SUPPLEMENTARY APPENDIX

Lymphocyte activation gene 3: a novel therapeutic target in chronic lymphocytic leukemia

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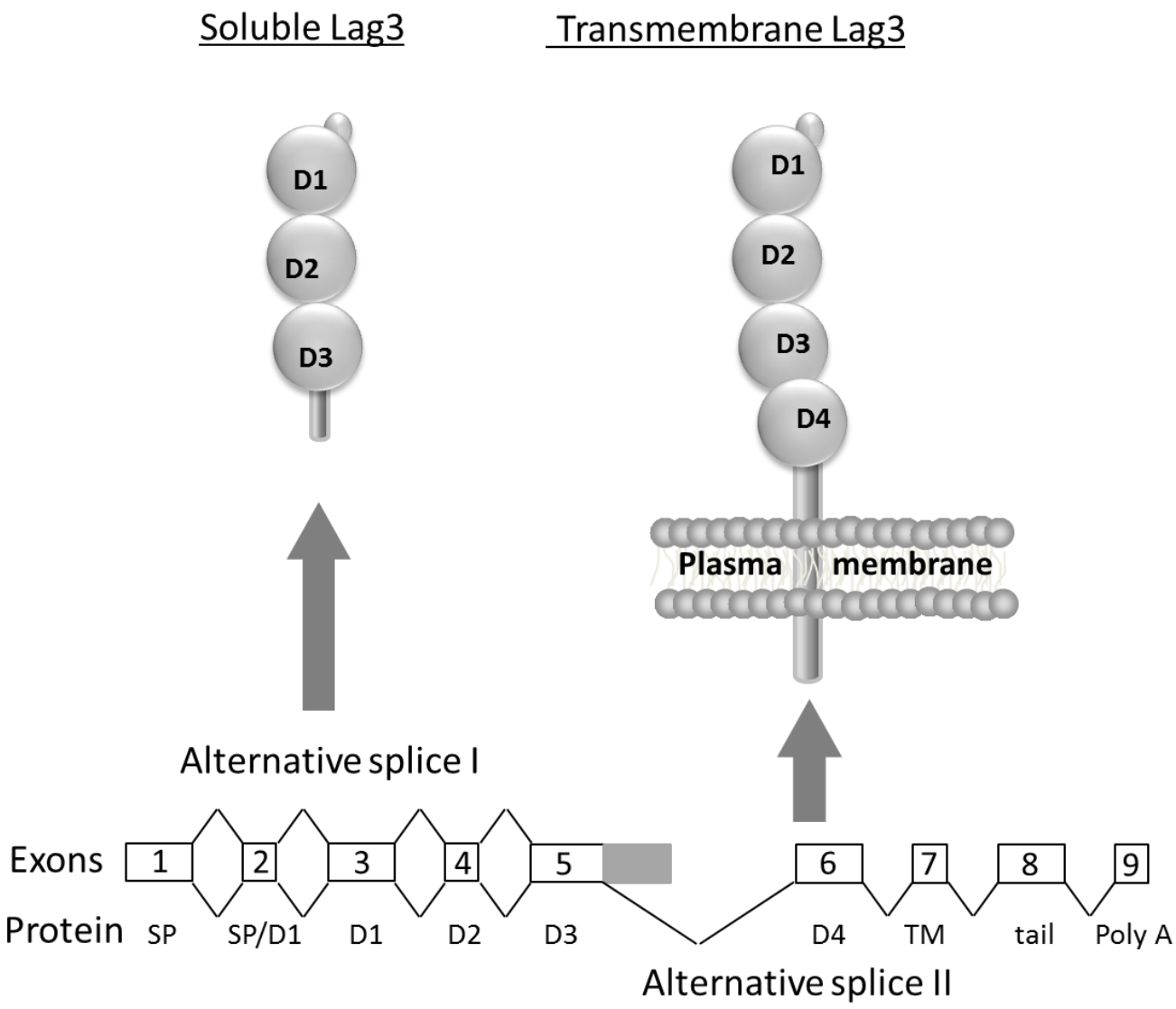
Supplementary Figure 1. Illustration of the membrane-expressed and secreted LAG-3 molecules.



Supplementary Material and Methods

Patients and samples

Mononuclear cells from patients with CLL and healthy controls were isolated by density-gradient centrifugation over Ficoll-Paque PLUS (GE healthcare, NJ, USA). Viably frozen cells were kept in fetal calf serum (FCS) containing 10% dimethyl sulfoxide and stored in liquid nitrogen. Before use, frozen cells were thawed and cultured at 37°C, 5% CO_2, in RPMI medium 1640 (Gibco, Life Technologies, CA, USA) supplemented with 10% FCS, penicillin, streptomycin, and L-glutamine all purchased from Biological Industries (BI, Kibbutz Beit Haemek, Israel). Serum samples from patients with CLL and healthy controls were also stored in -80°C for ELISA analysis.

Reagents and antibodies

Monoclonal antibodies (MoAb) anti-human CD19-APC (SJ25C1), CD86-PE (IT2.2), CD69-PE (L78), CD4-APC (RPA-T4), CD8-BV421 (RPA-T8), CD5-APC (L17F12), CD23-FITC (M-L233), AKT (pS473)-PE (M89-61), ERK1/2 (pT202/pY204)-PE (20A), cytofix fixation buffer, and Phosflow perm/wash buffer I were purchased from BD Biosciences (San Jose, CA, USA), Mouse IgG1 k isotype control PerCP-eFluor® 710 (P3.6.2.8.1), anti-Human CD223 (LAG3) PerCP-eFluor® 710 (3DS223H), CD8a-PE (HIT8a), CD4 FITC (RPA-T4), Anti-Human CD279 (PD-1) PE-Cyanine7 (J105) Mouse IgG1 κ isotype control PE- Cyanine7 (P3.6.2.8.1), anti-Human CD274 (PD-L1, B7-H1) (M1H1), anti-human CD279 (PD-1) (J116) and mouse IgG1 K Isotype control purified (P3.6.2.8.1) and polyclonal anti-Human IgG (Fc gamma-specific) PE, were purchased from eBioscience (San Diego, CA, USA). PE Mouse IgG1, κ Isotype control antibody was from BioLegend (San Diego, CA, USA). Anti-human Bcl-2 (50E3), Bcl-xL (54HS), Cleaved PARP (D64E10) and Polyclonal Mcl-1 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-LAG3 (17B4) was purchased from Abcam (Cambridge, UK). Purified anti-human Actin was from MP Biomedicals (Illkirch, France). Goat anti-Rabbit IgG (H+L)-HRP conjugate and Goat anti-Mouse IgG (H+L)-HRP conjugate were from Bio-Rad Laboratories (Richmond, CA). Recombinant soluble human LAG3-Ig fusion protein (LAG3-Fc) and human IgG1-Fc control were purchased from Enzo Life Sciences. Anti-LAG3 (blocking) (17B4) (PF) was purchased from AdipoGen (Liestal, Switzerland). All antibodies utilized in

the study were used in concentrations according to the manufacturer's instructions. Dynabeads Human T-Activator CD3/CD8 was purchased from Invitrogen (Oslo, Norway). PD-98059 and Syk Inhibitor VI, R406 were purchased from Merck Millipore's Calbiochem (Darmstadt, Germany). Ibrutinib was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA), CAL-101 (Idelalisib, (GS-1101) was from Selleckchem (TX, USA), and Wortmannin was purchased from Sigma-Aldrich (St. Louis, MO, USA). FcR Blocking Reagent was from Miltenyi Biotec Inc. (Auburn, CA, USA).

IGHV gene analysis

Analysis of IGHV gene status was performed as described in Wiestner et al.[18]. Amplification of the immunoglobulin heavy chain variable (IGHV) gene was performed as described by Hamblin et al.[19]. Briefly, cDNA was amplified by polymerase chain reaction (PCR) using a mixture of 5′ oligonucleotides specific for each leader sequence of the VH1 to VH7 IGHV families as forward primers and a 3′ oligonucleotide complementary to either the consensus sequence of the joining region or the constant region of the IgM locus as reverse primer. PCR was performed in 25 μL reactions with FastStart Taq (Roche) and 20 pmol of each primer. Products were purified (QIAquick Kit; Qiagen, Valencia, CA) and sequenced directly with the appropriate 3′ and 5' oligonucleotides by HyLab Sequencing Service (Rehovot, Israel). Nucleotide sequences were aligned to the VBase sequence directory (http://www.ncbi.nlm.nih.gov/igblast/). Sequences with 2% or less deviation from any germline IGHV sequence were considered unmutated.

Western blotting

Purified CLL cells were lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing Phosphatase Inhibitor Cocktail 2 and protease inhibitor Cocktail (Sigma-Aldrich, St. Louis, Missouri, USA). Extracts from cell lysates were separated on 10% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose paper. The nitrocellulose paper was incubated with the designated antibodies and HRP-conjugated secondary antibodies according to the manufacturer's instructions, detected using myECL™ Imager and quantified using MyImageAnalysis Software (both from Thermo Fisher Scientific, Waltham, MA USA).

Flow cytometry

For surface staining, cells were incubated for 30 min at 4°C with the appropriate antibodies, and intracellular staining was performed using Cytofix fixation buffer and Phosflow Perm/Wash buffer I, according to the manufacturer's instructions. Samples were acquired by the BD FACSCanto II and analyzed using BD FacsDiva software (Becton Dickinson, San Jose, CA).

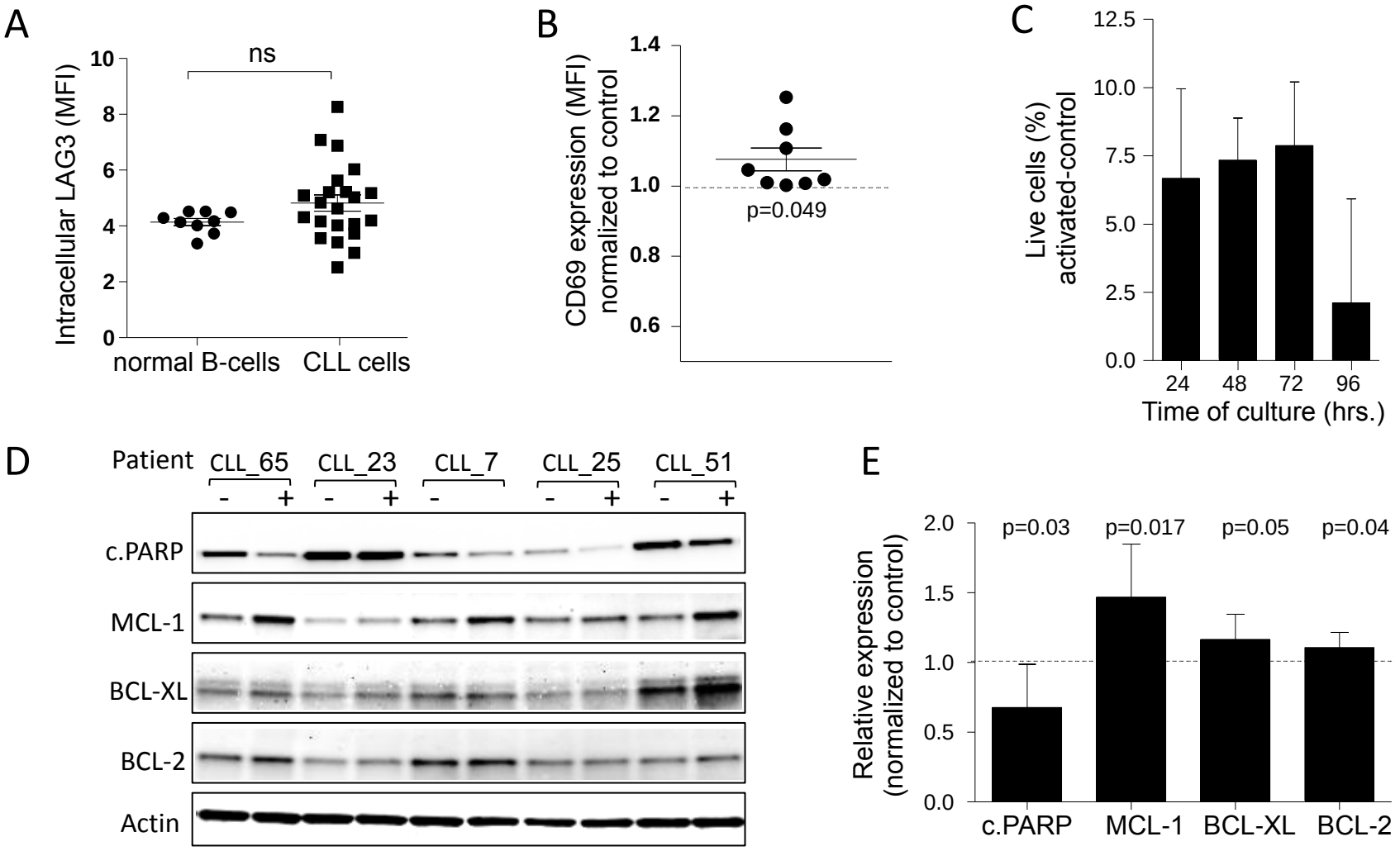
Quantitative PCR primers

GAPDH forward 5'-ATGGGGAAGGTGAAGGTCG
and reverse 5'-GGGGTCATTGATGGCAACAATA,
LAG3 forward 5'-TCACAGTGACTCCCAAATCCT
and reverse 5'-GCTCCACACAAAGCGTTCTT,
sLAG3 forward 5'-GACCTCCTGGTGACTGGAGA
and reverse 5' TCCCACCTGAGGCTGACC**.**
All primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA).

Supplementary Table 1. Patient characteristics

Study number	Gender/ Age (y)	Binet stage	ALC x1000/uL	IgHV gene	FISH	Treatment
CLL_01	M/69	A	18.3	M	Normal	-
CLL_02	F/85	C	59.9	UM	13q-	-
CLL_03	F/68	C	148.4	M	13q-	-
CLL_04	M/69	C	113.0	UM	normal	+
CLL_05	M/57	B	15	UM	normal	+
CLL_06	M/71	A	54.7	UM	13q-, +12	-
CLL_07	F/75	A	268.2	UM	+12	+
CLL_08	M/64	B	165.4	M	nd	-
CLL_09	M/54	B	172.6	UM	13q-	+
CLL_10	F/42	B	37.6	UM	17p-	+
CLL_11	F/81	A	210.4	M	nd	-
CLL_12	F/81	A	41.5	M	nd	-
CLL_13	M/55	B	70.2	UM	13q-	+
CLL_14	F/75	A	47	M	nd	-
CLL_15	M/65	B	88.6	UM	13q-	+
CLL_16	M/50	B	53.3	UM	13q-, +12	-
CLL_17	F/68	C	85.6	M	normal	+
CLL_18	M/64	B	38.7	M	normal	+
CLL_19	F/62	A	181.2	UM	normal	+
CLL_20	F/71	A	178	M	13q-	+
CLL_21	83/F	A	48.6	M	nd	-
CLL_22	F/67	B	36.45	UM	13q-	+
CLL_23	F/79	A	17.2	M	nd	-
CLL_24	F/60	A	34.6	M	normal	-
CLL_25	M/57	B	49.2	UM	13q-	+
CLL_26	F/64	A	32.6	M	nd	-
CLL_27	F/74	A	135.3	M	nd	-
CLL_28	F/59	A	36.7	M	13q-	-
CLL_29	M/61	A	59.5	UM	13q-	-
CLL_30	F/83	B	74.2	M	nd	+
CLL_31	F/68	B	121.7	M	normal	-
CLL_32	M/52	A	6.2	UM	13q-	+
CLL_33	F/68	A	83.1	UM	13q-, +12	+
CLL_34	M/76	C	142.6	M	13q-, 17p-	-
CLL_35	M/65	B	146.7	UM	11q-	+
CLL_36	F/66	A	16	M	13q-	-
CLL_37	M/70	B	50.7	M	13q-	+
CLL_38	тM/81	A	36.9	ND	13q	-
CLL_39	F/56	A	59.5	UM	13q-	-
CLL_40	F/61	C	46.7	UM	+12	+
CLL_41	M/45	B	87.8	UM	11q-	+
CLL_42	F/69	C	9.3	UM	13q-	-
CLL_43	F/79	A	62.6	UM	nd	-
CLL_44	M/91	A	19	ND	nd	-
CLL_45	M/82	B	22.5	UM	nd	+
CLL_46	M/61	A	135	M	13q-	-
CLL_47	M/80	B	104.9	UM	17p-	+
CLL_48	F/72	A	11.9	UM	13q-, +12, 17p-	+
CLL_49	F/70	A	80.8	UM	+12	-
CLL_50	F/63	A	23.3	M	normal	-
CLL_51	F/72	A	11.8	M	normal	-
CLL_52	F/80	A	47.2	UM	nd	-
CLL_53	M/50	A	39.3	M	normal	-
CLL_54	M/64	B	9.5	M	13q-	-
CLL_55	M/73	B	54.2	M	nd	+
CLL_56	M/69	B	38.6	UM	17p-	+
CLL_57	F/67	C	26.4	UM	normal	+
CLL_58	M/64	B	211.3	M	13q-	+
CLL_59	M/45	C	15.6	UM	17p-	+
CLL_60	F/78	B	7	ND	nd	+
CLL_61	M/58	C	53.5	UM	17p-	+
CLL_62	M/58	C	218.1	UM	17p-	+
CLL_63	M/63	B	35.5	UM	normal	+
CLL_64	F/72	A	80.2	M	nd	+
CLL_65	F/70	B	66.5	UM	+12	+

Supplementary Figure 2



A. Summary of intracellular LAG3 MFI normalized to Isotype control in B-cells (left, n=8) compared to CLL cells (right, n=22) isolated from peripheral blood of healthy controls and CLL patients, ns stands for not significant. **B.** Statistical analysis of CD69 surface expression on CLL cells (n=8) after sLAG3 activation: CLL cells were incubated with either Ig-Fc as control or LAG3-Fc for 24 hours and surface CD69 expression was analyzed on $CD5^+/CD23^+$ gated CLL cells, by flow cytometry**. C.** Differences in percentage of live CLL cells in the presence of LAG3-Fc compared to Ig-Fc (control), after 24, 48, 72 or 96 hours incubation (summary of 6-9 independent experiments). **D.** Representative Western blot analysis showing the levels of cleaved PARP, MCL-1, BCL-XL and BCL-2 in CLL cells after 48 hours incubation with Ig-Fc (control) or with LAG3-Fc. Actin was used to verify equal loading. **E.** Cumulative results of 7 independent experiments, performed as described in Fig 4, presenting quantified levels of cleaved PARP, MCL-1, BCL-XL and BCL-2 in LAG3-Fc activated CLL cells, normalized to control (Ig-Fc), after 48 hours incubation.