Functional invariant natural killer T-cell and CD1d axis in chronic lymphocytic leukemia: implications for immunotherapy

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Online Supplementary Design and Methods

Isolation and storage of peripheral blood mononuclear cells

Eighty milliliters of peripheral blood were collected into heparinized tubes, and diluted 1:1 in Dulbecco's phosphate-buffered saline (PBS). The peripheral blood mononuclear cell (PBMC) fraction was separated by density-centrifugation (using Lymphoprep; Axis-Shield, Oslo, Norway) according to the manufacturer's instructions. PBMC were aspirated, washed and resuspended in 90% fetal calf serum (SAFC Biosciences, Lenexa, KS, USA) with 10% dimethylsulfoxide (Sigma-Aldrich, St Louis, MO, USA) and placed in a controlled-rate freezing container for cryopreservation at -180°C in gaseous phase liquid nitrogen until use.

B-cell enrichment and depletion

B cells were positively selected and depleted using anti-CD19-coated immunomagnetic beads (Dynabeads CD19 pan-B; Invitrogen Dynal AS, Oslo, Norway) to achieve a residual level of chronic lymphocytic leukemia (CLL) cells of <5% in B-cell-depleted PBMC (confirmed by flow cytometry). Immunomagnetic beads were subsequently detached using anti-Fab antibodies specific for the bead-bound anti-CD19 antibody (DETACHaBEAD CD19; Invitrogen Dynal AS). For some experiments, CLL cells were sorted by fluorescence-activated cell sorting (FACS) based on CD5/CD19 co-expression on a BD FACSVantage DiVa (Becton Dickinson, San Jose, CA, USA), to ≥98.5% purity.

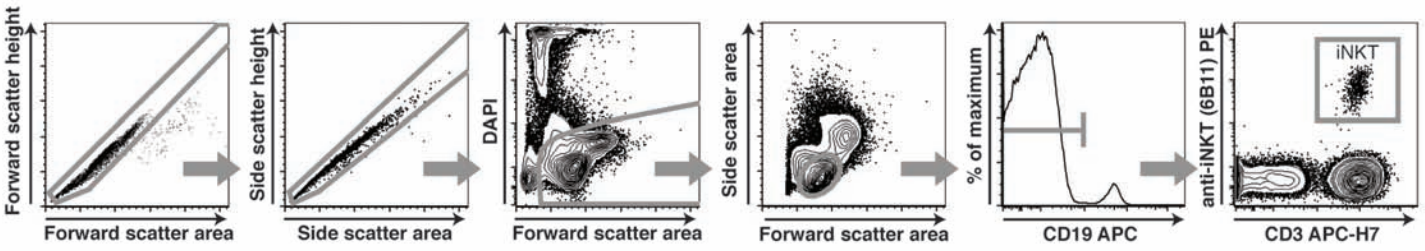
Flow cytometric analysis

Fc receptors were blocked with 2 mg/mL polyclonal human IgG (Intragam P, CSL Limited, Broadmeadows, Australia). For surface staining, cells were incubated for 20 min at 4°C with the fluorescent antibodies indicated at predetermined optimal concentrations. Cells were stained with a viability dye [LIVE/DEAD Fixable Blue; Molecular Probes, Eugene, or 4',6-diamidino-2-phenylindole (DAPI); Molecular Probes] to allow exclusion of dead cells. Data were acquired on an LSRII flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star Incorporated, Ashland, OR, USA). Automated compensation was performed using the appropriate fluorophore-labeled antibodies bound to anti-mouse Ig coated particles (BD Compbeads; BD Biosciences, Franklin Lakes, NJ, USA) or stained/unstained cell populations as required.

The fluorescent antibodies used were: CD25 FITC, CD161 FITC, Lin1 FITC, IFN-γ FITC, granzyme B FITC, HLA Class I FITC, anti-iNKT cell PE (clone 6B11; specific for Vα24Jα18 TCRa chain), CD1d PE (clone CD1d42), CD14 PE, CD19 PE, CD86 PE, CD80 PE, HLA-DR PerCP, CD5 APC, CD11c APC, CD14 APC, CD19 APC, CD3 APC-H7 from BD Biosciences (Franklin Lakes, NJ, USA); CD1c PE, CD4 Pacific Blue, IL-4 APC, CD8 Alexa Fluor 700, ZAP-70 PE from Biolegend (San Diego, CA, USA); CD23 FITC from Beckman Coulter (Brea, CA, USA).

Intracellular cytokine staining

PBMC were resuspended to $1x10^6$/mL in Iscove's modified Dulbecco's medium (Invitrogen, Auckland, New Zealand) with 5% human AB serum (Invitrogen) (cIMDM). Ionomycin 1 μg/mL (Merck, Manukau City, New Zealand), phorbol 12-myristate 13-acetate 10 ng/mL (PMA; Sigma-Aldrich, Auckland, New Zealand) and brefeldin A (eBioscience, San Diego, CA, USA) 2 μg/mL were added, and cells incubated at 37°C for 4 h before washing, blocking and surface staining. Cells were fixed, permeabilized, stained and washed using a fixation/permeabilization kit (BD Cytofix/Cytoperm; BD Biosciences) before flow cytometric analysis.



Online Supplementary Figure S1. Serial flow cytometric gating strategy used to identify iNKT cells.

Online Supplementary Table S1. Characteristics of healthy controls compared to untreated and treated patients

	Controls	Untreated CLL	Treated CLL	*P*
Study participants, n.	30	30	10	-
Male, n. (%)	12 (40%)	14 (47%)	7 (70%)	0.26
Age, median (range), years	64 (43-86)	63 (47-86)	62 (47-79)	0.75
iNKT cell frequency, median (range), % of T cells	0.022 (0.004-0.302)	0.010 (0.001-0.113)	0.011 (0.001-0.031)	0.08
iNKT cell absolute number, median (range), per mL blood	195.3 (33.9-1324)	329 (17.7-3082)	66.1 (14.3-1108)	<0.01
mDC absolute number, median (range), $x10^3$/mL blood	10.7 (2.5-40.8)	9.4 (0.4-47.9)	3.0 (0.1-11.2)	<0.01

Online Supplementary Table S2. Characteristics of patients with CLL.

Patient	Age (years)	Sex	Time from diagnosis to phlebotomy (months)	Binet (Rai) stage	ZAP-70 status	Prior treatment	Time since last treatment (months)	iNKT cells (% of T cells)	iNKT cells (absolute number per mL blood)	mDC (x 10^3/mL blood)
D008	62	F	15	A(I)	Negative	None	-	0.092	3081.6	29.34
D012	71	M	18	B(II)	Negative	None	-	0.008	213.2	27.13
D015	56	M	7	A(I)	Positive	None	-	0.010	322.9	6.32
D017	61	F	48	A(0)	Negative	None	-	0.010	586.5	41.67
D020	47	M	21	A(I)	Positive	None	-	0.052	1118.9	33.76
D022	61	M	87	B(II)	Negative	None	-	0.051	2452.1	47.88
D025	62	M	88	A(0)	Negative	None	-	0.010	335.0	19.79
D026	54	F	77	A(0)	Negative	None	-	0.036	417.2	0.42
D028	86	M	16	C(IV)	Positive	None	-	0.007	243.9	5.24
D029	71	M	1	B(I)	Positive	None	-	0.030	344.4	11.49
D030	68	M	69	A(0)	Negative	None	-	0.005	88.1	8.47
D034	70	F	71	A(0)	Negative	None	-	0.010	202.4	18.10
D036	51	M	14	B(II)	Positive	None	-	0.015	576.5	4.82
D037	60	M	19	A(0)	Negative	None	-	0.028	990.2	8.95
D038	61	M	116	A(0)	Negative	None	-	0.004	187.7	13.95
D039	79	F	9	A(0)	Negative	None	-	0.007	245.0	3.69
D041	69	F	20	A(0)	Negative	None	-	0.006	132.4	17.72
D046	55	F	20	A(0)	Negative	None	-	0.031	1072.6	23.83
D049	66	M	159	A(I)	Negative	None	-	0.029	594.6	5.95
D052	76	F	122	A(0)	Negative	None	-	0.001	17.7	0.66
D054	48	F	28	A(0)	Negative	None	-	0.012	296.3	4.57
D055	78	F	61	A(I)	Positive	None	-	0.006	154.7	6.52
D058	65	F	53	A(I)	Negative	None	-	0.010	262.7	13.25
D059	71	M	46	A(0)	Negative	None	-	0.005	97.4	47.86
D060	67	F	137	A(0)	Negative	None	-	0.023	527.1	9.01
D061	51	F	52	A(0)	Negative	None	-	0.024	1616.0	18.19
D063	50	M	62	A(I)	Positive	None	-	0.007	87.5	9.32
D067	56	F	139	A(0)	Negative	None	-	0.040	1575.3	6.27
D069	86	F	256	A(0)	Negative	None	-	0.005	97.7	9.56
D071	64	F	60	A(0)	Positive	None	-	0.113	2939.7	4.40
D072	62	M	100	A(I)	Negative	FCR	4	0.023	77.2	2.76
D073	52	M	139	A(0)	Positive	Chl, FC	22	0.005	86.7	10.09
D074	47	M	61	A(0)	Negative	FC, FCR	25	0.018	180.4	6.87
D075	63	M	188	C(IV)	Negative	Chl, FC	67	0.005	53.0	0.13
D076	72	M	142	C(IV)	Negative	Chl, F, FC	32	0.001	14.3	3.02
D077	61	M	153	A(0)	Negative	Chl	88	0.042	1107.8	6.58
D079	57	M	108	A(I)	Negative	CHOP, FC	13	0.004	18.6	1.61
D082	79	F	130	C(IV)	Negative	Chl, F	3	0.030	49.3	11.18
D084	54	F	64	A(I)	Negative	F	49	0.031	184.4	1.82
D085	68	F	302	A(0)	Negative	Chl, F, FC	17	0.011	55.0	3.46

FCR: fludarabine, cyclophosphamide and rituximab; Chl: chlorambucil; F: fludarabine; FC: fludarabine and cyclophosphamide; CHOP: cyclophosphamide, doxorubicin, vincristine and prednisone; mDC: myeloid dendritic cells.