The monocytic population in chronic lymphocytic leukemia shows altered composition and deregulation of genes involved in phagocytosis and inflammation

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Online Supplementary Design and Methods

Samples

Peripheral blood mononuclear cells (PBMC) were isolated from 31 patients with chronic lymphocytic leukemia (CLL) and 13 healthy donors by density gradient centrifugation (Ficoll, Pharmacia LKB Biotechnology, Piscataway, NY, USA). To enrich for peripheral blood monocytes, PBMC were incubated with CD14-specific microbeads and separated by AutoMACS throughout passage on two subsequent columns (Miltenyi Biotech, Bergisch Gladbach, Germany), obtaining a purity >97% as assessed by flow cytometry using APC-conjugated monoclonal antibody specific for CD14 (Miltenyi Biotech). Plasma samples were obtained by blood centrifugation at 2000 rpm for 15 min and then stored at -80°C.

Flow cytometry

Samples were analyzed using a CyFlow ML flow cytometer (Partec, Munster, Germany). Data were acquired in list mode by using FloMax (Partec) software, and then analyzed by FlowJo 9.5.2 (Treestar Inc., Ash- land, OR, USA) under MacOS 10. Samples were compensated by software, after acquisition, by using single stainings and Fluorescence Minus One (FMO) controls, as described elsewhere.[1] Monocytes were divided into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) subsets. Percentages of $Tie2^{+}$ cells among $CD14^{+}$ monocytes were determined by gating on the $DUMP^{-}$ channel, as previously defined.[2] Tie2-expressing monocytes (TEM) were enumerated by converting the percentage of positive cells into the absolute number of TEM/μL using the following formula: percentage of positive cells x white blood cell (WBC) count /100.

Genome-wide expression profiling

Fluorescence data were analyzed with Feature Extraction Software v10.5 (Agilent Technologies). Agglomerative two-dimensional clustering and supervised analysis based on t-tests were performed using Gene Spring GX v11.5 (Agilent) software. Genes were defined as differentially expressed between groups at a level of significance of $P<0.05$ and with a fold-change cut-off of ± 2 in all the pair-wise comparisons. The Gene Ontology Tool (*http://www.geneontology.org/*) and PANTHER classification system (**P**rotein **AN**alysis **T**Hrough **E**volutionary **R**elationships, *http://www.pantherdb.org/*) were used to unravel biological functions and pathways represented in gene lists.

Real-time polymerase chain reaction

For real-time polymerase chain reaction (PCR), RNA (100 ng) was reverse transcribed using a Transcription High Fidelity cDNA Synthesis kit (Roche Applied Science, Penzberg, Germany). All samples were analyzed in real time on a LightCycler 480 v.2 (Roche) in duplicate. Amplification of the sequence of interest was normalized to an housekeeping reference gene (glyceraldehyde 3-phosphate dehydrogenase, *GAPDH*) and compared to a calibrator sample (Universal Human Reference RNA; Stratagene, Cedar Creek, TX, USA). Differences in gene expression were determined by the comparative method ($2^{-\Delta\Delta CT}$). To exclude non-specific amplification and primer-dimer formation when using SYBR Green, a dissociation curve analysis was performed and then PCR products were tested by agarose gel electrophoresis.

Conditioned media from chronic lymphocytic leukemia cells and sample collection

Conditioned media (CM) of CLL samples used to treat monocytes had been obtained by centrifuging supernatants of CLL cells purified from the peripheral blood of untreated patients by depletion of non-B cells (B-Cell Isolation kit -BCLL-Miltenyi Biotech) after 48 h of culture at $1x10^{6}$ cells/mL.

After 24 h of treatment, monocytes were lysed into wells by using RLT/β-mercaptoethanol buffer (QIAGEN, Valencia, CA, USA) to obtain RNA samples for real-time PCR. CM were also collected from monocyte cultures by centrifuging supernatants at 1600 rpm for 10 min and stored -80°C. The control was defined as CM obtained from monocytes cultured without any stimulus. To evaluate soluble factors secreted by monocytes, we performed enzyme-linked immunosorbent assays. Each sample was tested in duplicate. To obtain an evaluation of monocyte-derived proteins, each value was subtracted from

protein levels of the investigated factor measured in CM obtained without monocytes, thus excluding the contribution of both medium and CLL cells.

Functional assays

To test monocyte migration, chemoattractants (CM CLL or 10% fetal bovine serum) were placed in serum-free Iscove's modified Dulbecco's medium (600 μL) in the bottom compartment and 200 μL of cell suspension (10^6 cells/mL) were added to inserts. Ten percent fetal bovine serum was used as a positive control. The chambers were incubated at 37°C in humidified air with 5% CO_2 for 2 h. Migrated cells were labeled with 8 μM calcein-AM (Sigma-Aldrich, St. Luis, MO, USA) for 45 min and then detached from inserts by pre-warmed trypsin 0.05%-EDTA 0.02% solution. The migratory cells were quantified in a fluorescence plate reader, Infinite200 (Tecan, Männedorf, Switzerland), at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

To test T-cell proliferation in response to monocytes, a fraction of purified T cells was labeled with 1 μM carboxyfluorescein succinimidyl ester (CFSE, eBioscience) following the manufacturer's protocol. We then cultured T cells on autologous monocytes (n=3 CLL, n=3 healthy donors disease) for 5 days (ratio T:M 2:1) in 48-well plates in an autologous mixed reaction. In alternative, monocytes from three CLL and three healthy donors were cultured with normal T cells (n=2) in an allogeneic lymphocyte reaction. Cell proliferation was monitored using a yellow tetrazolium MTT cell proliferation assay (Trevigen, Gaithersburg, MD, USA). In addition, a CFSE dilution assay was used to trace cell division by flow cytometry. CFSE-labeled $CD3^+$ T cells cultured in the presence of CD2/CD3/CD28 MACSiBead Particles (Miltenyi Biotech) were used as the positive control. Cells were collected from culture supernatants and incubated for 10 min at room temperature with APC-conjugated anti-CD3 (Becton Dickinson). Viable T cells were gated based on their characteristic position in the forward *versus* side scatter plot.

Immunohistochemistry and confocal microscopy

For immunohistochemical analysis, following antigen retrieval, sections were incubated with goat anti-human Tie2 (R&D System, dilution 1:20) or mouse anti-human CD68 (Novocastra, Leica Microsystems, Wetzlar, Germany, dilution 1:200). Binding of the primary antibodies was revealed using specific alkaline phosphatase- or peroxidase-conjugated secondary antibodies (Sigma-Aldrich) and either Fast Red (Thermo Scientific, Waltham, MA, USA) or 3-3'diaminobenzidine (DAB, Thermo Scientific) chromogens. Sections were counterstained with hematoxylin and analyzed under a Leica DM2000 optical microscope (Leica Microsystems). Microphotographs were collected using a Leica system. For confocal microscopy, slides were analyzed using a TCS SP5 laser scanning confocal microscope with four lasers (Leica Microsystems); images were acquired with LAS AF Version Lite 2.4 software (Leica Microsystems) and processed with Photoshop (Adobe Systems).

References

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2. Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol. 2008;8(4): 247-58.

Online Supplementary Table S1. Characteristics of CLL patients (n=26) at the time of flow cytometric analysis

Characteristic	N. of Patients	%
Age, years		
Median	69	
Range	34-85	
White blood cell count (10^9/L)		
Median	26	
Range	9-75	
Lymphocytes (%)		
Median	73	
Range	21-97	
β2 microglobulin (mg/L) (n=19)		
Median	2.2	
Range	0.9-3.8	
Sex		
Male	19	73
Female	7	27
Binet stage		
A	23	88
B-C	3	12
IGHV mutational status		
Mutated (≥2%)	14	54
Unmutated (<2%)	12	46
CD38 (n=18)		
CD38 negative (<30%)	12	67
CD38 positive (≥30%)	6	33
ZAP-70 (n=16)		
ZAP-70 negative (<20%)	8	50
ZAP-70 positive (≥20%)	8	50
FISH stratification (n=23)		
Low risk	16	70
Intermediate/high risk	7	30
Trisomy 12	2	9
Deletion 17p	3	13
Deletion 11q	2	9
Follow up (months)		
Median	38	
Range	9-122	

NOTE. Normal controls (n=13) included in our study were characterized by median age: 60 years (range, 48-77); gender: 7 female and 7 male. Low risk FISH = no abnormalities or 13q-; Intermediate/high risk FISH = 11q-,17p- or trisomy 12. FISH: fluorescence in situ *hybridization;* IGHV*: variable region of immunoglobulin heavy chain genes; ZAP-70: zeta-chain-associated protein kinase 70.*

Online Supplementary Table S2. Clinical and biological characteristics at sample collection of the five CLL patients included in the GEP analysis.

Code	Sex	Age	Binet	*IGHV* status	CD38	ZAP70	FISH	WBC (x10^9/L)	L(%)	Treatment
CLL1	M	78	A	mutated	neg	neg	none	43.9	86	untreated
CLL2	F	67	A	mutated	neg	neg	na	30.5	78	untreated
CLL3	M	42	B	mutated	neg	neg	none	17.0	81	untreated
CLL4	M	68	A	unmutated	neg	pos	none	10.2	59	untreated
CLL5	M	71	B	unmutated	neg	neg	none	42.0	83	untreated

IGHV*: variable region of immunoglobulin heavy chain genes; ZAP-70: zeta-chain-associated protein kinase 70; FISH: fluorescence* in situ *hybridization; WBC: white blood cell count; L: lymphocytes; na: not available*

Table 1. List of selected differentially expressed genes between CLL-derived and normal monocytes

Gene symbol	Fold change	Gene name
RAP1GAP	6.5	RAP1 GTPase activating protein
TMEM45B	5.8	Transmembrane protein 45B
DACH1	4.9	dachshund homolog 1
KIR2DS2	4.5	killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2
PLA2G4A	4.4	phospholipase A2, group IVA
LPAR6	4.0	lysophosphatidic acid receptor 6
CDC45L	3.7	CDC45 cell division cycle 45-like
ATP10D	3.7	ATPase, class V, type 10D
XYLT1	3.6	Xylosyltransferase I
TMEM35	3.5	transmembrane protein 35
COL14A1	3.3	collagen, type XIV, alpha 1
PGM2L1	3.2	phosphoglucomutase 2-like 1
CYP2D6	3.1	cytochrome P450, family 2, subfamily D, polypeptide 6
TMEM14A	3.0	transmembrane protein 14A
LPIN3	2.9	lipin 3
SEC24D	2.7	SEC24 family, member D
ZNF546	2.7	zinc finger protein 546
ARHGEF12	2.6	Rho guanine nucleotide exchange factor (GEF) 12
CPEB3	2.5	cytoplasmic polyadenylation element binding protein 3
JDP2	2.5	Jun dimerization protein 2
PRKCE	2.4	protein kinase C, epsilon
EDEM3	2.4	ER degradation enhancer, mannosidase alpha-like 3
PTPRO	2.4	protein tyrosine phosphatase, receptor type, O
USP46	2.3	ubiquitin specific peptidase 46
NAT2	2.3	N-acetyltransferase 2 (arylamine N-acetyltransferase)
RNF121	2.3	ring finger protein 121
KLHL8	2.3	kelch-like 8
DBF4B	2.2	DBF4 homolog B
GGCX	2.2	gamma-glutamyl carboxylase
FOXI3	2.2	forkhead box I3
CCDC106	2.2	coiled-coil domain containing 106
LPIN2	2.1	lipin 2
KLHL2	2.1	kelch-like 2
CSNK1G3	2.1	casein kinase 1, gamma 3
C8A	2.1	complement component 8, alpha polypeptide
MCM8	2.0	minichromosome maintenance complex component 8
SLC25A17	2.0	solute carrier family 25
NAT1	2.0	N-acetyltransferase 1 (arylamine N-acetyltransferase)
CYP3A43	2.0	cytochrome P450, family 3, subfamily A, polypeptide 43
TLR4	2.0	Toll-like receptor 4
TUBB3	-12.3	tubulin, beta 3
CCNA1	-6.2	cyclin A1
RTN4R	-4.2	reticulon 4 receptor (RTN4R)
CCL5	-3.8	chemokine (C-C motif) ligand 5
FUT1	-3.7	fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase,H blood group)
NR1D1	-3.5	nuclear receptor subfamily 1, group D, member 1
PTGR2	-3.2	prostaglandin reductase 2
TUBB2A	-3.0	tubulin, beta 2A
KLF3	-2.9	Kruppel-like factor 3
ARG2	-2.8	arginase, type II
ARL4A	-2.5	ADP-ribosylation factor-like 4A
SLC9A9	-2.5	solute carrier family 9
ARL4A	-2.5	ADP-ribosylation factor-like 4A
ZNF692	-2.4	zinc finger protein 692
RTN1	-2.4	reticulon 1
SMPD1	-2.4	sphingomyelin phosphodiesterase 1, acid lysosomal
HSD17B4	-2.3	hydroxysteroid (17-beta) dehydrogenase 4
LENG8	-2.3	leukocyte receptor cluster (LRC) member 8
TAF4B	-2.2	TAF4b RNA polymerase II
NDRG3	-2.2	NDRG family member 3
RASSF4	-2.2	Ras association (RalGDS/AF-6) domain family member 4
SERTAD2	-2.2	SERTA domain containing 2
RBM47	-2.1	RNA binding motif protein 47
SNN	-2.1	stannin
SMYD3	-2.0	SET and MYND domain containing 3
DPAGT1	-2.0	dolichyl-phosphate (UDP-N-acetylglucosamine) N-acetylglucosaminephosphotransferase 1
CDC42EP3	-2.0	Cdc42 effector protein 3
RUNDC2A	-2.0	RUN domain containing 2A
FTMT	-2.0	ferritin mitochondrial
DUSP3	-2.0	dual specificity phosphatase 3

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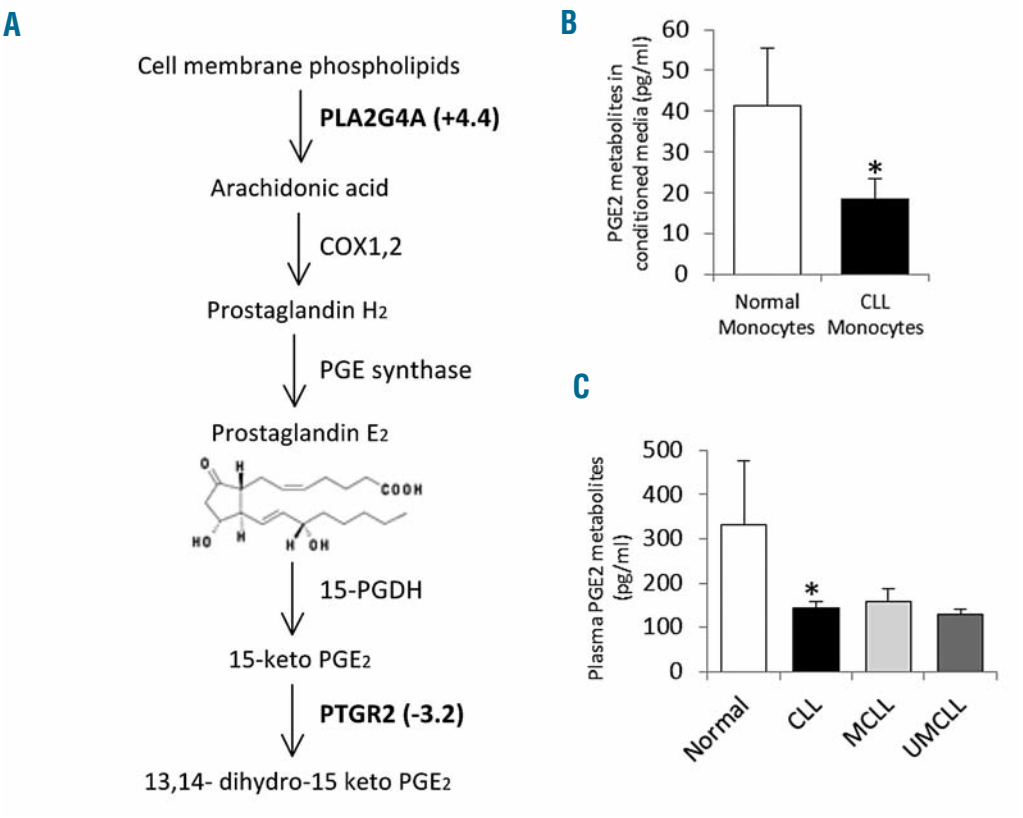
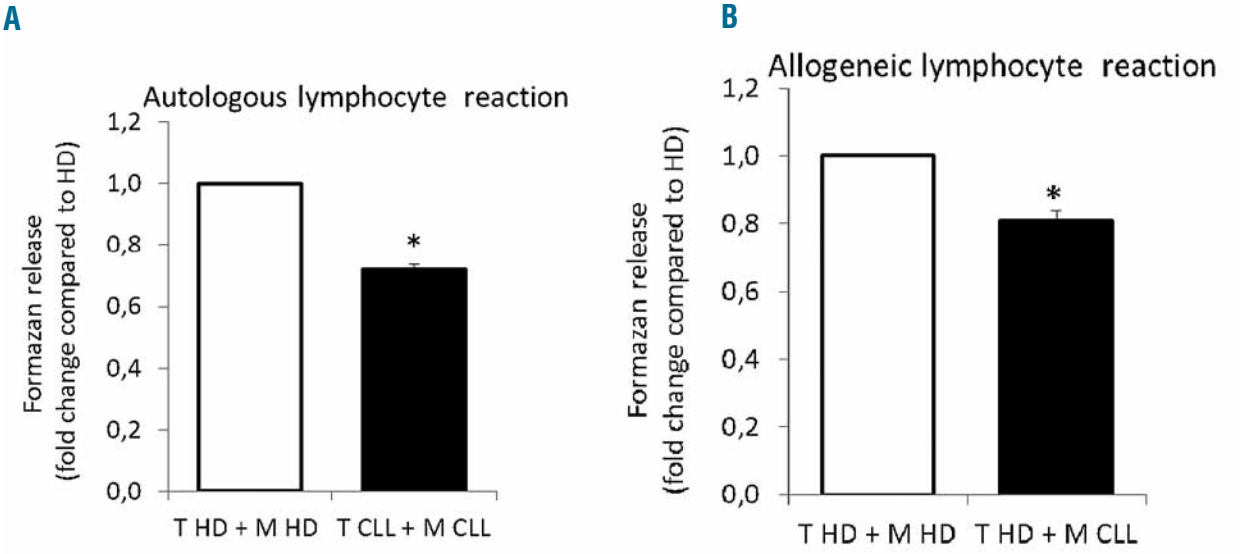
Genes are ranked ordered according to the fold difference in gene expression levels between CLL and normal monocytes both in the up-regulated and down-regulated lists.



Online Supplementary Figure S1. Detection of 13, 14-dihydro-15-keto PGE2 in CLL. (A) Scheme of the biosynthesis and catabolism of prostaglandin E2 (PGE2). Histograms represent protein levels in pg/mL of PGE2 metabolites as 13, 14-dihydro-15-keto PGE2 detected in conditioned media collected from CLL-derived (n=6) and normal (n=8) monocytes after 24 h culture (B) and in plasma samples from CLL patients (n=16) and healthy donors (n=8) (C). **P*<0.05 in CLL compared to normal control, Mann-Whitney test). CLL patients carrying mutated (MCLL) and unmutated (UMCLL) *IGHV* genes are also indicated. No difference was detected in plasma levels between MCLL and UMCLL.



Online Supplementary Figure S2. Autologous and allogeneic T-cell proliferation in response to CLL monocytes and normal monocytes. (A) MTT proliferation assay with T cells from three CLL patients and three normal controls (HD) cultured for 5 days together with autologous monocytes. (B) MTT proliferation assay with normal T cells cultured for 5 days together with allogeneic monocytes from three CLL patients and three healthy donors. Results are expressed as fold-change compared to controls (HD) of MTT-derived formazan by proliferating T cells. Data summarize three different experiments (*P*<0.05, Mann-Whitney test).