

T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers

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Methods and Materials

Flow cytometric analysis of PBMC

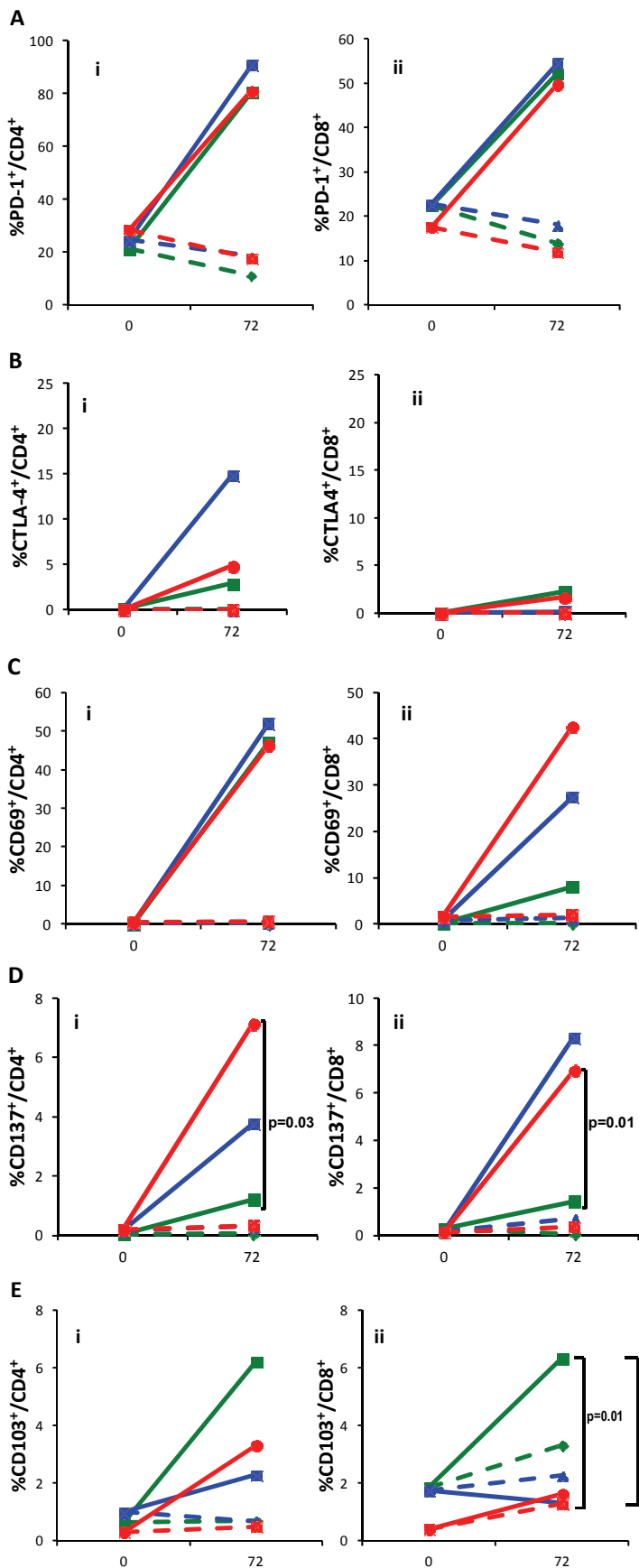
For intracellular staining, cells were washed in CSB, incubated for 30 min at 4°C with Fix/Perm (1:4) solution, washed with Perm/Buffer solution (1:10) and incubated with the anti-CTLA-4 or isotype control for 25 min at RT. Excess of antibodies was removed by washing twice with Perm/Buffer. The cells were then resuspended in 500 µl CSB and analyzed by flow cytometry using a FACSCanto II flow cytometer (BD-Biosciences) and analyzed by FACSDiva version 6.1.3 (BD Biosciences) or FlowJo version 8.8.2 (TreeStar) softwares. The frequency of unstimulated cells was subtracted from those of cultured cells.

To assess Ki-67 expression, freshly isolated CLL cells were fixed with 1.6% paraformaldehyde and permeabilized with 80% methanol. The cells (4×10^6) were then stained with AF647-anti-Ki-67 or isotype control (Bio-Legend).

Statistical Analyses

Comparisons of marker expression between different groups of patients were done either with the unpaired T-test or the non-parametric Mann-Whitney U test depending on data distribution. Simple regression analyses and the Spearman rank test were used to estimate the relationship between the expression levels of the different markers and white blood cell (WBC) counts.

Supplementary Figure 1. Effect of PHA stimulation on T-cell surface expression of immune checkpoints and activation markers. PBMCs from CLL patients and controls were cultured for 72 hours in medium in the absence or in the presence of PHA. The horizontal axis represents the time of PHA stimulation expressed in hours. Color lines represent median values from 8 healthy controls (green), 9 non-progressive (blue) and 8 progressive (red) CLL patients in the absence (dashed line) or presence (unbroken line) of PHA in the culture medium. **(A)** PD-1 expression in CD4⁺ (i) and CD8⁺ (ii) cells. **(B)** CTLA-4 expression in CD4⁺ (i) and CD8⁺ (ii) cells. **(C)** CD69 expression in CD4⁺ (i) and CD8⁺ (ii) cells. **(D)** CD137 expression in CD4⁺ (i) and CD8⁺ (ii) cells. **(E)** CD103 expression in CD4⁺ (i) and CD8⁺ (ii) cells. Standard deviation values are reported in the Tables aside.



A - i

PD-1 ⁺ /CD4 ⁺	healthy		non-progressive		progressive	
timepoint	control	PHA	control	PHA	control	PHA
0 hrs	5,32	-	8,97	-	12,60	-
72 hrs	3,36	24,81	13,04	14,11	12,01	12,71

A - ii

PD-1 ⁺ /CD8 ⁺	healthy		non-progressive		progressive	
timepoint	control	PHA	control	PHA	control	PHA
0 hrs	7,39	-	11,68	-	11,85	-
72 hrs	4,03	19,90	8,61	16,50	10,15	14,88

B - i

CTLA-4 ⁺ /CD4 ⁺	healthy		non-progressive		progressive	
timepoint	control	PHA	control	PHA	control	PHA
0 hrs	0,21	-	0,03	-	0,42	-
72 hrs	1,07	6,86	0,84	20,15	0,21	6,76

B - ii

CTLA-4 ⁺ /CD8 ⁺	healthy		non-progressive		progressive	
timepoint	control	PHA	control	PHA	control	PHA
0 hrs	0,90	-	0,04	-	0,10	-
72 hrs	1,91	12,01	0,35	3,59	0,23	7,48

C - i

CD69 ⁺ /CD4 ⁺	healthy		non-progressive		progressive	
timepoint	control	PHA	control	PHA	control	PHA
0 hrs	0,06	-	0,45	-	0,86	-
72 hrs	0,44	35,26	0,33	26,38	0,89	23,10

C - ii

CD69 ⁺ /CD8 ⁺	healthy		non-progressive		progressive	
timepoint	control	PHA	control	PHA	control	PHA
0 hrs	0,18	-	2,13	-	2,35	-
72 hrs	1,14	28,24	1,87	25,75	3,77	22,76

D - i

CD137 ⁺ /CD4 ⁺	healthy		non-progressive		progressive	
timepoint	control	PHA	control	PHA	control	PHA
0 hrs	0,05	-	0,81	-	0,85	-
72 hrs	0,08	3,20	0,44	10,92	0,24	7,73

D - ii

CD137 ⁺ /CD8 ⁺	healthy		non-progressive		progressive	
timepoint	control	PHA	control	PHA	control	PHA
0 hrs	0,18	-	0,19	-	0,46	-
72 hrs	0,41	5,26	0,55	5,85	0,94	11,57

E - i

CD103 ⁺ /CD4 ⁺	healthy		non-progressive		progressive	
timepoint	control	PHA	control	PHA	control	PHA
0 hrs	0,12	-	1,96	-	0,42	-
72 hrs	0,31	12,94	0,40	16,41	0,82	5,02

E - ii

CD103 ⁺ /CD8 ⁺	healthy		non-progressive		progressive	
timepoint	control	PHA	control	PHA	control	PHA
0 hrs	0,47	-	2,69	-	3,50	-
72 hrs	1,21	3,58	2,85	3,75	5,87	1,50

Supplementary Table 1. Absolute T cell counts in CLL patients compared to controls

		Controls (n=9)	Non-progressive (n=39)	Progressive untreated (n=22)	Progressive treated (n=19)
CD3 ⁺ /μL	Median (range)	1340 (750 – 2360)	2440* (620 – 9200)	3135* (530 – 14570)	3130** (980 – 8380)
CD4 ⁺ /μL	Median (range)	913 (462 – 1440)	1344 (340 – 3792)	1195 (290 – 7120)	1040 (410 – 3400)
CD8 ⁺ /μL	Median (range)	380 (170 – 1070)	650 (60 – 5304)	1180 (90 – 6480)	1530*** (260 – 4650)

*p<0.05, ** p<0.005, *** p<0.0005

Supplementary Table 2. Median percentage values for each T cell subpopulation in CLL patients compared to controls

	Controls	Non-progressive	Progressive untreated	Progressive treated
% CD3 ⁺ / lymph	74,6	8,6	2,6	5,5
% CD4 ⁺ / lymph	44,8	4,3	1,2	1,6
% naive / CD4 ⁺	45,8	37,8	27,3	4,6
% T _{CM} / CD4 ⁺	18,8	27,8	24,6	16,4
% T _{EM} / CD4 ⁺	20,9	26,1	41,5	69,5
% T _{EMRA} / CD4 ⁺	7,4	4,8	5,2	4,6
% CD8 ⁺ / lymph	21,6	2,3	1,1	2,8
% naive / CD8 ⁺	17,2	15,9	5,9	1,8
% T _{CM} / CD8 ⁺	3,5	2,3	1,4	0,9
% T _{EM} / CD8 ⁺	18,9	24,3	33,8	26,5
% T _{EMRA} / CD8 ⁺	54,5	60,1	49,7	64,3
% PD-1 ⁺ / CD4 ⁺	21,1	24,0	34,3	56,0
% naive / PD1 ⁺ CD4 ⁺	4,4	4,3	3,5	1,0
% T _{CM} / PD1 ⁺ CD4 ⁺	28,7	31,1	25,2	12,9
% T _{EM} / PD1 ⁺ CD4 ⁺	61,3	53,7	62,7	77,6
% T _{EMRA} / PD1 ⁺ CD4 ⁺	6,2	6,4	6,3	3,7
% PD-1 ⁺ / CD8 ⁺	33,0	24,8	35,7	31,3
% naive / PD1 ⁺ CD8 ⁺	3,5	3,0	1,9	1,3
% T _{CM} / PD1 ⁺ CD8 ⁺	5,9	2,7	2,5	1,2
% T _{EM} / PD1 ⁺ CD8 ⁺	48,4	42,8	56,2	43,5
% T _{EMRA} / PD1 ⁺ CD8 ⁺	40,2	52,5	38,4	50,4
% i.c. CTLA-4 ⁺ / CD4 ⁺	15,0	20,4	26,5	30,3
% i.c. CTLA-4 ⁺ / CD8 ⁺	1,8	2,9	3,8	3,8
% CD69 ⁺ / CD4 ⁺	2,1	0,9	2,4	2,1
% CD69 ⁺ / CD8 ⁺	5,4	1,9	3,4	2,4
% Th1 / CD4 ⁺	15,0	24,7	41,9	62,6
% Th2 / CD4 ⁺	58,9	47,7	33,8	23,5
% Th17 / CD4 ⁺	14,5	11,3	9,7	5,4
% Tregs / CD4 ⁺	4,0	4,8	3,8	4,2
% Ki67 ⁺ CD4 ⁺	1,2	1,25	1,8	3,7
% Ki67 ⁺ CD8 ⁺	0,9	1,30	1,6	2,2