Hematopoiesis in patients with mature B-cell malignancies is deregulated even in patients with undetectable bone marrow involvement

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SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Clinical samples

Between February 28, 2012 and April 24, 2014 we analyzed 125 bone marrow (BM) samples from newly diagnosed patients with mature B cell malignancies, including CLL (n=21, median 66 , range 40-79), MCL (n=27, median 65, range 48-79), DLBCL (n=35, median 65, range 29-81), FL (n=24, median 63, range 40-82), and MM (n=18, median 65, range 30-73). 22 BM samples were obtained from healthy volunteers. Of these, 13 age-matched BM samples (median age 63, range 29-78) were used for comparison to the whole PT cohort (n=125), 15 (younger) age-matched BM samples were used for comparison to the younger patients (i.e. <45 years, median age 27, range 22-45), and 7 (older) age-matched BM samples were used for comparison to the elderly patients (≥45 years, median age 70, range 63-78). All samples were obtained after written informed consent according to the Helsinki Declaration of 1975 (revised in 1985). The study was approved by Ethics Committee of the Charles University General Hospital in Prague. Bone marrow mononuclear cells (BMMC) were obtained by a standard Ficoll-Hypaque gradient centrifugation. Cells were then twice washed in PBS and incubated with 1% human immunoglobulin at room temp for 10 minutes and then stained ready for fluorescence activated cell sorting (FACS) analysis.

Antibodies, Cell Staining, sorting and storage

Human HSPCs, namely HSC ($Lin^-CD34^+CD38^-CD90^+CD45RA^-$), multipotent progenitors (MPP, $Lin^-CD34^+CD38^-CD90^-CD45RA^-$), multi-lymphoid progenitors (MLP, $Lin^-CD34^+CD38^-CD90^-CD45RA^+$), and proB cells ($CD34^+CD38^+CD10^+CD19^+$) were stained and subsequently sorted using a FACS Aria IIu (BD Biosciences, San Jose, CA, USA) and evaluated using BD FACS Diva 6.2 software.[1-4]

To exclude lineage-positive cells BMMCs were stained with a biotinylated lineage antibody cocktail (Miltenyi Biotech,Bergisch-gladbach, Germany) followed by APC-Cy7 streptavidin (Biolegend, Fell, Germany). Lineage-negative cells were then stained with PE-anti-CD34 (8G12), Pe-Cy7-anti-CD38 (HIT2), FITC-anti-CD90 (eBio 5E10) and APC-anti-CD45 (HI100), and sorted to obtain HSC, MPP and MLP populations. For the analysis of the proB population, BMMCs were stained with PE-anti-CD34 (8G12), Pe-Cy7-anti-CD38 (HIT2), FITC-anti-CD19 (HIB19), and APC-anti-CD10 (ebioOCB). Relevant isotype matched controls and fluorescence minus one (FMO) controls were used to determine the level of background staining and as gating controls, respectively. Exclusion of non-viable cells was done using propidium iodide (PI) staining excited by UV laser and detected at Hoechst detector after filtering by Long pass filter 600LP

and 610/20 dichroic filter. HSC, MPP, MLP and proB cells underwent a second round of sorting for purity using the same gates from first round. The Appropriate number of cells were then sorted directly into CellsDirect One-Step qRT-PCR cells lysis solution (Invitrogen) and stored at -80°C pending specific target amplification (STA).

Specific Target Amplification (STA)

Pooled Taqman assays ("Taqman Master Mix") were first prepared by mixing all the gene-specific 0.2x TaqMan Assay Mix (Applied Biosystems) in one tube and diluted with 1 x Tris-EDTA buffer so that each assay concentration had final concentration of 0.2X according to the protocol by BioMark Dynamic Array (Fluidigm, CA, USA).[5] Before quantitative real-time PCR (qPCR) analysis, proB or HSC cells underwent specific target amplification. Briefly, 200 cells sorted into 2μL of lysate solution were mixed with the 0.2x pooled Taqman Master Mix plus 2x reaction mix (CellsDirect™, Invitrogen) and Superscript III/Platinum Taq mix (Invitrogen). The final volume for specific target amplification (STA) was 20μL. Samples were then run on a Mastercycler Gradient (Eppendorf) with the temperature settings of 50°C for 15 minutes for reverse transcription (RT), and then 2 minutes at 95°C after RT. STA was then carried out with 14 cycles of 95°C for 15 seconds and 60°C for 4 minutes.[5] The final STA product was then diluted with Tris-EDTA buffer in ratio 1:5 to final volume of 100μL and stored at -80°C until qPCR).[6-8] All taqman assays were obtained from Applied Biosystems.

Real-time quantitative PCR (qPCR)

Quantitative PCR was done on ABI 7900HT Fast Real-Time PCR System (Life Technologies) with Human GAPDH as an internal control. Taqman gene expression assays for all the genes analyzed were obtained from Life Technologies. Duplicate sample amplifications were done in 8 μL volumes and the data was analyzed using SDS 2.4 software (Applied Biosystems) by the comparative Ct method. The data is displayed as mean ± standard deviation (SD).

Statistical analysis

Statistical significance of differences between sample means was evaluated using Student´s t-test and mean ± SD was reported. Correlation analysis with 95% confidence interval was done and all linear regression show the 95% confidence of the best fit line. All statistical analyses were done on Prism software (Version 5, GraphPad Software, La Jolla, CA, USA). P-values <0.05 were considered statistically significant

Supplemental References

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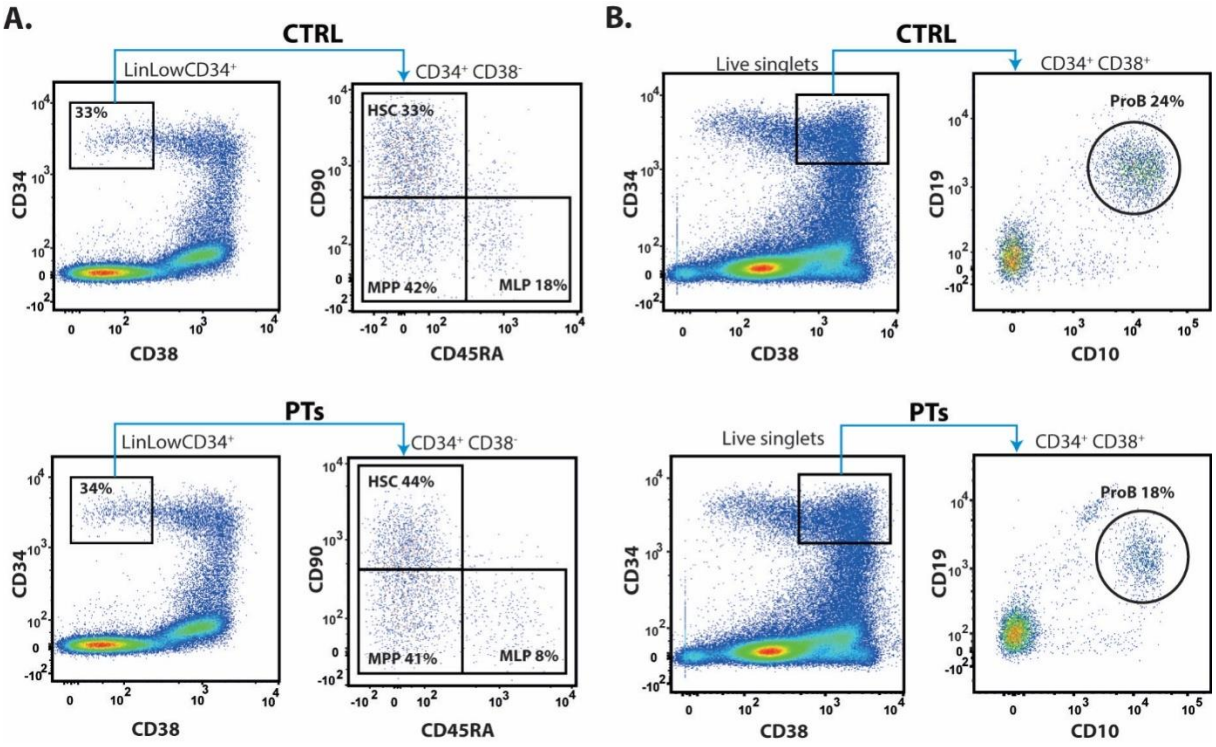
Supplemental Table

	GENE SYMBOL	GENE EXPRESSION AMONG THE PT SAMPLES (%)	RELATIVE GENE EXPRESSION (MEAN, 10^{-3})	SD (+/-)	GENE EXPRESSION AMONG THE CTRL SAMPLES (%)	RELATIVE GENE EXPRESSION (MEAN, 10^{-3})	SD (+/-)	P-VALUE
GROUP 1A	RUNX1	81	198.7	137.5	77	16.6	13.0	0.0001
	CD44	92	193.1	152.3	100	28.6	34.0	0.0003
	MCL1	83	89.8	71.5	77	7.7	5.5	0.0007
	IKAROS	83	48.7	38.9	85	7.4	3.9	0.0009
	PROM1	89	103.5	84.1	92	18.0	15.9	0.0009
	BCL11A	87	59.1	48.3	69	4.6	3.7	0.0013
	GATA2	86	83.7	69.7	85	15.8	9.4	0.0021
	BCL2L1	87	40.3	24.5	69	14.5	14.9	0.0032
	FOXP1	73	4.6	2.3	31	1.0	0.5	0.0038
	ABCB1	75	27.3	20.4	46	3.6	3.3	0.0069
	PU.1	75	40.4	29.7	46	6.5	4.1	0.0080
	MYC	81	43.6	39.1	54	2.7	0.9	0.0084
GROUP 1B	IRF8	24	52.7	61.0	54	7.7	9.1	0.0702
	BMI1	79	89.3	59.9	23	25.8	30.5	0.0761
	FOXO1	56	11.2	8.9	15	2.0	0.5	0.1587
	CD34	94	67.0	94.4	100	41.8	56.7	0.3587
	SOX11	90	7.2	6.9	85	5.5	2.7	0.4444
GROUP 2	CCND1	27	3.2	2.4	0	0	0	0
	NOTCH	40	6.5	10.7	0	0	0	0
	ZAP70	38	3.2	2.6	8	0	0	0

Supplemental Table 1. Gene expression analysis of HSCs obtained from patients (PT, n=63) and controls (CTRL, n=13)-derived BM samples. Relative expression levels +/- standard deviations (SD) and frequencies of the gene expression among the PT and CTRL HSC samples are shown together with P-values calculated from unpaired t-tests for the difference in relative expression mean values between PT and CTRL.

Supplemental Figure



Supplemental Figure 1. Flow cytometry analysis workflow of BM-derived HSPC populations. A representative gating strategy for: (A) HSC, MPP and MLP analysis for CTRL (top) and PT (bottom), (B) proB analysis for CTRL (top) and PT (bottom).