Higher levels of reactive oxygen species are associated with anergy in chronic lymphocytic leukemia

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Supplementary materials and methods
Supplementary Table 1. Characteristics of CLL samples used in the study.
Supplementary Figure 1. Analysis of reactive oxygen species in primary CLL.

Supplementary Materials and methods

Patient Samples

Heparinized peripheral blood mononuclear cell (PBMC) samples were collected from 33 CLL patients attending clinics at the Southampton General Hospital, The Royal Berkshire and Royal Wolverhampton NHS trusts (all UK). All samples were obtained following informed consent in accordance with local Ethics Committee approval and the Declaration of Helsinki. The majority of samples were obtained at time of diagnosis prior to treatment for CLL. In the 6 cases where treatment was given, this was at least 12 months prior to sample collection. Tumor *IGHV* use and homology to germline was determined by aligning the tumor *IGHV-D-J* rearrangement to the IMGT database (http://imgt.cines.fr/).[1] Mutational status was determined using the conventional 98% homology cut-off.[2] Expression of cell surface CD5 and CD19 were determined as previously described.[3,4] Surface IgM (sIgM) signaling capacity was determined by measuring the percentage of cells with increased intracellular calcium following stimulation with soluble goat $F(ab')_2$ anti-IgM and using a cut-off value of ≥5% responding cells to define samples as sIgM responsive.[4] PBMCs were cryopreserved and recovered as previously described.[4]

Quantification of reactive oxygen species (ROS)

ROS were quantified using the fluorescent probe CM-H_2DCFDA (Life Technologies, Paisley, UK) as described before.[5] Cells were diluted to $1x10^6$ cells/ml in complete media and incubated with CM-H_2DCFDA (1 μM) for 1 hour

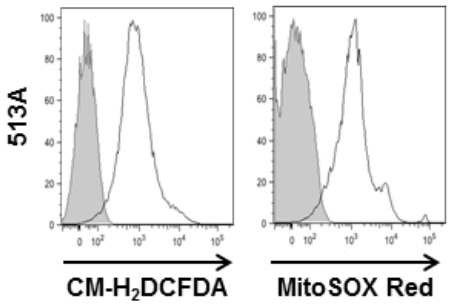
at 37°C in the dark. Cells were then washed twice and resuspended in pre-warmed phosphate buffered saline. Fluorescence was analyzed using a FACS Canto (BD Biosciences, Oxford, UK). Plots were overlaid using FlowJo (v9.5.2, Tree Star, Ashland, OR, USA) and GeoMFI values were determined using FACS Diva (v6.1.3, BD Biosciences). For quantitation of ROS levels, CM-H_2DCFDA fluorescence in live, singlet cells (based on FSC/SSC distribution) was determined as the difference between the GeoMFI of CM-H_2DCFDA stained and control, unstained cells. FSC/SSC analysis was also used to determine the overall percentage of viable cells in the sample. In experiments to quantify mitochondria-derived ROS, cells were incubated with MitoSOX-red (5 μM; Life Technologies) instead of CM-H_2DCFDA.[6] For combined analysis of ROS and phenotypic markers, cells were first incubated with CM-H_2DCFDA for 45 minutes, washed and then resuspended in FACS buffer containing APC-conjugated anti-CD19, PerCP Cγ5-conjugated anti-CD5 and/or PE-conjugated anti-CXCR4 (all from Biolegend, London, UK). Cells were incubated on ice in the dark for 15 minutes followed by analysis using a FACS Canto (BD Biosciences). ROS and CXCR4 expression was determined following gating of live, singlet cells that were positive for both CD19 and CD5.

Statistical Analysis

Statistical comparisons were performed using GraphPad Prism (v6, GraphPad Software Inc., La Jolla, CA, USA) or IBM Statistical Package for the Social Sciences (SPSS) software v.22.0 (Chicago, IL, USA). Time to progression requiring first treatment (TTFT) was defined as the time elapsed from date of diagnosis to date of first treatment. The accepted indications to initiate treatment were based on the National Cancer Institute criteria.[7] Survival analysis was performed by Kaplan-Meier method using log rank statistics to test for significant associations. Identification of the optimal cut-off value for ROS levels was determined by receiver operating characteristic analysis and Youden's t-test, using treatment as a state variable.

Sample	*IGVH* gene	*IGHV* status[a]	CLL (% cells)[b]	Binet stage at diagnosis	Prior treatment	sIgM (GeoMFI)	Ca^{2+} mobilization (% cells)	ROS[c] (GeoMFI)
281a	3-23	M	82	A	N	11	6	18482
341	5-51	M	96	A	Y	531	39	2416
343a	3-48	U	89	A	Y	104	77	1913
348	3-15	M	95	A	N	73	19	11875
368a	3-15	M	92	B	Y	39	15	3956
382	3-07	M	79	NA[d]	NA	129	0	13311
388	4-34	M	75	A	N	43	2	19084
393	1-69	U	92	C	Y	60	41	8345
414	4-59	M	95	A	N	19	0	8356
476a	1-69	U	93	A	N	47	16	4999
480	4-34	M	92	A	Y	49	33	18275
493	4-34	M	85	A	N	29	6	1429
494	3-15	M	95	A	Y	29	6	7355
500a	3-48	U	79	A	N	58	9	2860
504a	1-2	U	89	A	N	25	23	2426
505a	1-69	U	96	A	N	44	17	1363
513	1-69	U	99	A	N	79	62	818
520a	3-33	U	81	A	N	18	7	2540
525a	3-23	M	95	A	N	10	6	3581
526	3-30	U	96	B	N	27	7	3987
542	3-23	M	93	A	N	21	2	21391
550a	4-34	U	58	A	N	40	100	4230
551	3-33	U	95	B	N	124	46	570
558	4-31	M	95	B	N	16	39	10225
560	4-39	M	91	A	N	10	4	15367
561	3-48	M	94	A	N	39	50	6878
564	4-4	M	88	A	N	1480	78	1164
566	3-11	U	96	A	N	67	7	2203
567	3-33	M	85	A	N	96	45	1157
570	4-34	M	79	A	N	13	3	10561
618	3-11	U	86	A	N	23	42	3940
635	3-21	U	87	A	N	23	82	5523
636a	4-34	M	87	A	N	16	2	3768

Supplemental Table 1: Characteristics of samples. [a]M, mutated; U, unmutated; [b]CLL cells were defined as the percent of $CD19^+CD5^+$ cells of all lymphocytes in the test sample; [c]Reactive oxygen species (relative CM-H_2DCFDA fluorescence); [d]NA, not available.



Supplementary Figure 1. Analysis of mitochondrial ROS in primary CLL.
CLL PBMCs were recovered from cryopreservation, rested for 3 hours and then stained with CM-H_2DCFDA or MitoSOX Red for 1 hour at 37°C prior to flow cytometric analysis. Representative results for from 6 samples analyzed. Shaded – unlabeled, control cells. Open - CM-H_2DCFDA or MitoSOX Red-labeled cells. Note that both CM-H_2DCFDA and MitoSOX Red stained cells show increase fluorescence compared to unstained cells demonstrating that at least a proportion of CLL ROS are mitochondrial derived.

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