The PD-1/PD-L1 axis contributes to T-cell dysfunction in chronic lymphocytic leukemia

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Online Supplementary Design and Methods

B-cell purification

PBMC were obtained by Ficoll-Hypaque (GE Healthcare, Milan, Italy) centrifugation. B lymphocytes were purified by negative selection by using anti-CD3, -CD14, and -CD16 antibodies and sheep anti-mouse Dynal magnetic beads (Invitrogen, Monza, Italy), with a purity constantly over 95%.

Immunohistochemistry and immunofluorescence microscopy

Sections were deparaffinized and endogenous peroxidase activity was blocked. Epitope retrieval was performed in 0.01M citrate buffer, pH 6.0 (40 min, 98°C). Antibodies used were goat polyclonal anti-PD-1 (2.5 μg/mL) and anti-CD23 (10 μg/mL) both from R&D Systems, rabbit polyclonal anti-PD-L1 (3 μg/mL, Novus Biologicals, Segrate, Italy) and anti-CD2 (1.3 μg/mL, Sigma, Milan, Italy), mouse monoclonal anti-CD3 (1:200, clone PS1) and anti-CD4 (1:10, clone 4B12) both from Novocastra (Milan, Italy). Goat HRP-polymer detection system (Biocare Medical, Milan, Italy), anti-rabbit HRP-conjugated Abs and 3,3′-diaminobenzidine (EnVision™ System, Dako, Milan, Italy) were used to visualize the reaction. Slides were analyzed using a DMI 3000 B optical microscope (Leica Microsystems), equipped with a DCF 310 FX digital camera (Leica Microsystems). Quantification of staining intensity was performed using the LAS Version 3.8 software (Leica Microsystems) by comparing the percentage of PD-L1+ areas in 20x magnification images of PC to other parts of the same slide. At least 6 independent images of PC *versus* 6 of other areas were considered for each sample. For immunofluorescence, tissue sections were incubated with the following secondary Abs: AlexaFluor-633-conjugated goat anti-mouse IgG (Life Technologies), DyLight-488-conjugated bovine anti-goat IgG and DyLight-594-conjugated donkey anti-rabbit IgG (both from Jackson Immunoresearch, West Grove, PA, USA). Samples were counterstained with 4,6-diamidino-2-phenylindole and mounted in Slow-Fade Gold reagent (both from Life Technologies).

Immunofluorescent slides were analyzed using a TCS SP5 laser scanning confocal microscope equipped with 4 lasers (Leica Microsystems); images were acquired with LAS AF Version Lite 2.4 software (Leica Microsystems) and processed with Photoshop (Adobe Systems, San Jose, CA, USA). Staining quantification was performed by comparing PD-L1 mean pixel intensity in 63x magnification images of $CD3_{high}$ *versus* $CD3_{low}$ areas. At least 10 independent images of the different areas per slide were analyzed using ImageJ software (National Institutes of Health. Available from: http://rsbweb.nih.gov/ij/).

After labeling with carboxyfluorescein diacetate N-succinimidyl ester (CFSE, 5 μM final concentration, Invitrogen), purified CLL cells or peripheral blood normal B lymphocytes were incubated ($2.5x10^5$/well) in 96-well plates in the presence of CpG ODN2006K (1 μg/mL; TibMolBiol, Genoa, Italy) and recombinant human IL-2 (100 IU/mL; R&D Systems). The proliferative response was measured after five days by staining with anti-CD19-PE and anti-PD-L1-PE-Cy7 mAbs (eBioscience). PBMC were similarly labeled and incubated with anti-CD3 (0.5 μg/mL) and anti-CD28 (0.5 μg/mL, both soluble) mAbs for 3-5 days. Cells were stained with anti-CD8-PE, -CD4-PE-Cy5 and -PD-1-PE-Cy7 before being analyzed by flow cytometry.

ELISpot assay

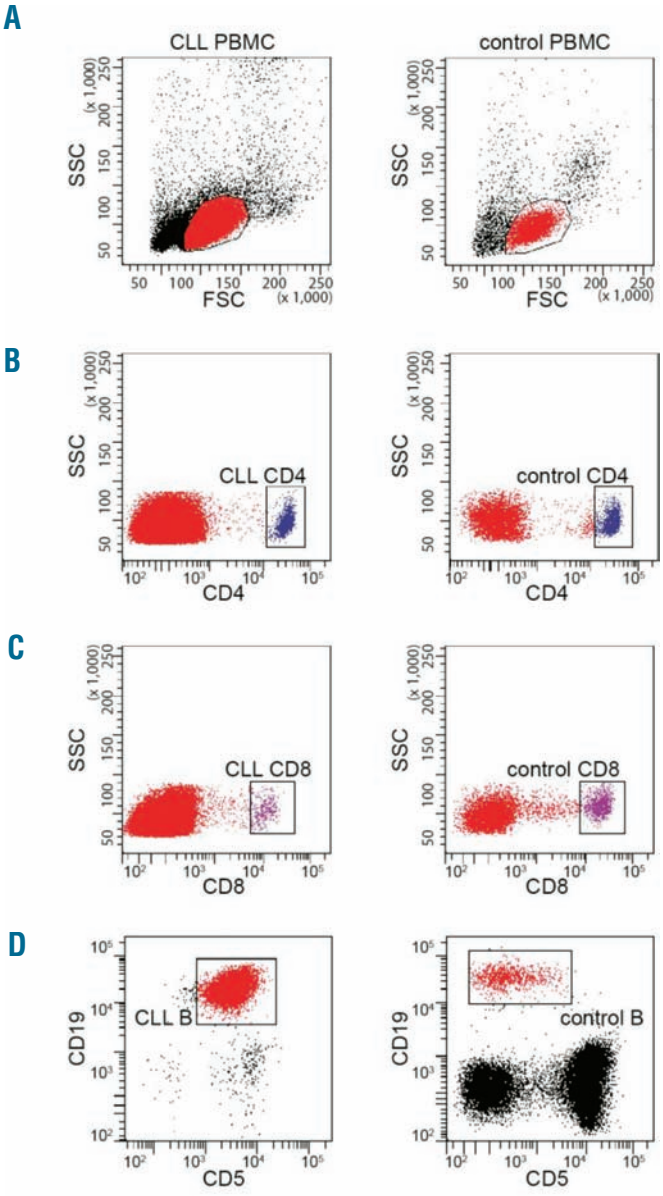
Nitrocellulose membrane 96-well microtiter plates (Multiscreen, Millipore, Milan, Italy) were coated with an anti-IFN-γ Ab (Biolegend). $2.5 x10^6$ PBMC from CLL patients or control donors were cultured for five days with a mitogenic combination of anti-CD3 and anti-CD28 antibodies (see above). Soluble PD-L1 (from R&D System; 10 μg/mL) or blocking anti-PD-1 and anti-PD-L1 antibodies (both from eBiosciences; final concentration 5 μg/mL) were used to interfere with the PD-1/PD-L1 axis. After five days cells were recovered and seeded ($2.5x10^5$) in triplicate wells in the presence of the indicated treatments. After 24 h, cells were lysed with distilled water and a biotinylated anti-IFN-γ mAb was added to the wells, followed by streptavidin-horse radish peroxidase-conjugated (both from Biolegend). The substrate solution (3-amino-9-ethylcarbazole, Sigma) was added for 30 min and the reaction stopped by rinsing with tap water. Red spots (indicative of reactive lymphocytes) were detected with the AID Elispot-Reader (Bioline Amplimedical, Milan, Italy).

Online Supplementary Table S1. Characteristics of CLL patients and of controls.

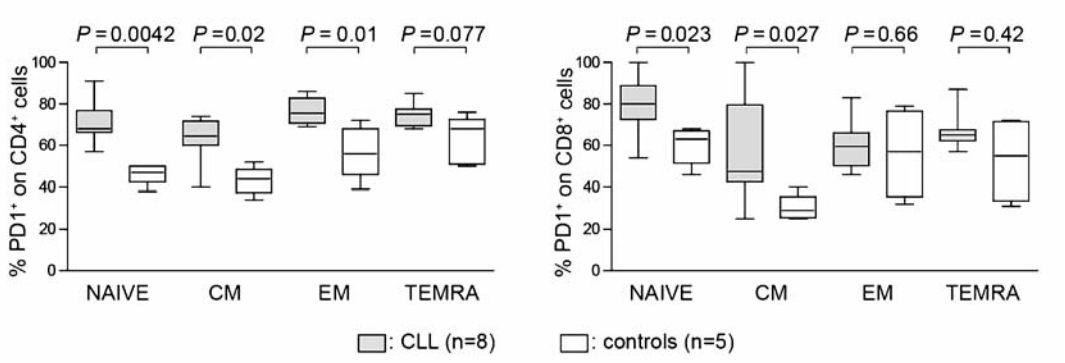
Variable	N. (%)
Controls	33
Mean age (years)	65
Sex (males)	18 (54)
Patients	117
Mean age (years)	63
Sex (males)	60 (51)
Binet stage at diagnosis, n. 98	
A	76 (78)
B/C	22 (22)
Treatment, n. 94	
Treated	38 (40)
Fludarabine-based	20 (53)
Campath-based	3 (8)
Other	15 (39)
IgVH gene mutation status, n. 90	
UM (<2%)	35 (39)
Genomic aberrations, n. 76	
deletion 11q/17p	12 (16)
deletion 11q only	4 (5)
deletion 17p only	11 (14)
deletion 13q	35 (46)
trisomy 12	18 (24)
normal	20 (26)

Online Supplementary Table S2. Clinical characteristics of patients undergoing lymph node biopsy.

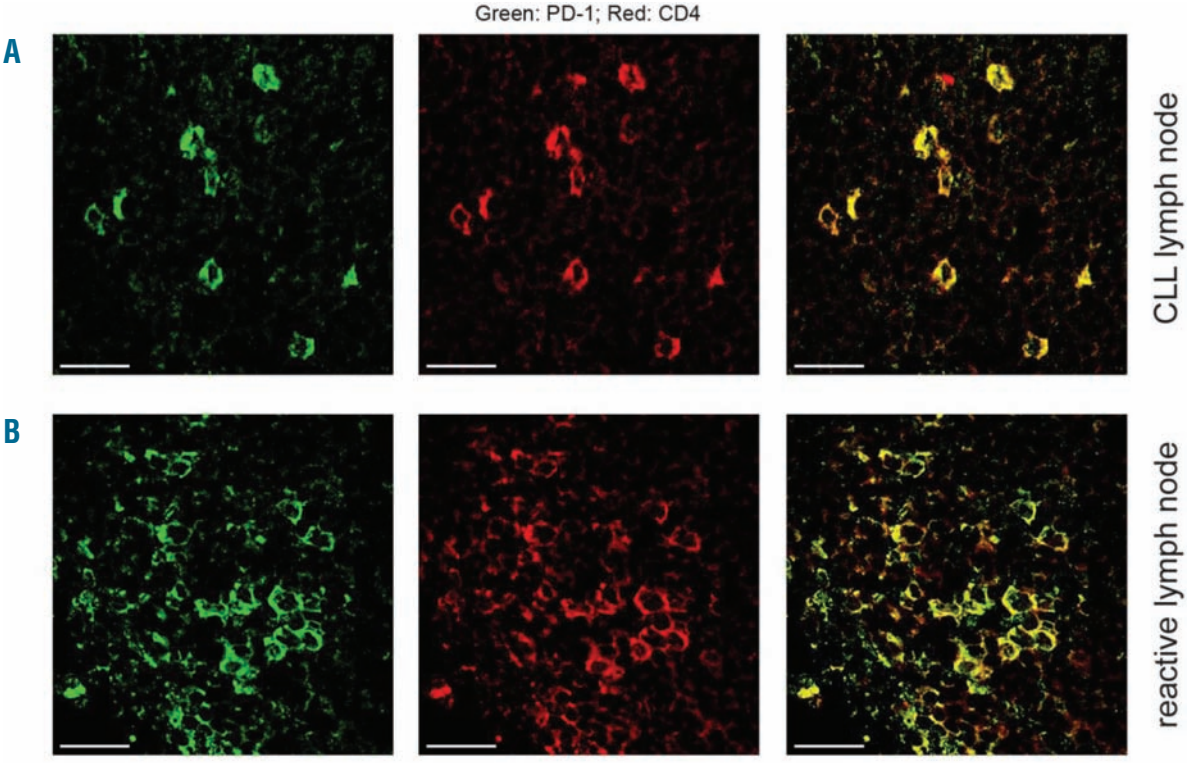
Variable	N. (%)
CLL lymph nodes	20
Median age at diagnosis (years), n.12	64
Binet stage at diagnosis, n. 12	
A	2 (17)
B/C	10 (83)
Treatement, n. 12	
Treated	8 (66)
Genomic aberrations, n. 8	
deletion 11q/17p	4 (50)
deletion 17	0 (0)
deletion 13	0 (0)
trisomy 12	1 (12.5)
normal	3 (37.5)



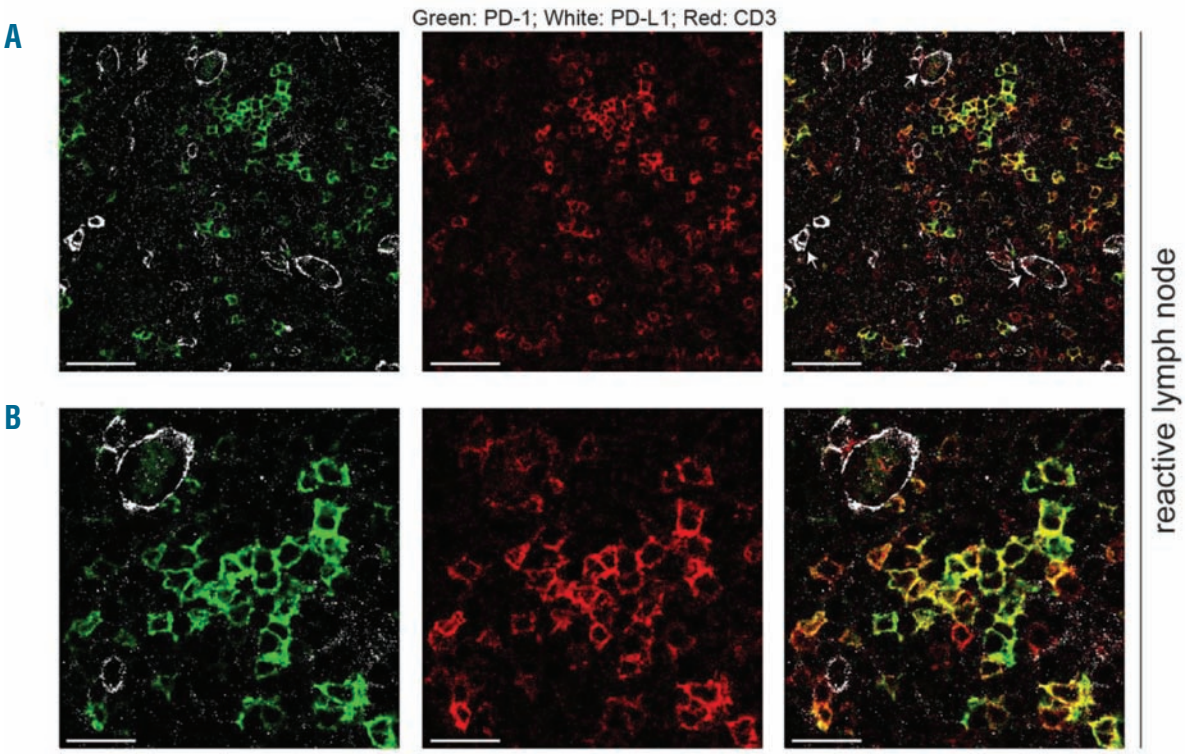
Online Supplementary Figure S1. Sequential gating strategy using multicolor flow cytometry. (A) Lymphocytes from PBMC preparations from CLL patients (left) or age- and sex-matched controls (right) were first identified based on a combination of forward and side scatter (FSC and SSC). $CD4^+$ (B) or $CD8^+$ (C) T-lymphocyte subsets were next gated on the basis of SSC and stained with a specific antibody. Double CD19/CD5 staining (D) was used to gate on $CD5^+$ CLL or CD5- normal B lymphocytes.



Online Supplementary Figure S2. Comparative analysis of PD-1 expression on $CD4^+$ and $CD8^+$ T-cell subpopulations in CLL patients and in age- and sex-matched donors. Cumulative data showing the distribution of PD-1 surface levels in $CD4^+$ (left) or $CD8^+$ (right) lymphocyte subpopulations in 8 CLL patients (gray box plots) and 5 matched controls (open bars).



Online Supplementary Figure S3. Immunofluorescence analysis of PD-1 expression on $CD4^+$ T lymphocytes. (A) Double staining of a representative CLL LN section with anti-PD-1 (green) and anti-CD4 (red) indicates that PD-1 expressing cells are $CD4^+$ T lymphocytes. (B) Double staining of a representative germinal center from a reactive LN tissue section with anti-PD-1 (green) and anti-CD4 (red). Original magnification 63x, zoom factor of 2. Scale bar represents 25 μm. Images were acquired using a TCS SP5 laser scanning confocal microscope (Leica Microsystems) with an oil immersion 63x/1.4 objective lenses equipped with the LAS AF software and processed with Adobe Photoshop (Adobe Systems).



Online Supplementary Figure S4. Immunofluorescence analysis of PD-1, PD-L1 and CD3 expression in reactive lymph node samples. (A-B) Triple staining of a representative reactive LN section with anti-PD-1 (green), anti-PD-L1 (white) and anti-CD3 (red), showing that PD-L1 expressing cells are restricted to endothelial cells (white arrows), while PD-1–positive elements are predominantly $CD3^+$. Original magnification 63x. Zoom factor of 2 for the images below (B). Scale bars represent 50 μm (A) and 25 μm (B). Immunofluorescence was analyzed using a TCS SP5 laser scanning confocal microscope (Leica Microsystems) with an oil immersion 63x/1.4 objective lenses, images were acquired with the LAS AF software and processed with Adobe Photoshop (Adobe Systems).