was monitored by flow cytometry and was above 80% in all experiments.

In vitro culture

The modeling of early human B-cell development *in vitro* was previously described[1]. Briefly, BM or CB $CD34^+$ cells were cultivated in 96 wells plates at 10^5 cells/ml in Iscove's medium supplemented with insulin, transferrin, nonessential amino acids, glutamine, and reduced glutathione and 10% FCS with the addition of human IL-6, stem cell factor (SCF), Flt3-L (25 ng/ml each; all Immunotools). After 7 days, cells were harvested from original wells and re-plated at 1×10^5 cells/ml and cultivated in the presence of human IL-7 (20 ng/ml, Immunotools), SCF, and Flt3-L (25 ng/ml each). From day 14 on, cells were maintained in cytokine-free medium that was changed twice a week. Cells were analyzed weekly by flow cytometry and collected for RNA analysis, when sufficient material was available. To generate primary stroma cells, BM cells were isolated by Ficoll gradient and cultivated at the concentration of 10^6 cells/ml in Mesencult medium (Stemcell Technologies) supplemented with 10% Mesencult™ MSC Stimulatory Supplement (Stemcell Technologies). After 7 days, non-adherent cells were removed and adherent cells

spitted once they reached 70% confluency. Stroma cells were used for experiment at week 4-6 of culture to support the development of FACS sorted HD pro-B-cell (defined as $CD34^+$, $CD10^+$, $CD19^-$, IgM^-). In this setting, stroma cells were seeded at 500 cells/ml, in 96 wells flat bottom and pro-B-cell at 50.000/ml in enriched medium as described above. Cytokines were added as indicated. After 7 days of culture, the development of IgM^+ cells was analyzed by flow cytometry.

Flow cytometry

Phenotype of cultured cells was determined by flow cytometry using anti-CD34 PE-Cy7 (clone 581, Biolegend), anti-CD10 FITC (clone HI10a Biolegend, W8E7 BD), anti-CD38 PB (clone HIT2 Exbio), anti-CD19 BV510 (clone HIB19,Biolegend) or anti-CD19 APC-Cy7 (clone HIB19 Biolegend), anti-CD33 PerCP-Cy5.5 (clone WM53, Biolegend), anti IgD PE (Southern Biotech) or anti IgD PE-Cy7 (clone IA6-2, Biolegend), anti IgM AF647 (Jackson Immuno Research). Dead cells were identified by Live/Dead fixable Near IR Dead (APC-H7,Life Technologies GmbH), zombie NIR™ Fixable Viability Kit (APC-Cy7, Biolegend) or zombie Aqua™ Fixable Viability Kit (AmCyan, Biolegend). By means of timed acquisition on the flow cytometer, absolute counts of cells per well were collected. Data were acquired using a FACS Canto II (BD) and analyzed by FlowJo Software. The antibody panel used in diagnostic to define B cell subpopulations is based on a surface markers backbone composed by anti-CD10 APC (Beckton Dickinson), anti-CD3/CD16/CD33 mix PerCP-Cy5.5 (all 3 Biolegend), CD19 PE-Cy7 (Beckman Coulter), CD20 Pacific Blue (Biolegend). Intracellular markers were stained using the IntraPrep Kit (Beckman Coulter). In one staining the addition of surface anti-CD34 FITC (Beckton Dickinson), and intracellular anti-CD79 alpha PE (Beckman Coulter) helped identifying pro-B-cell and pre-BI cells, and in a second staining anti-surface IgM PE (Biozol), and intracellular anti-IgM FITC (Dako) allowed identifying pre-BI, pre-BII and immature B-cell.

Quantitative PCR

Total RNA was extracted using TRIzol (ThermoFisher Scientific) following manufacturer instructions and quantified with the Nanodrop 2000c (Thermo Scientific). For cDNA synthesis, 100 ng of RNA were reverse-transcribed using random hexamer primers (ThermoFisher Scientific) and SuperScript III reverse transcriptase (ThermoFisher Scientific). Quantitative PCR was performed using commercially available Taqman primer/probes kits: CD79alpha (HS00998119, Applied Biosystems), E2A (HS00413032, Applied Biosystems), EBF1 (HS01092694, Applied Biosystems), RAG2 (HS00379177, Applied Biosystems), FOXO1 (HS01054576_m1, Applied Biosystems), PAX5 (HS00277134_m1, Applied Biosystems), TdT (HS00958490_m1, Applied Biosystems), IgM (Hs00941538_g1, Applied Biosystems). 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 $2^{-\Delta Cq}$ method with cDNA concentrations standardized to the reference lineage specific gene *CD79alpha.*

Supplemental Figure Legend

Supplemental figure 1. *In vitro* CD34$^+$ culture in B-cell differentiating conditions results in immature B-cell development. (A) Dynamic of B-cell development starting from CB derived CD34$^+$ cells. The plot depicts the frequency of pro-B (ProB), pre-B (PreB) and immature-B (ImmB) cells in the CD10$^+$ gate. Each dot represent a replicate well (n=5 wells per time point). (B-E) Isolated BM CD34$^+$ cells were cultivated in B-cell differentiating conditions. At indicated time cells were harvested and stained as described. (B, C) Cells were identified via FSC and SSC, within this gate, single cells were identified, and then dead cells excluded from the analysis. Lymphoid precursors were identified by the expression of CD10. Within the gate of CD10$^+$ cells, pro-B-cell (ProB) and common lymphoid progenitors (CLP) were identified as CD19$^-$IgM$^-$ cells, pre-B-cell were identified as CD19$^+$IgM$^-$ cells and immature (ImmB) B-cell were identified as CD19$^+$IgM$^+$ cells. During the course of the study we used 2 different CD19 antibodies, the first conjugated with AmCyan and the second with APC-Cy7. Accordingly the live/dead cell exclusion was APC-Cy7 or AmCyan respectively. Hence, the panel (B) was used for P1-9 and HD 6-8 and the panel (C) was used for P11-15, BTK1, BTK2, Ikaros and HD 1-5 and HD 9. (D) IgM$^+$ cells appearing at day 49 of culture were further analyzed for different surface markers. As shown, IgM$^+$ cells were also CD19$^+$, CD38$^+$, partially IgD$^+$ and CD33$^-$. (E) Expression levels of IgM (heavy chain) transcripts by quantitative PCR during the culture supports the occurrence of two waves of B cell development. Data are expressed in relation to CD79α. Mean (line) and standard error of mean (shadow) are shown and evaluated on healthy donors (grey).

Supplemental figure 2. *In vitro* developmental dynamics of *BTK*- or *IKZF1*- (Ikaros) mutated CD34$^+$ cells reproduce patients BM B cell defect. Isolated BM CD34$^+$ cells were cultivated in B-cell differentiating conditions. At indicated time cells were harvested and stained as described. (A) Counts of pre-B-cell (PreB) and immature B (ImmB) cells at the indicated time points in healthy donors (HD), *BTK*- and *IKZF1*- (Ikaros) mutated patient. Symbols represent mean and standard error of mean of four to ten replicates per time point for each patient and HD, by timed acquisition on the flow cytometer. Each symbol represents an individual patient or HD. (B) Expression levels of indicated transcription factors driving B cell differentiation were evaluated by quantitative PCR. Data are expressed in relation to CD79α. Mean (line) and standard error of mean (shadow) are shown and evaluated on healthy donors (grey) and 2 BTK patients (yellow).

Supplemental Figure 3. **Expression dynamic of CD19 and IgM over time in live CD10$^+$ cells.** Common lymphoid progenitors (CLP) and pro-B-cell (ProB), pre-B-cell (PreB) and immature B-cell (ImmB) in CD10$^+$ gate of indicated patients (P) or healthy donor (HD). Each plot is representative of 1 replicates per time point.

Supplemental Figure 4. Dynamic of development of pro-B and pre-B-cell from BM CD34$^+$ cells of CVID patients. Percentage of common lymphoid progenitors (CLP) and pro-B-cell (ProB) and percentage of pre-B-cell (PreB) within the CD10$^+$ gate at day 14, 21 and 49 in healthy donors (HDs, grey) and CVID patients. CD34$^+$ cells derived from CVID patients with normal BM analysis *in vivo* are in blue (group 1); CVID patients with altered early B-cell development *in vivo*: P2, P6 and P11: red (group 2); P5, P7, P8, P14 and P15: purple (group 3); P3 and P4: green (group 4). Mean and standard error of mean of three to ten replicates are represented. Each symbol represents one patient. Filled symbols represent *NFkB1* deficient (P8 in purple and P13 in blue). (B) Expression levels of transcription factors driving B-cell specification and commitment. EBF1, RAG2, FOXO1 and TdT were evaluated by quantitative PCR. Mean (line) and standard error of mean (shade) are shown and evaluated on seven HD, three CVID patients with normal BM analysis *in vivo* (group 1, in blue), two patients from group 2 (in red), three patients from group 3 (in purple) and one patient from group 4 (in green). RNA expression relative to CD79α is shown.

Supplemental Figure 5. Rapid development of IgM$^+$ immature B-cells but failure to generate a second wave of immature B-cells in group 4 CVID patients. (A) Counts of CD10$^+$ cells, (B) common lymphoid progenitors (CLP) and pro-B-cell (ProB), (C) pre-B-cell (PreB) and (D) immature B-cell (ImmB) from day 14 to day 49 of culture in healthy donors (HD, grey) and CVID patients with impaired BM development *in vivo* group 3 (purple, patients P5, P7, P8, P14 and P15) and group 4 (green, P3 and P4). Each symbol represents a distinct patient. Mean and standard error of mean of three to ten replicates. Filled symbols represent NFkB1-deficient (P8 in purple and P13 in blue) patients. (E) Comparison of IgM$^+$ cells counts at day 14 and at day 49 of culture. Nonparametric Kruskal-Wallis test for multiple comparisons (**** p<0,0001; * p<0,05, n.s. : non-significant).

Supplemental Figure 6. Non-permissive BM environment in CVID: defective B-cell reconstitution upon B-cell depletion or hematopoietic stem cell transplantation and insufficient support to B-cell development from CVID stroma cells *in vitro*. (A) Patient P7 and P14 (group 3) were treated with rituximab (RTX), and P3 (group 4) with allogenic

hematopoietic stem cell transplantation (HSCT) at indicated time point and peripheral B cell repopulations analyzed by flow cytometry in peripheral blood. B-cell counts per microliter are indicated, grey shade indicates normal range. (B) To isolate stroma cells from HD and CVID P5 BM, BM cells were seeded in Mesencult medium for 4-6 weeks. Stroma cells were then used as feeders for HD FACS sorted pro-B-cell. Read-out of the experiment was the development of immature B-cell (ImmB), one week after seeding. (C) Development of immature B-cells (defined as double positive surface IgM^+, and cytoplasmic (cy) IgM^+) in presence of HD or P5 stroma cells only. (D) Results of the culture in presence of IL-7 and SCF.

Supplemental Table S1

	Sex	Age at BM analysis (years)	Time since diagnosis at BM analysis (years)	Infection susceptibility	Immunological dysregulation	BM T cells infiltrates	Immuno suppressive treatment before BM analysis
P1	F	36	4	Respiratory tract infections	Splenomegaly, lymphadenopathy, cytopenia, organ autoimmunity	yes	Steroids
P2	M	22	7	Chronic CMV infection, urinary and gastrointestinal tract infections, parotitis	Splenomegaly, lymphadenopathy, gut involvement, cytopenia, other autoimmunity (suspected hepatitis and diabetes), malignancy (plasmoblastic lymphoma)	yes	Steroids, mycophenolate mofetil
P3	M	40	3	Respiratory and gastrointestinal tract infections	Splenomegaly, lymphadenopathy, cytopenia, other autoimmunity (thyroiditis), malignancy (large granular lymphocytic leukemia)	yes	Steroids, Cyclosporine A
P4	M	53	22	Respiratory tract infections	Splenomegaly, gut involvement, lung involvement, cytopenia	yes	
P5	M	45	21	CMV, sinusitis, respiratory, gastrointestinal tract infections	Splenomegaly, lymphadenopathy, gut involvement, lung involvement, cytopenia, lentigo maligna	yes	
P6	M	34	0	Sinusitis, otitis, respiratory tract infections, (Herpes, EBV)	Splenomegaly, lung involvement, granuloma, cytopenia, oligoarthritis	yes	
P7	M	23	0	Respiratory tract infections, meningitis, gluteal abscess	Splenomegaly, cytopenia, CNS vasculitis	no	Steroids, Cyclosporine A
P8	F	29	1	Sinusitis, respiratory tract infections	Gut involvement	no	
P9	F	35	0	Respiratory, intestinal tract infections, H. pylori, Herpes zoster	Splenomegaly, lymphadenopathy, gut involvement, lung Involvement, granuloma, cerebellitis / neurosarcoidosis, B-cell lymphoma	no	
P10	M	41	0	Throat infections, sinusitis, respiratory tract infections	Cytopenia, spondylitis	yes	
P11	M	71	3	Respiratory, gastrointestinal tract infections, H. pylori	Splenomegaly, lymphadenopathy, gut involvement, gastric tubular adenoma with severe dysplasia, perianal epithelial papilloma with severe dysplasia	yes	
P12	F	62	2	Respiratory tract infections	Gut involvement	no	
P13	F	29	13	Respiratory tract infections	Splenomegaly, lymphadenopathy, gut involvement (chronic diarrhea), lung Involvement, cytopenia	n.d.	Steroids
P14	F	39	5	Respiratory, intestinal tract infections, H zoster, meningitis	Lymphadenopathy, gut involvement, lung involvement, cytopenia, other autoimmunity	no	Steroids
P15	F	37	0	Sinusitis, respiratory tract infections	Gut involvement, other autoimmunity	no	

BTK 1	M	43	40	Respiratory tract infections	Crohn-like disease, angiosarcoma of liver	yes	
BTK 2	M	40	37	Respiratory tract infections, lambliasis, chronic HCV	polyarthritis, CD8+-T cell lymphoma	yes	
IKZF1	M	31	3	EBV reactivation, Herpes, therapy-resistant HPV infection	Splenomegaly, lymphadenopathy, hepatomegaly, pancytopenia, recurrent fever	yes	

neg=negative; na=not assessed

Supplemental Table S2. Genetic data

Patient	Genetic analysis performed:	Detected mutation		
		Gene	Mutation	Zygosity
P1	whole exome sequencing	-	-	-
P2	panel sequencing *****	-	-	-
P3	panel sequencing *	-	-	-
P4	panel sequencing **	-	-	-
P5	whole exome sequencing	-	-	-
P6	n.d.	-	-	-
P7	n.d.	-	-	-
P8	panel sequencing ****	NFkB1	c.1066+1G>C (g.IVS11+1G>C)	heterozygous
P9	whole exome sequencing	-	-	-
P10	n.d.	-	-	-
P11	n.d.	-	-	-
P12	n.d.	-	-	-
P13	panel sequencing ****	NFkB1	c.835+2T>G (g.IVS9+2 T>G)	heterozygous
P14	panel sequencing ****	-	-	-
P15	panel sequencing ***	-	-	-
BTK1[#]	Sanger sequencing	BTK	c.215G>A (p.R28H)	hemizygous
BTK2[#]	Sanger sequencing	BTK	c.1750+5G>A (g.IVS17+5G>A)	hemizygous
Ikaros[§]	panel sequencing ******	IKZF1	c.1342 GC>AG insertion/deletion variant rs765655969 and rs750934235	heterozygous

n.d.: not done

	Genes included:
*	TNFRSF13B, NFKB2, NFKB1, IKZF1, CR2, ATP6AP1. CD19, CD81, PIK3CD, PIK3R1, LRBA
**	TNFRSF13B, CD19, CXCR4, NFKB2, ICOS, CR2, CD81, PRKCD, TNFRSF13C, MS4A1, TNFSF12, DNMT3B, NFKB1, LRBA
***	TNFRSF13B, CD19, CXCR4, NFKB2, ICOS, CR2, CD81, PRKCD, TNFRSF13C, MS4A1, TNFSF12, DNMT3B, NFKB1, LRBA
****	AKT1, GATA2, NFKB2, PRKCD, TFRC, BCL6, ICOS, NFKBIA, RAG1, TNFRSF13B, BTK, ICOSLG, PDCD1, RAG2, TNFRSF13C, CD274, IKBKB, PDCD1LG2, RELA, TNFRSF17, CD28, IKBKG, PIK3AP1, SH2D1A, TNFRSF4, CD80, LRBA, PIK3CD, STAT1, TNFSF13, CD86, NFKB1, PIK3R1, STAT3, TNFSF13B, CTLA4
*****	AKT1, CD79B, ICOS, MS4A1, RAG1, TNFRSF13B, APCS, CD80, ICOSLG, NFKB1, RAG2, TNFRSF13C, BCL6, CD81, IGHM, NFKB2, RELA, TNFRSF17, BLNK, CD86, IGLL1, NFKBIA, SH2D1A, TNFRSF18, BTK, CDX1, IKBKB, PDCD1, STAT1, TNFRSF4, CD19, CLEC16A, IKBKG, PDCD1LG2, STAT3, TNFSF10, CD27, CR2, IL21, PIK3AP1,

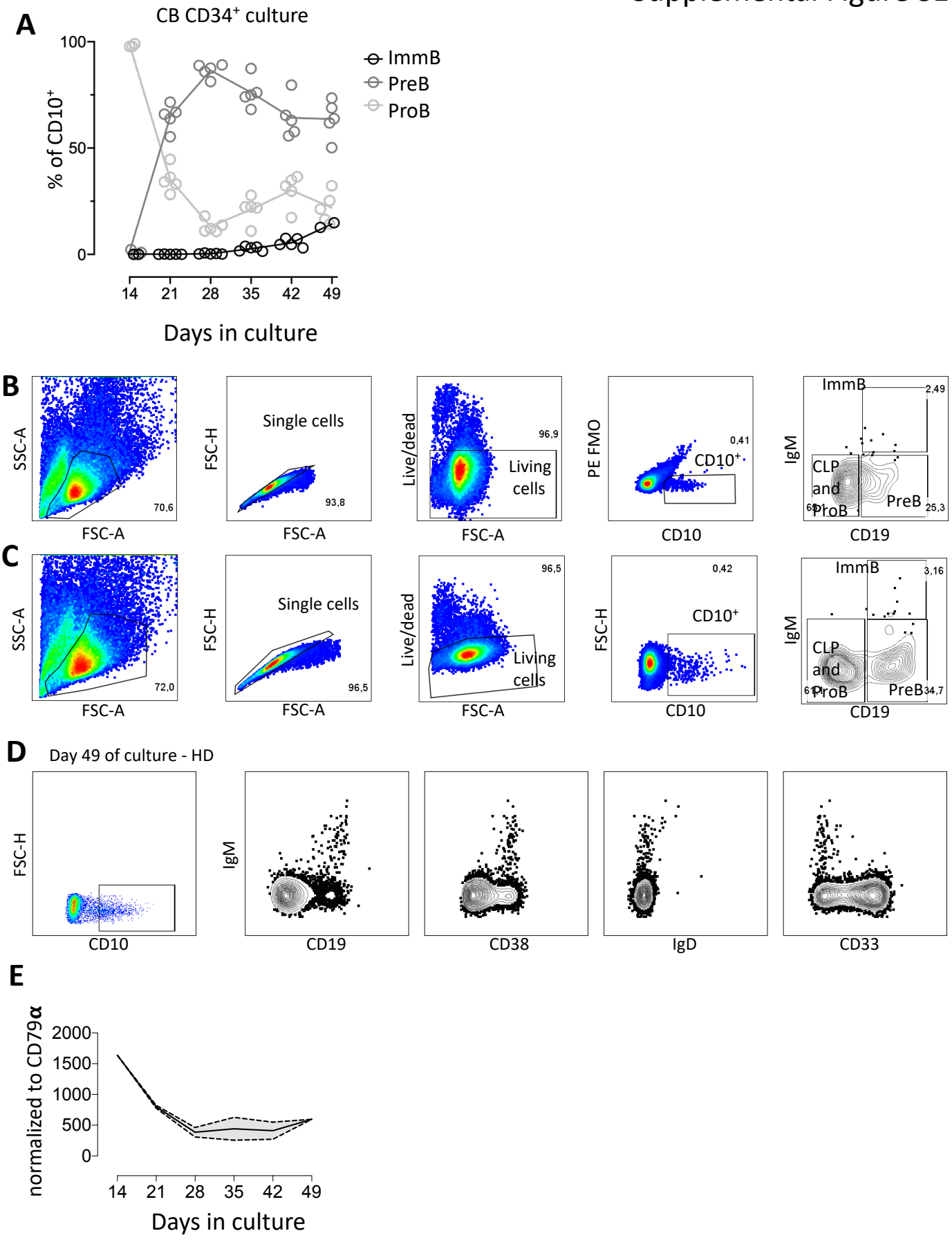
	TCF3, TNFSF13, CD274, CTLA4, IL21R, PIK3CD, TFRC, TNFSF13B, CD28, CXCL12, IRF4, PIK3R1, TGFB1, VAV1, CD40, CXCR4, LRBA, PRDM1, TGFB2, VAV2, CD40LG, DCLRE1C, MLH1, PRKCD, TGFB3, CD79A, GATA2
******	AICDA; AIRE; BBX; BTK; CARD11; CARD9; CD274; CD28; ADA2; CR2; CTLA4; DKC1; DOCK8; FCHO1; FOXP3; ICOS; IKZF1; IL10RA; IL10RB; IL17A; IL17RA; ITSN2; LRBA; MICALL2; MYH9; MYO5B; NCF2; NFKB1; NFKB2; NFKBIA; NOD2; P2RX7; PDCD1; PGM3; PIK3C2A; PIK3CD; PIK3R1; PIK3R4; PTEN; RAG1; RAG2; REL; RELA; RELB; RLTPR; SEC61A1; SH2D1A; SPINK5; STAT1; STAT3; STXBP2; TNFRSF13B; TNFRSF13C; TNFRSF17; TNFSF10; TNFSF13; TNFSF13B; TYK2; WAS; XIAP; ZNF341

[#] BTK1 has a missense mutation c215 G>A located on exon 2 coding for the PH domain, this mutation destroys the ability of BTK protein to bind to IP4[2,3]. BTK1 $CD34^+$ cells show a severe impairment in development.
BTK2 has a published missense mutation c1750+5G>A in an intronic region resulting in exon skipping and the truncation of the carboxyterminal part of the protein[4], and a shorter protein. The SH1, SH2 domains are maintained. BTK2 cells show a residual ability to develop into CD10 and pre-B-cell. The mutations have both been associated with agammaglobulinemia, but the effect of the first seems to be more severe. Indeed our differentiation assay cold be helpful to assess the severity of the impact of mutations on B cell development. The clinical phenotype and peripheral B-cell numbers are similar in both patients.
[§] IKZF1 mutation: insertion/deletion variant c.1342-1343insdelAG, rs765655969 and rs750934235 in the *IKZF1 gene*[5], coding for Ikaros.

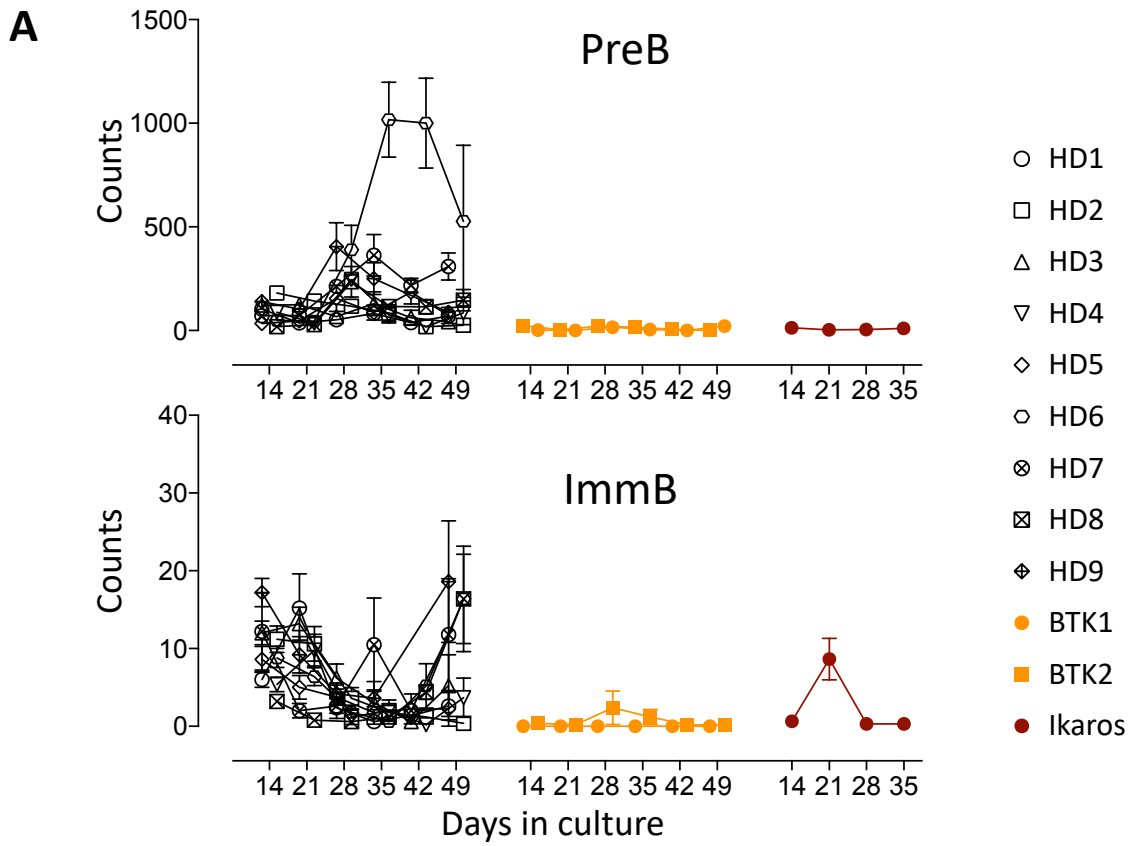
References

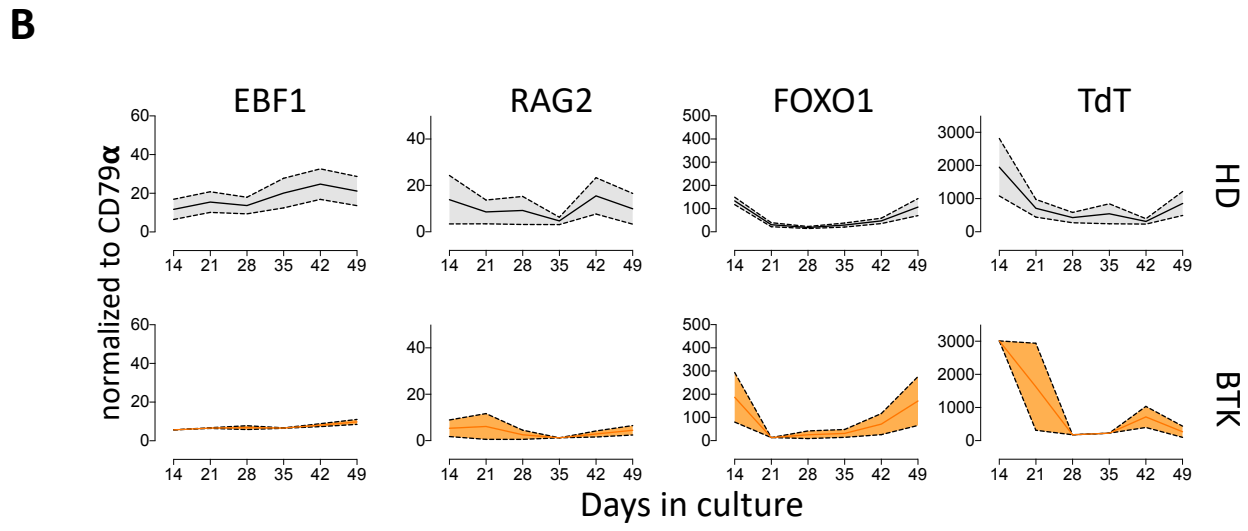
1. Kraus H, Kaiser S, Aumann K, et al. A Feeder-Free Differentiation System Identifies Autonomously Proliferating B Cell Precursors in Human Bone Marrow. *The Journal of Immunology*. 2014;192(3):1044-1054.
2. Fiorini M, Franceschini R, Soresina A, et al. BTK: 22 novel and 25 recurrent mutations in European patients with X-linked agammaglobulinemia. *Hum Mutat*. 2004;23(3):286.
3. Lee PP, Chen TX, Jiang LP, et al. Clinical characteristics and genotype-phenotype correlation in 62 patients with X-linked agammaglobulinemia. *J Clin Immunol*. 2010;30(1):121-131.
4. Duriez B, Duquesnoy P, Dastot F, Bougneres P, Amselem S, Goossens M. An exon-skipping mutation in the btk gene of a patient with X-linked agammaglobulinemia and isolated growth hormone deficiency. *FEBS Lett*. 1994;346(2-3):165-170.
5. Eskandarian Z, Fliegauf M, Bulashevska A, et al. Assessing the Functional Relevance of Variants in the IKAROS Family Zinc Finger Protein 1 (IKZF1) in a Cohort of Patients With Primary Immunodeficiency. *Front Immunol*. 2019;10:568.

Supplemental Figure S1

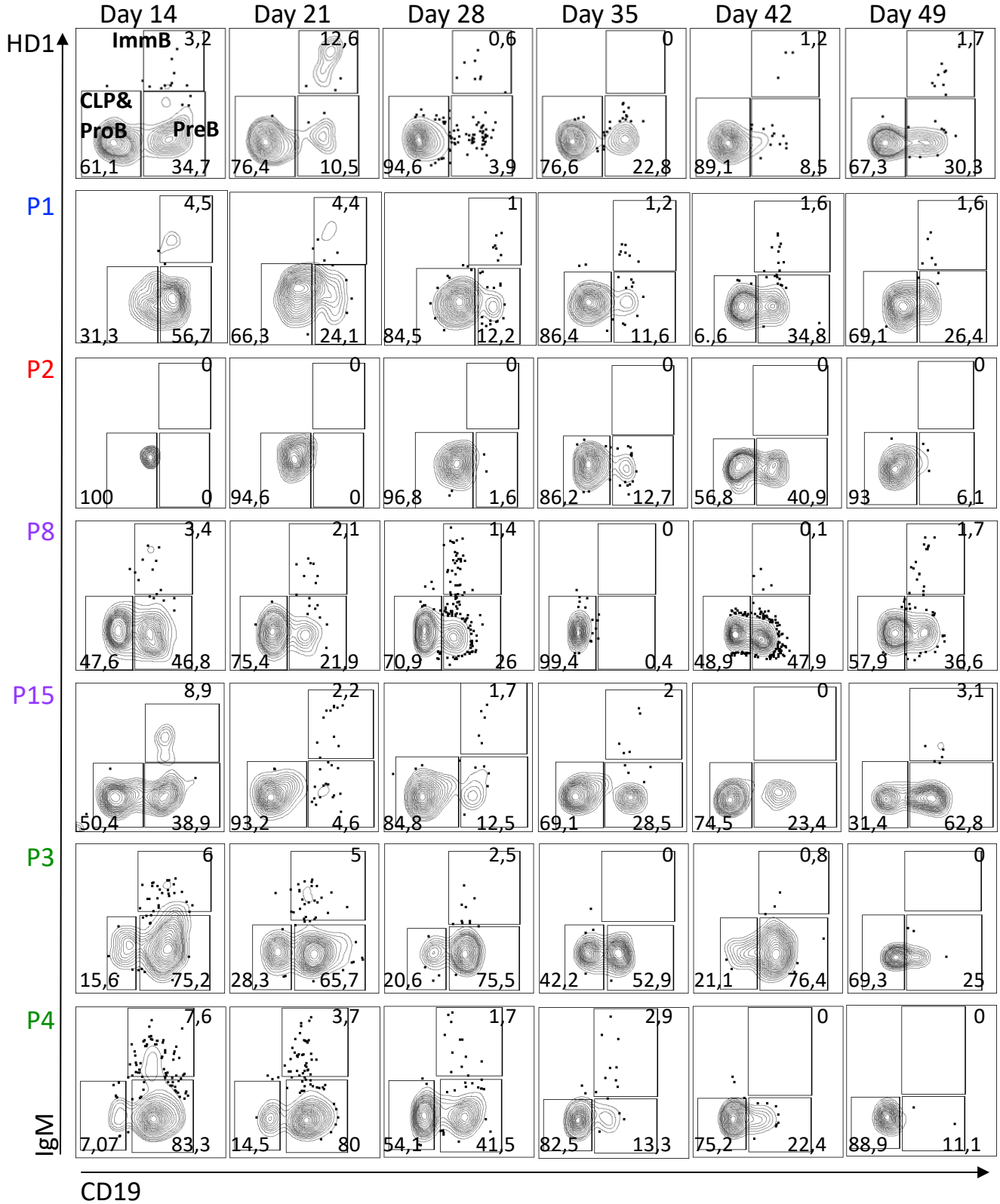


Supplemental Figure S2

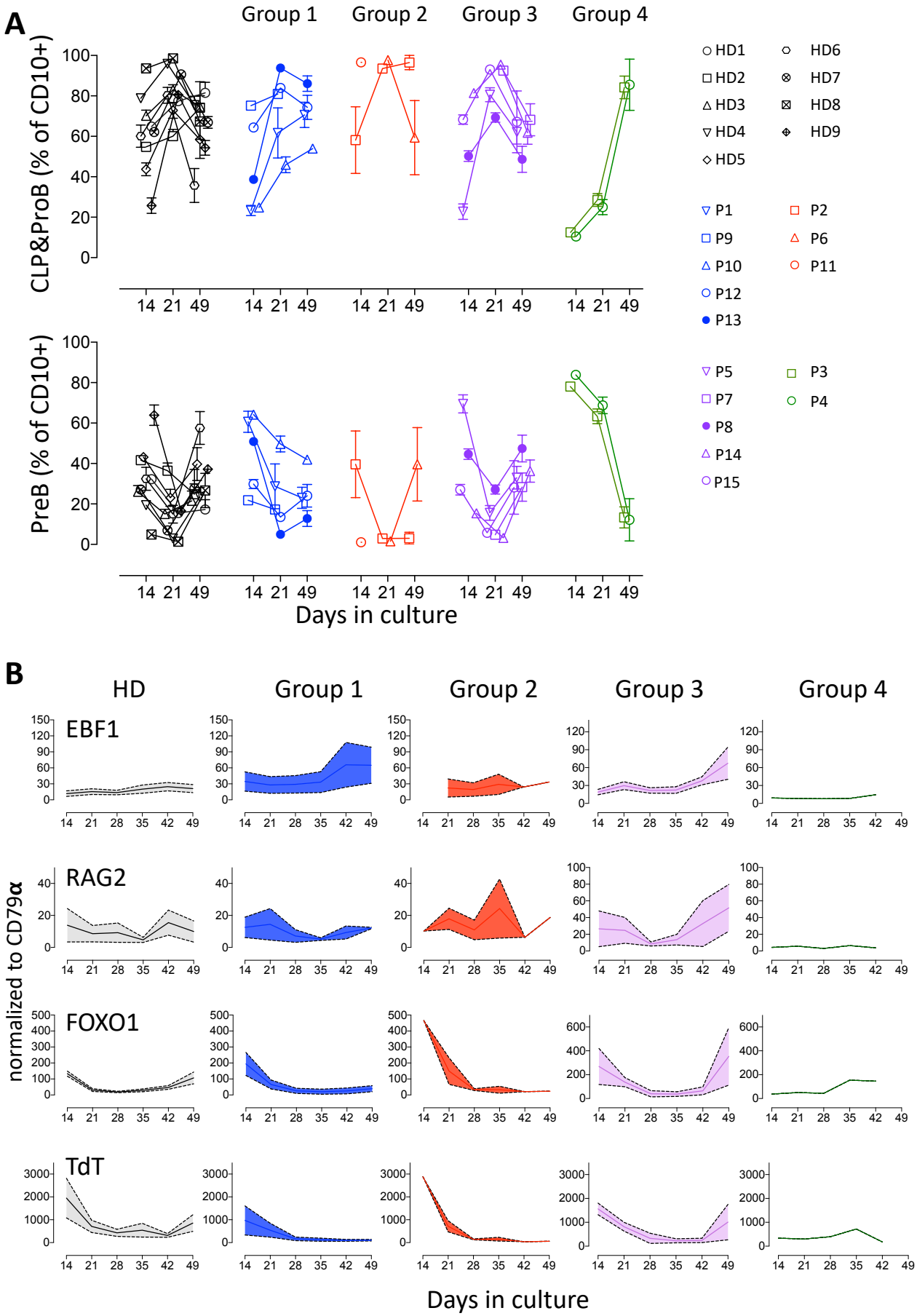




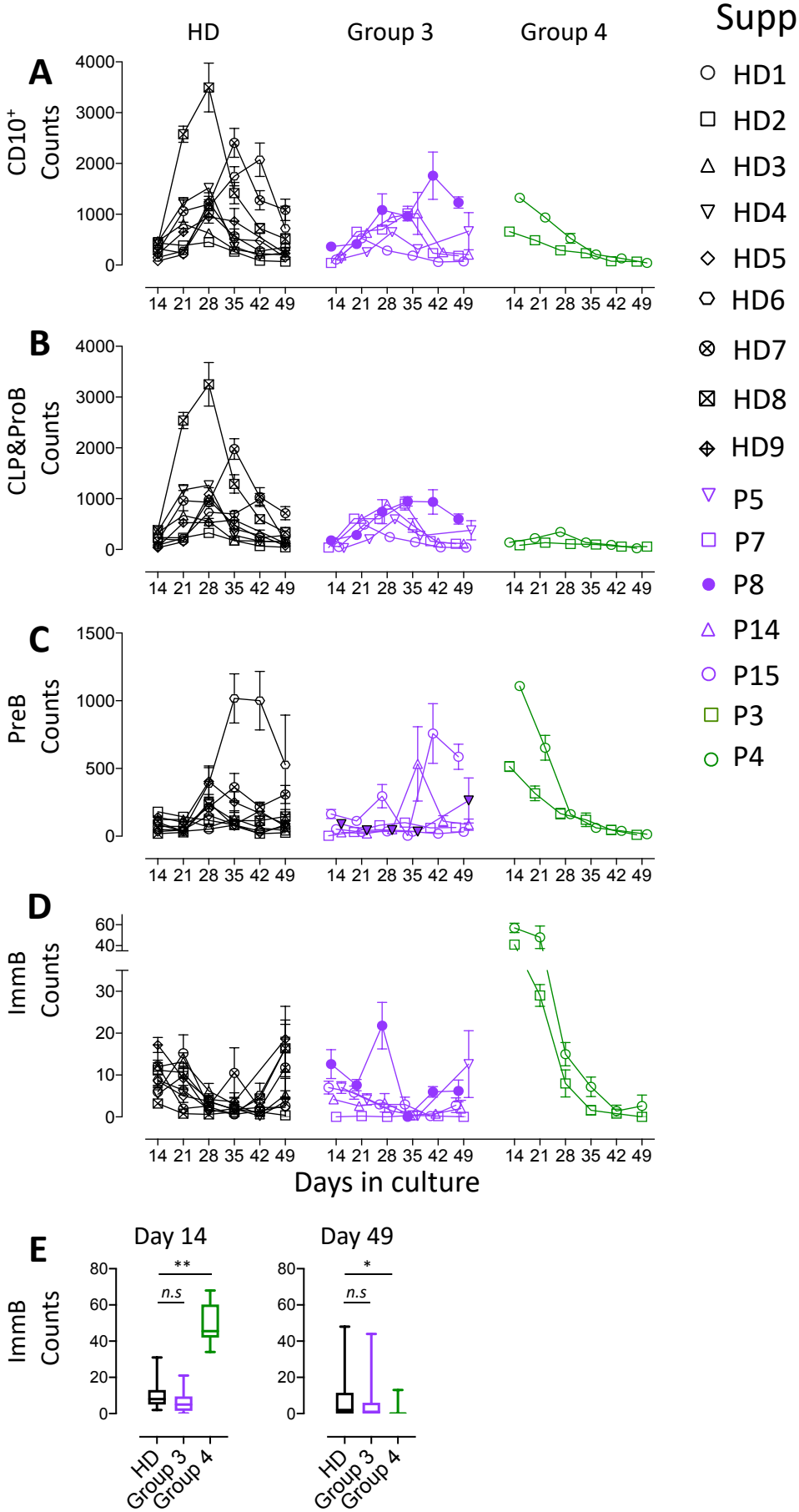
Supplemental Figure S3

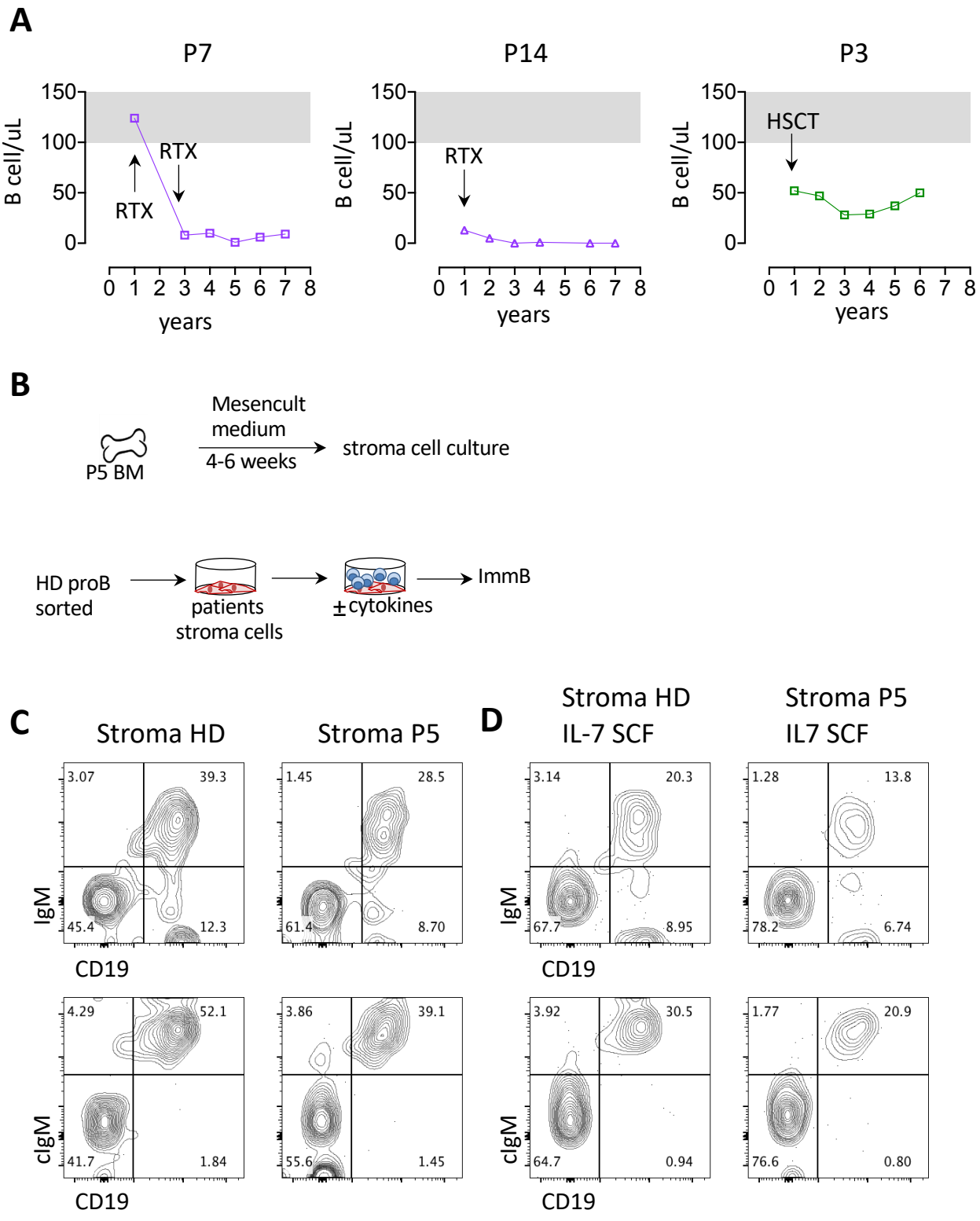


Supplemental Figure S4



Supplemental Figure S5




A
P7
P14
P3
B cell/uL
150
100
50
0
RTX
RTX
RTX
HSCT
0 1 2 3 4 5 6 7 8
years
B
P5 BM
Mesencult medium
4-6 weeks
stroma cell culture
HD proB sorted
patients stroma cells
±cytokines
ImmB
C
Stroma HD
Stroma P5
IgM
clgM
CD19
3.07
39.3
45.4
12.3
1.45
28.5
61.4
8.70
4.29
52.1
41.7
1.84
3.86
39.1
55.6
1.45
D
Stroma HD IL-7 SCF
Stroma P5 IL7 SCF
3.14
20.3
67.7
8.95
1.28
13.8
78.2
6.74
3.92
30.5
64.7
0.94
1.77
20.9
76.6
0.80