SUPPLEMENTARY APPENDIX

Lenalidomide interferes with tumor-promoting properties of nurse-like cells in chronic lymphocytic leukemia

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 doi:10.3324/haematol.2014.113217
Manuscript received on July 10, 2014. Manuscript accepted on November 12, 2014.
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Online Supplementary File

Manuscript no. HAEMATOL/2014/113217

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Supplementary Design and Methods

Samples and treatments

Blood samples from untreated CLL patients were obtained from the Hematology Unit of Modena Hospital. To enrich for monocytes, peripheral blood mononuclear cells (PBMCs) were incubated with CD14 Microbeads (Miltenyi Biotec, Germany), obtaining a purity >97%. Lenalidomide was purchased from Celgene (San Diego, CA) and was dissolved in dimethyl sulfoxide (DMSO), which was used as a vehicle control in all experiments. For long-term treatment, NLCs were generated with lenalidomide 0.5μM or vehicle, provided at set up and every 3 days. In some experiments, NLCs were generated from PBMCs and then treated with lenalidomide before evaluations.

Actin polymerization

Actin polymerization was inspected with Rhodamine-Phalloidine-based F-actin Visualization Biochem Kit (Cytoskeleton, Denver, CO, USA) according to manufacturer's instructions. CD14+ monocytes from 9 CLL patients were plated and after 2 hours of adhesion treated with lenalidomide at 0.5, 1 or 10μM for 20 minutes or vehicle. After treatment, monocytes were fixed on coverslip by incubating with fixative solution for 10 minutes and then permeabilized for 5 minutes. After wash, Rhodamine-Phalloidine was loaded on coverslip and incubated for 30 minutes. The actin polymerization was visualized with Leica DMRA2 fluorescence microscopy (Leica Microsystems, Wetzlar, Germany) equipped with a DC350 FX camera and then quantified with ImageJ software v.1.45s by evaluating the fluorescence of every individual cell in 5 different fields at 400X magnification. Results are expressed as mean fluorescence intensity of these 5 fields. In

some experiments, adherent monocytes (n=8) were treated for 30 minutes with Rac1 inhibitor (Calbiochem, EMD Millipore, Merck, Darmstadt, Germany) at 50μM, or Y27632 dihydrochloride, a specific inhibitor of ROCK, at 10μM or GGTI298 trifluoroacetate salt hydrate, a specific Rap1 inhibitor (Sigma-Aldrich, St. Luis, MO, USA) at 10μM.

Chemotaxis assays

Migration assays on CLL monocytes (n=5) treated or not with 0.5μM lenalidomide were performed using 5-μm pore size PET inserts (Millipore, Billerica, MA, USA). After 4 hours, migrated cells were labeled with 4μM Calcein-AM (Sigma-Aldrich) and quantified by Infinite200 reader (Tecan, Männedorf, Switzerland). Recombinant human C-C chemokines CCL2/MCP1 (R&D Systems, Minneapolis, MN, USA) and CCL3/MIP-1α (PeproTech, Rocky Hill, NJ, USA) at 10ng/mL and the CXC chemokine CXCL12/SDF-1α (PeproTech) at 200ng/mL were used as chemoattractants.

Gene expression analysis

Large-scale gene expression profiling (GEP) was performed on total RNA from NLCs treated with lenalidomide 0.5μM or vehicle (control) for 10 days (n=4) extracted by using RNeasy Midi kit Plus (QIAGEN, Valencia, CA, USA). High quality RNAs were amplified and Cy3-labeled using Low Input Quick Amp Labeling kit (Agilent Technologies, Palo alto, CA, USA). Agilent RNA One-Color Spike-In was added in each sample to provide positive controls for monitoring the microarray workflow from sample amplification and labelling to microarray processing. All cRNA products were purified using RNeasy columns (QIAGEN). Samples had to contain at least 6 pmole of cyanine dye/μg of cRNA to be considered suitable for subsequent hybridization. Cy3-labeled cRNA (1.65 μg) were fragmented to an average size of 50-100 nt by incubation at 60°C for 30 min using *in situ* Hybridization kit-plus (Agilent). Samples were hybridized for 17 hours at 65°C on 4×44K Whole Human Genome Microarray (Agilent) and then scanned using laser scanner (Agilent Technologies). Fluorescence data were analyzed with Feature Extraction Software v.10.5 (Agilent Technologies) and QC Chart tool v.1.3. Genes were defined as differentially expressed between groups (control NLCs vs. treated NLCs) at $p<0.05$ (paired t-test) and fold change ± 1.5 by using

Gene Spring GX v12.6 (Agilent) software. Data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://ncbi.nlm.nih.gov/geo/, GSE57861).

For Real-time PCR, RNA (100 ng) was reverse transcribed using Transcription High fidelity cDNA Synthesis kit (Roche Applied Science, Penzberg, Germany). All samples were analyzed in real time on LightCycler 480 v.2 (Roche) in duplicate. Amplification of the sequence of interest was normalized to an housekeeping reference gene (Glyceraldehyde 3-phosphate dehydrogenase, GAPDH) and compared to a calibrator sample (Universal Human Reference RNA; Stratagene, Cedar Creek, TX). Differences in gene expression were determined by comparative method ($2^{-\Delta\Delta CT}$). To exclude non-specific amplification and primer-dimer formation when using SYBR Green, a dissociation curve analysis was performed and then PCR products were tested by agarose gel electrophoresis.

Immunoblotting

Proteins (100µg/lane) were electrophoresed on 4% to 20% SDS-polyacrylamide gradient gels (Biorad laboratories, Hercules, CA, USA). Membranes were immunoblotted with primary antibodies listed in Supplementary Table 2, incubated with species-specific horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare, Uppsala, Sweden) and developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). Images were acquired and analyzed using Image Lab Software v.3.0 (Biorad Laboratories).

Phagocytosis

Phagocytosis was inspected by using CytoSelect™ 96-Well Phagocytosis assay (Cell Biolabs, San Diego, CA, USA). NLCs were generated culturing PBMCs from CLL patients (n=6) for 10 days without lenalidomide in 96-well plates. Then, we washed off CLL cells from adherent NLCs and we treated NLCs with lenalidomide 0.5µM or 1 µM or vehicle (control) for 4 hours before phagocytosis assays. This procedure was used to avoid a bias due to different number of NLCs generated in long-term culture with lenalidomide. After treatment with lenalidomide, opsonized zymosan particles were added to cells for 1 hour. After wash, NLCs were fixed by incubation with fixative solution for 5 minutes. External zymosan particles were blocked and cells were permeabilized.

Zymosan particles engulfed by NLCs were measured by colorimetric detection at 405nm. In some experiments, NLCs were treated with Rap1 inhibitor at 10 μM for 30 minutes followed by treatment with lenalidomide 0.5 μM.

FITC-DEXTRAN uptake and confocal microscopy of NLCs

For confocal microscopy experiments with NLCs, PBMCs from CLL patients were plated on glass coverslips in 24-wells plate in complete medium to generate NLCs as indicated. After 12-14 days, the coverslips were transferred in new wells and incubated for 15 minutes at 37 °C in PBS 5% FCS with 1 mg/ml of FITC-DEXTRAN (Sigma). Coverslips were then fixed (4% paraformaldehyde for 10 minutes at RT), permeabilized (0.1% saponin for 20 minutes at RT), blocked with goat serum (30 min at 4 °C) and incubated with anti-phalloydin-Alexa 568 (Invitrogen Life Technologies, 1:100, 1 hour at 4 °C) or with anti-caveolin-1 (BD Pharmigen, 1:50 overnight at 4 °C) followed by secondary antibody Goat anti-Rabbit-Alexa 594 (Invitrogen Life Technologies, 1:300, 1 hour at 4 °C). Samples were counter-stained with DAPI and mounted in SlowFade Gold reagent (both from Invitrogen). Slides were analyzed using a TCS SP5 laser scanning confocal microscope equipped with 4 lasers (Leica Microsystems, Milan, Italy), images acquired with the LAS AF software and processed with Adobe Photoshop (Adobe Systems, San Jose, CA). Pixel intensity analyses were performed using the ImageJ (downloadable at http://rsbweb.nih.gov/ij/) and the LAS Application Suite (Leica Microsystems) softwares. Mean pixel intensity was calculated by defining a region of interest (ROI) and measuring green fluorescence pixel intensity.

NLCs activation and proliferation

NLCs (n=6) were generated in presence of lenalidomide 0.5μM or vehicle. After 5 days, NLCs activation was monitored using MTT assay (Trevigen, Gaithersburg, MD, USA) according to manufacturer's instructions. Results were normalized to NLCs number in the corresponding condition.

NLCs were cultured in presence of lenalidomide 0.5μM or vehicle control for 5 days, Ki-67 expression and cell cycle were analyzed by flow cytometry. Briefly, cells were detached with 5mmol/L EDTA in Dulbecco's PBS, stained with CD14 APC antibody and permeabilized using cold

70% ethanol for 1 hour at -20°C. Then cells were incubated for 20 minutes with anti-Ki-67 PE antibody or corresponding isotype control (Miltenyi Biotec) and analyzed by flow cytometry.

For cell cycle analysis, NLC cells were collected, suspended in PI cell cycle solution (citrate buffer plus 1 mg/mL PI and 1 mg/mL RNAse A) and incubated in the dark for 30 minutes at 37°C. Then, cells were immediately acquired by flow cytometry excluding debris and selecting single cells according to FL2-A vs FL2-W plot, data were analyzed using FlowJo software (Tree Star Inc.).

For CFSE dilution assay, PBMCs were stained with CFSE 0.5μM (eBioscience). CFSE-labeled PBMCs (n=5) were cultured for 5 days in complete medium either in presence of lenalidomide (0.5μM) or vehicle. Cell division was measured by staining NLCs with APC-conjugated anti-CD14 (Becton Dickinson).

Mixed lineage reaction (MLR)

To perform allogeneic mixed lineage reactions, we generated NLC culturing PBMCs from CLL patients for 10 days without lenalidomide. Then, we washed off CLL cells from adherent NLCs and we treated NLCs with lenalidomide 0.5μM or vehicle (control) overnight. In addition, we isolated T lymphocytes from buffy coats of 5 healthy donors (HD) from the blood bank of Modena Hospital by Pan-T cell isolation kit (Miltenyi Biotec) obtaining a purity >99%. T lymphocytes were stained with CFSE (0.5μM) for 10 minutes at 37°C and then cultured on NLCs in fresh new medium or alone or stimulated with αCD3/CD2/CD28-coated beads (Miltenyi Biotec). The proliferative response was measured after 7 days gating the CD3+ alive cells. Proliferation was defined as the percentage of cells showing low CFSE staining. Data were analyzed using FlowJo software (Tree Star).

Cytokine secretion assay (CSA)

To determine secretion of IL-2, NLCs were cultured for 10 days either in presence or absence of lenalidomide 0.5μM and analyzed using the cytokine secretion assay (CSA) for IL-2, according to the manufacturer's instructions (CSA Detection Kit; Miltenyi Biotec). NLCs, detached with EDTA in Dulbecco's PBS, were immunostained with IL-2 catch reagent and incubated for 1 hour at 37°C to allow cytokine secretion. After washes, cells were labeled with IL-2 Detection antibody conjugated

to APC and CD14 FITC Ab. An isotype control sample for each condition was acquired to exclude autofluorescence background.

Statistical analyses

Data were analyzed using SPSS version 20.0 (SPSS, Chicago, IL, USA). In some experiments, results were normalized on control (100%) (vehicle-treated samples). Normalization was performed by dividing the value of a particular sample treated with lenalidomide to the value of the corresponding sample treated with vehicle DMSO. *P* values were calculated by Student t test (*$p<0.05$, **$p<0.01$). Data are presented as mean and standard error of the mean (SEM) is depicted as error bars.

Supplementary Table 1. Primers used in real-time PCR

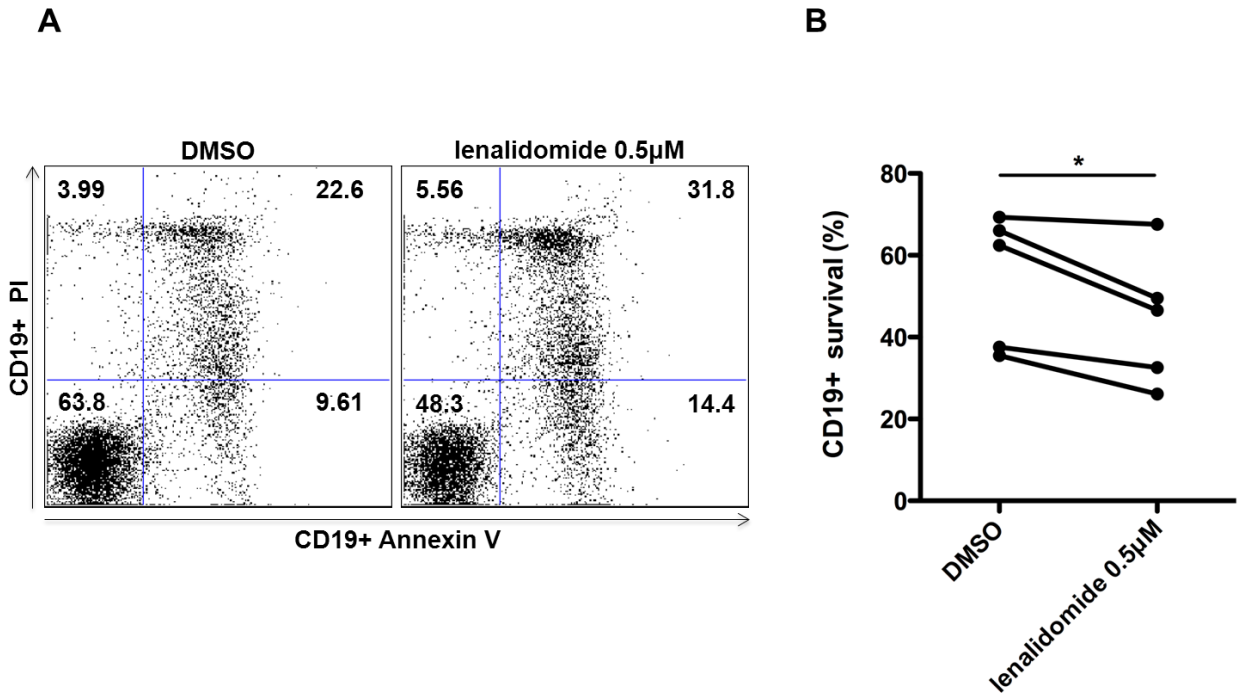
Gene	Forward primer	Reverse primer
CCR1	5'-AGTACCTGCGGCAGTTGTTC-3'	5'-AAGGGGAGCCATTTAACCAG-3'
CCR2	5'-CCCATCATCTATGCCTTCGT-3'	5'-GGCAATCCTACAGCCAAGAG-3'
CXCR4	5'-GGATATAATGAAGTCACTATGGGAAAA-3'	5'-GGGCACAAGAGAATTAATGTAGAAT-3'
CORO1B	5'-TGCGGGCCATCTTCCTGGCAGAT-3'	5'-GGCCATGGGTTCCTCGAGGT-3'
RHOH	5'-TTGATTTCCGGAGTCAGTCA-3'	5'-GTTCTCCACGGCTTCAGTTT-3'
IL10	5'-CATAAATTAGAGGTCTCCAAAATCG-3'	5'-AAGGGGCTGGGTCAGCTAT-3'
IL8	5'-AGACAGCAGAGCACACAAGC-3'	5'-ATGGTTCCTTCCGGTGGT-3'
IDO1	5'-GTGTTTCACCAAATCCACGAT-3'	5'-CTGATAGCTGGGGGTTGC-3'
IL2	5'-AAGTTTTACATGCCCAAGAAGG-3'	5'-AAGTGAAAGTTTTTGCTTTGAGCTA-3'

Supplementary Table 2. Antibodies used in immunoblotting

Antibody	Manufacturer
Anti-Akt rabbit	Cell Signaling Tech, Beverly, MA
Anti-phospho-Akt rabbit	Cell Signaling Tech, Beverly, MA
Anti-Coronin-1B (C terminus) rabbit polyclonal	ECM Biosciences
Anti-RHOH rabbit polyclonal	Abcam
Anti-ILK1 rabbit	Cell Signaling Tech, Beverly, MA
Anti-βactin mouse	Abcam

Supplementary Figures

Supplementary Figure 1. Lenalidomide overcomes NLCs mediated CLL cell protection. (A) PBMCs from 5 CLL patients were cultured for 10 day in presence or absence of lenalidomide. Dot plot exemplifies viability of CD19+ CLL cells in one representative case. (B) Values of CLL viability in presence or absence of lenalidomide are connected by lines (Student T test, *p<0.05, **p<0.01).



Supplementary Figure 2. Lenalidomide stimulates NLCs proliferation. (A) Box plots display Ki67 expression in NLCs treated or not with lenalidomide for 5 days relative to 6 independent experiments. Whiskers depict min and max values (Student t test, **p<0.01). (B) Bar diagrams summarize the percentage of NLCs in each phase of cell cycle in presence or not of lenalidomide for 5 days (Student t test, n=5, *p<0.05, **p<0.01). (C) Displayed are cell cycle profiles of NLCs after 5 days in two representative samples.