
Idelalisib impairs T-cell-mediated immunity in chronic lymphocytic leukemia

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doi:10.3324/haematol.2017.187070*

Supplementary Methods

Patients and samples

Peripheral blood mononuclear cells (PBMCs) collected from n=40 untreated CLL patients diagnosed at the Hematology Division of Modena and preserved in liquid nitrogen, were separated in autoMACS (Miltenyi Biotec) by two different methods: i) incubation with CD19 microbeads (Miltenyi Biotec) to keep the negative fraction depleted from leukemic B-cells ii) incubation with Pan T-cell Isolation kit (Miltenyi Biotec) to obtain purified T-cells. By the first method, we had a mix of T-cells and antigen presenting cells (APCs) proper for ELISpot and cytokine secretion assays. For all the other tests, we used T-cells purified by the second method, reaching 97.5% median purity (range from 96.0% to 98.2%). PBMCs were also collected from 10 age-matched healthy donors (HD) afferent to the Transfusional Division of Modena and separated by the first method. All patients provided informed consent in accordance with the local institutional review board requirements and the Declaration of Helsinki Principles.

Western Blot

T-cells purified from n=6 CLL patients were treated or not with 5 μ M idelalisib (Selleckchem) for 1 hour, followed or not by stimulation with 500ng/ml antiCD3 antibody (Mabtech) for 30 min. A mixed pool of T-cells tested for each condition was lysed in buffer with dithiothreitol and protease inhibitor cocktail (BioVision). 30 μ g proteins were electrophoresed on SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Bio-Rad Laboratories). Membranes were immunoblotted with anti-phosphoAkt (ser473) (#4060; Cell Signaling), anti-Akt (#9272; Cell Signaling) and anti- β actin (ab6276; Abcam). Finally, they were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare) and developed by using Western BrightTM Sirius detection kit (Advansta). The blot images were acquired by Chemidoc XRS+ (Bio-Rad Laboratories) and analyzed with Image Lab Software v.5.2.1 (Bio-Rad Laboratories).

Gene Expression Profile

Large-scale gene expression profiling was performed on total RNA extracted from T-cells (RNeasy Mini kit Plus, QIAGEN) purified from n=4 CLL patients, by hybridization on 4X44K Whole Human Genome Microarray (Agilent Technologies). T-cells were pre-treated or not with 5 μ M idelalisib for 1 hour and stimulated with 500ng/ml antiCD3 antibody for 4 hours. Fluorescence data were analyzed with Feature Extraction Software v.10.7 (Agilent Technologies). Supervised analyses based on Anova test were performed using Gene Spring GX Software (Agilent Technologies).

Real-Time PCR

RNA (50ng) extracted from T-cells (RNeasy Mini kit Plus, QIAGEN) purified from n=5 CLL patients was reverse transcribed using SuperScript® VILO cDNA Synthesis Kit (ThermoFisher). IL-2, TNF α and IFN γ mRNAs were amplified in Real-Time PCR by using specific primers and SYBR Green Master MIX (ThermoFisher). T-cells pre-treated or not with idelalisib and stimulated with antiCD3 antibody as previously described for gene expression profiling, were tested and compared. All samples were run in duplicate on LightCycler 480 v.2 (Roche). Amplification of the sequence of interest was normalized to the housekeeping reference gene GAPDH. Differences in gene expression were determined by comparative method ($2^{-\Delta\Delta CT}$).

ELISpot

ELISpot for IFN γ detection was performed by using Human IFN- γ ELISpotPRO (ALP) (Mabtech), according to manufacturer's instructions. 100000 cells of the negative fraction obtained from PBMCs of n=5 CLL patients and n=4 healthy donors (HD) were pre-treated or not with 5 μ M idelalisib for 1 hour, followed or not by stimulation with 500ng/ml antiCD3 antibody for 18 hours. In parallel, experiments were done by stimulating cells with 100ng/ml PepMIX HCMVA (pp65) (peptide mix of CMV phosphoprotein 65) (JPT) or 100ng/ml CEF (peptide mix of CMV, Epstein Barr and Influenza virus) (Mabtech), in presence or in absence of 5 μ M idelalisib. T samples cultured without treatment or stimuli were used as controls (CTRL and CTRL+idelalisib). Only HD and CLL cases responding to stimulations were included in the final analysis.

Cytokine Secretion Assay

Cytokine secretion assay (CSA) for IFN γ was performed by using IFN- γ Secretion Assay Detection Kit (FITC) human (Miltenyi Biotec), following manufacturer's instructions. 150000 cells of the negative fraction obtained from PBMCs of n=5 CLL patients were pre-treated or not with 5 μ M idelalisib for 1 hour and then stimulated or not with 500ng/ml antiCD3 antibody for 1 hour. Afterwards, cells were stained with catch reagent, incubated for 1 hour at 37°C under continuous rotation, and then labeled with IFN γ detection antibody (FITC). Finally, they were counterstained with antiCD3 (PE), antiCD8 (APC) and antiCD4 (PerCP) antibodies (BD Biosciences), in order to identify CD3+CD8+ and CD3+CD4+ lymphocytes secreting IFN γ (IFN γ +). T samples cultured without treatment or stimuli were used as controls (CTRL and CTRL+idelalisib). For all tested conditions, about 100000 events were acquired inside the "lymphocytes" gate by flow cytometer FACSCalibur (BD Biosciences) and relative data were analyzed with FlowJo Software v.10.2 (LLC).

Migration

Migration assay was performed in 24-well plates containing 5 μm pore size PET inserts (Millipore). From 300000 to 500000 T-cells purified from n=5 CLL patients were pre-treated or not with 5 μM idelalisib for 1 hour and placed on top of the inserts. In some experiments, 100ng/ml CXCL10 (PeproTech) was added under the inserts. After 2 hours of incubation, migrated T-cells were collected from well-bottoms and stained with 4 μM Calcein-AM (Sigma-Aldrich) for 45 min. Finally, they were quantified using Infinite200 (Tecan) fluorescence plate reader at 485 nm excitation and 520 nm emission.

Proliferation

T-cells purified from n=3 CLL patients were stained with 0.5 μM CFSE (eBioscience) and treated or not with 5 μM idelalisib for 1 hour. Therefore, they were stimulated with a mix of antiCD3+antiCD28+antiCD2 antibodies (Cell Activation/ Expansion Kit human, Miltenyi Biotec) for 72 hours, in presence of 300U/ml IL-2 (PeproTech). Finally, cells were stained with antiCD3 antibody (APC) (BD Biosciences). For all tested conditions, about 10000 events were acquired inside the “lymphocytes” gate by flow cytometer FACSCalibur (BD Biosciences) and relative data were analyzed with FlowJo Software v.10.2 (LLC).

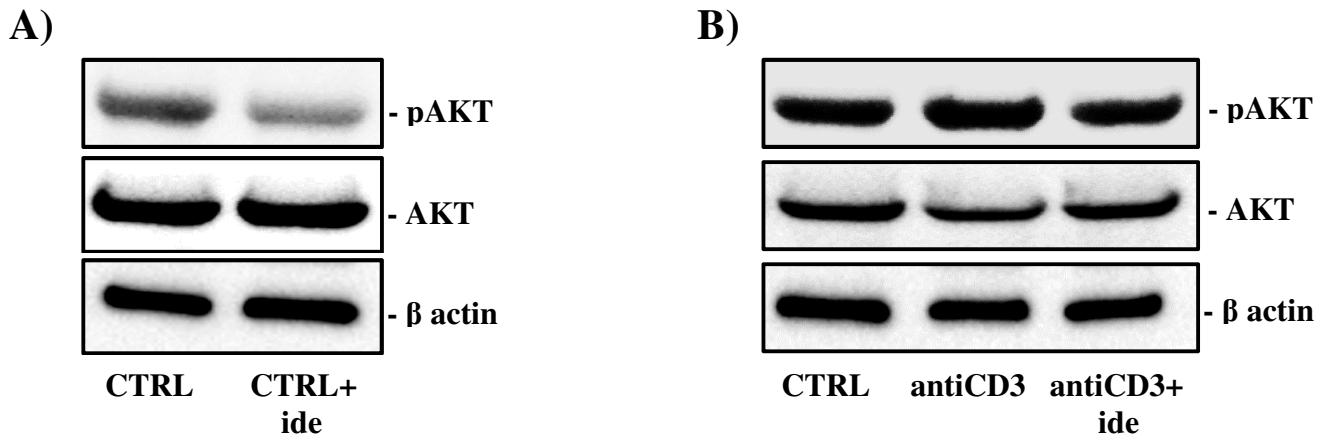
Vitality

Apoptotic T-cell death was analyzed in PBMCs of n=3 CLL patients cultured *in vitro* for 24 and 48 hours in presence or in absence of 1 μM or 5 μM idelalisib, by using antibodies for Annexin V (FITC), Propidium Iodide (PI) (eBioscience) and CD3 (APC) (BD Biosciences). Not treated PBMCs were used as controls. Survival rate was defined as the percentage of Annexin V negative/PI negative cells inside the CD3 positive gate. For all tested conditions, about 10000 total events were acquired by flow cytometer FACSCalibur (BD Biosciences) and relative data were analyzed with FlowJo Software v.10.2 (LLC).

Statistics

Data were analyzed using SPSS v.24 (SPSS) and compared by Student t-test. Statistical significance was considered with $p \leq 0.05$.

Supplementary Figure S1. PhosphoAKT levels are reduced by idelalisib in T-cells of CLL patients. Here are shown two representative Western blots. **A)** T-cells were incubated in presence or in absence of 5 μ M idelalisib (CTRL+ide and CTRL, respectively) for 1 hour. PhosphoAKT (pAKT) levels are decreased by idelalisib. **B)** T-cells were pre-treated or not with idelalisib and then stimulated with antiCD3 antibody for 30 min. Under stimulation, the amount of pAKT is increased but it is reduced to the value of CTRL in presence of the drug.



Supplementary Table S1. List of the 65 genes most modulated by idelalisib in T-cells of CLL patients stimulated with antiCD3 antibody (Anova $p \leq 0.01$ and $[FC] \geq 2$)

Probe Name	p-value	FC ([ANTICD3+IDE] vs [ANTICD3])	Regulation ([ANTICD3+IDE] vs [ANTICD3])	Gene Symbol	Description
A_23_P133408	3,05E-04	-18,732206	down	CSF2	Homo sapiens colony stimulating factor 2 (granulocyte-macrophage) (CSF2), mRNA [NM_000758]
A_23_P51534	0,00103415	-7,1689844	down	XCL2	Homo sapiens chemokine (C motif) ligand 2 (XCL2), mRNA [NM_003175]
A_23_P376488	7,26E-06	-6,883258	down	TNF	Homo sapiens tumor necrosis factor (TNF), mRNA [NM_000594]
A_24_P45476	7,00E-04	-6,8494635	down	XCL1	Homo sapiens chemokine (C motif) ligand 1 (XCL1), mRNA [NM_002995]
A_23_P251031	0,00384275	-6,425854	down	IL13	Homo sapiens interleukin 13 (IL13), mRNA [NM_002188]
A_23_P46936	2,55E-05	-6,421565	down	EGR2	Homo sapiens early growth response 2 (EGR2), transcript variant 1, mRNA [NM_000399]
A_23_P216225	3,60E-08	-6,407652	down	EGR3	Homo sapiens early growth response 3 (EGR3), transcript variant 1, mRNA [NM_004430]
A_24_P228130	0,00878732	-5,9981503	down	CCL3L3	Homo sapiens chemokine (C-C motif) ligand 3-like 3 (CCL3L3), mRNA [NM_001001437]
A_23_P151294	5,34E-05	-5,7383513	down	IFNG	Homo sapiens interferon, gamma (IFNG), mRNA [NM_000619]
A_23_P373017	0,00637871	-5,632913	down	CCL3	Homo sapiens mRNA for pLD78 peptide, complete cds. [D00044]
A_23_P30115	1,64E-04	-5,271836	down	IL2	Homo sapiens interleukin 2 (IL2), mRNA [NM_000586]
A_23_P320578	6,90E-04	-5,233278	down	RGS16	Homo sapiens regulator of G-protein signaling 16 (RGS16), mRNA [NM_002928]
A_23_P125278	1,53E-04	-5,0078936	down	CXCL11	Homo sapiens chemokine (C-X-C motif) ligand 11 (CXCL11), transcript variant 1, mRNA [NM_005409]
A_24_P50759	1,08E-06	-4,808019	down	TNF	Homo sapiens tumor necrosis factor (TNF), mRNA [NM_000594]
A_24_P319088	0,0035879	-4,787074	down	CCL23	Homo sapiens chemokine (C-C motif) ligand 23 (CCL23), transcript variant CKbeta8, mRNA [NM_005064]
A_23_P214080	1,05E-04	-4,7571044	down	EGR1	Homo sapiens early growth response 1 (EGR1), mRNA [NM_001964]
A_24_P20607	1,04E-04	-4,742346	down	CXCL11	Homo sapiens chemokine (C-X-C motif) ligand 11 (CXCL11), transcript variant 1, mRNA [NM_005409]
A_23_P30122	2,91E-04	-4,596041	down	IL2	Homo sapiens interleukin 2 (IL2), mRNA [NM_000586]
A_23_P217845	6,32E-04	-4,5937805	down	RGS16	Homo sapiens regulator of G-protein signaling 16 (RGS16), mRNA [NM_002928]
A_23_P207564	0,00217246	-4,563165	down	CCL4L2	Homo sapiens chemokine (C-C motif) ligand 4-like 2 (CCL4L2), transcript variant CCL4L2b2, mRNA [NM_001291470]

A_23_P18452	3,98E-04	-4,4868608	down	CXCL9	Homo sapiens chemokine (C-X-C motif) ligand 9 (CXCL9), mRNA [NM_002416]
A_32_P108156	0,00830117	-4,3211465	down	MIR155HG	Homo sapiens MIR155 host gene (non-protein coding) (MIR155HG), long non-coding RNA [NR_001458]
A_23_P128230	1,42E-04	-3,9884927	down	NR4A1	Homo sapiens nuclear receptor subfamily 4, group A, member 1 (NR4A1), transcript variant 1, mRNA [NM_002135]
A_23_P81898	0,00141192	-3,967373	down	UBD	Homo sapiens ubiquitin D (UBD), mRNA [NM_006398]
A_23_P167250	1,61E-05	-3,8578732	down	IL21	Homo sapiens interleukin 21 (IL21), transcript variant 1, mRNA [NM_021803]
A_23_P127288	9,09E-04	-3,497599	down	IL2RA	Homo sapiens interleukin 2 receptor, alpha (IL2RA), mRNA [NM_000417]
A_23_P250245	0,0030754	-3,2184224	down	CD72	Homo sapiens CD72 molecule (CD72), mRNA [NM_001782]
A_23_P306867	0,00876837	-3,163697	down	NR4A3	Homo sapiens nuclear receptor subfamily 4, group A, member 3 (NR4A3), transcript variant 4, mRNA [NM_173199]
A_23_P380318	3,61E-05	-3,090398	down	EGR4	Homo sapiens early growth response 4 (EGR4), mRNA [NM_001965]
A_23_P160720	0,00689807	-3,027223	down	BATF3	Homo sapiens basic leucine zipper transcription factor, ATF-like 3 (BATF3), mRNA [NM_018664]
A_23_P91390	0,00147491	2,831331	up	THBD	Homo sapiens thrombomodulin (THBD), mRNA [NM_000361]
A_23_P153320	0,00238952	-2,7707179	down	ICAM1	Homo sapiens intercellular adhesion molecule 1 (ICAM1), mRNA [NM_000201]
A_23_P328740	0,0023836	-2,6983771	down	NEURL3	Homo sapiens neuralized E3 ubiquitin protein ligase 3 (NEURL3), transcript variant 2, mRNA [NM_001285486]
A_23_P152002	0,00366031	-2,6672103	down	BCL2A1	Homo sapiens BCL2-related protein A1 (BCL2A1), transcript variant 1, mRNA [NM_004049]
A_23_P253783	0,00316453	-2,6510162	down	MFSD2A	Homo sapiens cDNA FLJ14490 fis, clone MAMMA1002886. [AK027396]
A_23_P87879	1,19E-05	-2,6060574	down	CD69	Homo sapiens CD69 molecule (CD69), mRNA [NM_001781]
A_23_P144872	0,00481669	2,5899246	up	GM2A	Homo sapiens GM2 ganglioside activator (GM2A), transcript variant 1, mRNA [NM_000405]
A_24_P303091	0,00167069	-2,5550523	down	CXCL10	Homo sapiens chemokine (C-X-C motif) ligand 10 (CXCL10), mRNA [NM_001565]
A_23_P130194	0,00674081	-2,5515745	down	PYCR1	Homo sapiens pyrroline-5-carboxylate reductase 1 (PYCR1), transcript variant 1, mRNA [NM_006907]
A_23_P70670	0,00216861	-2,5410705	down	CD83	Homo sapiens CD83 molecule (CD83), transcript variant 1, mRNA [NM_004233]
A_23_P394246	0,00958134	-2,5236053	down	HCAR1	hydroxycarboxylic acid receptor 1 [Source:HGNC Symbol;Acc:HGNC:4532] [ENST00000432564]
A_23_P77859	0,00112431	-2,519433	down	TMEM88	Homo sapiens transmembrane protein 88 (TMEM88), mRNA [NM_203411]
A_23_P156505	7,44E-04	-2,5194163	down	IRF4	Homo sapiens interferon regulatory factor 4 (IRF4), transcript variant 1, mRNA [NM_002460]

A_24_P230563	3,75E-04	-2,5140584	down	IL2RA	Homo sapiens interleukin 2 receptor, alpha (IL2RA), mRNA [NM_000417]
A_23_P214360	0,00353805	-2,4951487	down	IRF4	Homo sapiens interferon regulatory factor 4 (IRF4), transcript variant 1, mRNA [NM_002460]
A_23_P168828	0,00120156	-2,4770823	down	KLF10	Homo sapiens Kruppel-like factor 10 (KLF10), transcript variant 1, mRNA [NM_005655]
A_24_P917729	0,00304543	-2,4722307	down	AGK	acylglycerol kinase [Source:HGNC Symbol;Acc:HGNC:21869] [ENST00000492693]
A_23_P17065	0,00866759	-2,4378958	down	CCL20	Homo sapiens chemokine (C-C motif) ligand 20 (CCL20), transcript variant 1, mRNA [NM_004591]
A_24_P31875	2,91E-04	2,3628242	up	ZNF645	Homo sapiens zinc finger protein 645 (ZNF645), mRNA [NM_152577]
A_23_P383422	5,38E-04	-2,2963867	down	NFKBID	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta (NFKBID), mRNA [NM_139239]
A_23_P421423	0,00682906	-2,2845297	down	TNFAIP2	Homo sapiens tumor necrosis factor, alpha-induced protein 2 (TNFAIP2), mRNA [NM_006291]
A_24_P319113	0,00499864	-2,2739122	down	P2RX7	Homo sapiens purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7), transcript variant 1, mRNA [NM_002562]
A_23_P42386	0,00415557	-2,2302253	down	CGA	Homo sapiens glycoprotein hormones, alpha polypeptide (CGA), transcript variant 2, mRNA [NM_000735]
A_23_P62890	0,0013193	-2,2241695	down	GBP1	Homo sapiens guanylate binding protein 1, interferon-inducible (GBP1), mRNA [NM_002053]
A_23_P74290	7,73E-04	-2,2026606	down	GBP5	Homo sapiens guanylate binding protein 5 (GBP5), transcript variant 1, mRNA [NM_052942]
A_32_P107372	0,00138002	-2,1739028	down	GBP1	Homo sapiens guanylate binding protein 1, interferon-inducible (GBP1), mRNA [NM_002053]
A_24_P274270	1,91E-04	-2,1620374	down	STAT1	Homo sapiens signal transducer and activator of transcription 1, 91kDa (STAT1), transcript variant beta, mRNA [NM_139266]
A_23_P338479	0,00182896	-2,1572974	down	CD274	Homo sapiens CD274 molecule (CD274), transcript variant 1, mRNA [NM_014143]
A_24_P374943	0,00852437	-2,1347315	down	CXADR	Homo sapiens coxsackie virus and adenovirus receptor (CXADR), transcript variant 1, mRNA [NM_001338]
A_23_P56630	7,52E-04	-2,1210513	down	STAT1	Homo sapiens signal transducer and activator of transcription 1, 91kDa (STAT1), transcript variant alpha, mRNA [NM_007315]
A_24_P249626	0,00203505	-2,1069903	down	CDC42BPG	Homo sapiens CDC42 binding protein kinase gamma (DMPK-like) (CDC42BPG), mRNA [NM_017525]
A_24_P618401	0,00548602	2,0830195	up	CKMT2-AS1	Homo sapiens CKMT2 antisense RNA 1 (CKMT2-AS1), transcript variant 1, long non-coding RNA [NR_034121]
A_24_P188447	0,00889735	-2,0448742	down	ELAVL4	Homo sapiens ELAV like neuron-specific RNA binding protein 4 (ELAVL4), transcript variant 1, mRNA [NM_021952]

A_23_P42257	8,76E-04	-2,0092447	down	IER3	Homo sapiens immediate early response 3 (IER3), mRNA [NM_003897]
A_23_P128050	0,00993336	-2,0037158	down	BCL2L14	Homo sapiens BCL2-like 14 (apoptosis facilitator) (BCL2L14), transcript variant 2, mRNA [NM_030766]

Legend: *p-value=Anova p-value. FC=fold-change. ANTICD3+IDE=T-cells pre-treated with idelalisib and stimulated with antiCD3 antibody. ANTICD3=T-cells stimulated with antiCD3 antibody. down=downregulated. up=upregulated.*