SUPPLEMENTARY APPENDIX

Aminobisphosphonates prevent the inhibitory effects exerted by lymph node stromal cells on anti-tumor Vδ 2 T lymphocytes in non-Hodgkin lymphomas

Alessandra Musso,[1] Silvia Catellani,[2] Paolo Canevali,[3] Sara Tavella,[4] Roberta Venè,[1] Silvia Boero,[1] Ivana Pierri,[2] Marco Gobbi,[2] Annalisa Kunkl,[5] Jean-Louis Ravetti,[5] Maria Raffaella Zocchi[3]* and Alessandro Poggi[1]*

[1]Unit of Molecular Oncology and Angiogenesis, IRCCS AOU San Martino-IST, Genoa; [2]Laboratory of Hematology and Clinical Hematology, IRCCS AOU San Martino-IST, Genoa; [3]Division of Immunology, Transplants and Infectious Diseases, San Raffaele Scientific Institute, Milan; [4]Department of Experimental Medicine (DIMES), University of Genoa, and IRCCS AOU San Martino-IST, Genoa; and [5]Unit of Pathology, IRCCS AOU San Martino-IST, Genoa, Italy

**MRZ and AP contributed equally to this work.*

 doi:10.3324/haematol.2013.097311
Manuscript received on August 28, 2013. Manuscript accepted on October 18, 2013.
Correspondence: alessandro.poggi@istge.it

SUPPLEMENTAL METHODS

Patients. Fortyeight patients, diagnosed with non Hodgkin's lymphoma (NHL, 30 follicular, FL, and 18 diffuse large B cell lymphomas, DLCL, Suppl. Table 1) according to the WHO classification[32] at the Department of Oncology and Hematology, University of Genoa, were analyzed between January 2010 and August 2012. Lymph node (LN) bioptic specimens were obtained under conventional diagnostic procedures, provided informed consent and the study was approved by the institutional ethical committee (IRB approval 0026910/07, renewal 03/2009 and 12/2010). Paraffin-embedded LN samples were processed at the Pathology Department of the IRCCS AOU San Martino-IST in Genoa. Fifteen sentinel LN free of neoplastic disease, were also studied.

Monoclonal antibodies (mAbs) and reagents. The VioBlue-conjugated anti-CD3 monoclonal antibody (mAb), was from Miltenyi (Bergisch Gladbach, Germany). The anti-CD45RA, APC-anti-CD27, PE- or APC-anti-CCR7 mAbs were purchased from Biolegend Inc. (San Diego, CA). The anti-SH2 (CD105), the anti-SH3 (CD73a), producing hybridomas were from the American Type Culture Collection (ATCC, Manassas, VA). The anti-prolyl-4-hydroxylase (PH4) mAb (5B5) was from Dako Italia (Milan, Italy), the anti-NKG2D (MAB139) mAb from R&D System (Milan, Italy), the anti-trans-glutaminase (TG2) from Thermo Scientific (Fremont, CA), the FITC-goat anti-rabbit (GAR) antiserum was from Zymed, Life Technologies (Monza, Italy), while the FITC-anti-Vδ2 mAb γδ123 (IgG1, Miltenyi, Bergisch Gladbach, Germany).[23] The anti-MIC-A mAb AMO1 was from Immatics Biotechnologies (Tubingen, Germany) and the anti-ULPB3 mAb M551 was kindly provided by Amgen (Seattle, WA, M.T.A n.200309766-001).

Complete medium was composed of RPMI1640 (Biochrom, Berlin, Germany) with 10% fetal calf serum (FCS) supplemented with penicillin, streptomycin and L-glutamine (Biochrom); medium specific for LNMSC was prepared as described.[31] The carboxy-fluorescein diacetate succinimidyl ester (CFSE) was from Molecular Probes (Eugene, OR). Pamidronate (Pam) and mevastatin (Meva)

were from Sigma Chemicals Co. (St. Louis, MO) and recombinant IL2 (rIL2) from PeproTech EC (London, UK). Zolendronic acid was kindly provided as sodium salt (zoledronate, Zol) by Novartis Pharma (Basel, Switzerland, MTA 37318).

Isolation of LNMSC and co-cultures. LNMSC were obtained from LN fragments of NHL patients and cultured as described.[31] Vδ2 T cells were isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors using the anti-Vδ2 BB3 mAb[23] and EasySep custom kit (Stemcell Technologies, Vancouver, Canada) according to manufacturer's instructions. This method was chosen as it allows recovery and purity of Vδ2 T cells of >99%, while negative separation, in our hands, was not so efficient in isolating highly purified Vδ2 T cells (purity about 85%, recovery 75%). Indeed negative selection is based on the use of different antibodies, including some directed against molecules, such as CD8, CD56 and CD16, shared by a fraction of Vδ2 T cells with other T or non-T cell populations, so that a part of the Vδ2 effector T cells can be loss.

Cells were cultured in medium supplemented with 10ng/ml IL2, with or without LNMSC; for the autologous system, LN cell suspensions were frozen until LNMSC were obtained from the same specimens, then thawed and used for autologous co-cultures. LNMSC were obtained by culturing LN cell suspensions from NHL patients in six well plates ($5x10^6$ cells/well) in RPMI 1640 complete medium.[30,31] After 3d, non adherent cells were washed away and adherent cells cultured for additional 7d. On day 10 cells were transferred into $25cm^2$ flasks and expanded as described.[30,31] LNMSC expressed SH2/CD105, SH3/CD73, PH4, TG, MIC-A, ULBP3 (Suppl. Fig.1), CD90, HLA-I, collagen, vimentin, bone sialoprotein, osteopontin, SH4/CD73b, CD44, β1-integrin/CD29, ICAM1/CD54, alkaline phoshatase, but not CD45, CD31, CD34, CD33, CD3, CD2, CD16, CD14, ICAM2, ICAM3, CD80, CD86, CD83, and HLA-DR (not shown). This phenotype was superimposable in all the LNMSC derived from the 48 patients, with only slight differences in the intensity of expression, and was stable during culture.

In some experiments, LNMSC were pre-treated for 12h with Pam (5μM) or Zol (1μM); in other experiments, LNMSC were incubated with Meva (10μM) for 48h before adding Zol (1μM) during the last 12h; doses and time points were selected on the basis of preliminary experiments (Suppl. Fig.1 and Suppl. Fig.2). The doses of NPBs have been chosen as effective on γδ T cell proliferation (as determined in preliminary experiments) and absence of toxic effects according to the literature.[5,20] Co-cultures were performed using different ratios between LNMSC and Vδ2 T lymphocytes (1:5 to 1:50); the ratio of 1:10 was chosen, on the basis of previously published data.[31] After co-culture Vδ2 T cells were recovered (on day 5 for cytotoxicity, on day 5-10-14 for phenotype and proliferation) by gently harvesting non-adherent cells (LNMSC left adherent to plastic culture plates) and the purity assessed by FACS analysis using the FITC-anti-Vδ2 mAb γδ123 (>95%). In some experiments, the anti-TGFβ mAb (5μg/ml, clone 1D11, R&D System Inc. Milan, Italy) was added to the co-cultures. On day 5 or 10 (14 for autologous co-cultures), Vδ2 T cell proliferation or phenotype were evaluated. Proliferation was measured, after labelling of Vδ2 T cells with 1μM CFSE, by FACS analysis calculating the logarithmic decrease of green fluorescence intensity; data were analyzed using the Modfit 3.1 computer program (Verity Software House, Topsham, ME). To evaluate the effect of N-BPs on LNMSC viability, we performed the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, $2x10^4$ LNMSC were cultured in flat-bottom microwells in the absence or presence of N-BPs (Pam 10-5μM or Zol 10-5-1μM) for 6, 12 or 24h. Then, 10 μl/well of 5mg/ml MTT solution (Sigma) were added and incubated for 4h at 37°C in the dark, lysed and further incubated at 37°C for 1h. Absorbance at 570nm was measured using Victor X5 plate-reader (PerkinElmer Ltd., Beaconsfield, GB).

Cytofluorimetric analyses and cytokine measurement. Immunofluorescence on LNMSC or on Vδ2 T cells was performed as described.[31] For the identification of Vδ2 T cell subpopulations, the following mAbs were used: VioBlue-anti-CD3, FITC-anti-Vδ2 γδ123, anti-CD45RA followed by

FITC-conjugated anti-isotype GAM, APC-anti-CD27. Control aliquots were stained with FITC-, PE- or APC-labeled isotype-matched irrelevant mAbs. Samples were analyzed by CyAn ADP flow cytometer (Beckman Coulter Inc., Brea, CA). Results are expressed as log of mean fluorescence intensity (MFI) or percentage of positive cells, or as the ratio between the MFI of each sample and the negative control, as indicated in the figure legend.

Quantification of TGFβ, IL15, IL10, TNFα or IFNγ in the supernatants (SN), harvested from co-cultures at 48h, was performed by ELISA (eBioscience Inc., San Diego, CA or PeproTech EC, London, UK, respectively), compared to a standard curve of the respective cytokine and expressed as pg/ml/10^6 cells.

Cytotoxicity assay. Cytolytic activity of Vδ2 T cells, always washed before using in this assay, was analyzed in a 4-hour ^{51}Cr-release assay against the C1R human lymphoid cell line or the MICA transfected C1R-MICA cell line (kindly provided by Alexander Steinle, University of Frankfurt, Germany) labeled with ^{51}Cr, at an E:T ratio of 10:1, in V-bottomed microwells. Some experiments were also performed using the LYB8 and KARPAS lymphoma cell lines (ATCC) or untreated or N-BPs-pre-treated LNMSC as targets. In some samples, Vdelta2 T cells were exposed to saturating amounts (5μg/ml) of anti-NKG2D mAb before adding the C1R or the C1R-MICA$^+$ targets. One hundred microliters of supernatant were measured in a gamma counter and the percentage of ^{51}Cr-specific release was calculated as described previously. In some samples the effector cells were exposed to saturating amounts (5μg/ml) of the anti-NKG2D mAb at the onset of the cytotoxicity assay.[23] Redirected killing was performed using as target the P815 murine cell line in the presence of the anti-NKG2D or the anti-CD16 mAb (VD4, IgG1, 5μg/ml) as described.[33] An unrelated mAb, matched for the isotype (BD Pharmingen, BD Italia, Milan, Italy), was used as control. ADCC assay was performed using the anti-CD20 therapeutic antibody rituximab (20μg/ml, RTX, Roche, Les Ulis, France) and the CD20$^+$ lymphoid cell lines C1R or C1R-MICA$^+$ as target cells. In some

experiments, Vδ2 T cells were used as effector cells after 5 days of co-culture with either untreated or pre-treated LNMSC (12h with Pam 5μM or Zol 1μM).

Cytoskelekton analysis by confocal microscopy. LNMSC were pre-incubated with 10μM mevastatin for 48h; after extensive washes, LNMSC were analyzed for morphology and cytoskeleton rearrangement. In some experiments, 1mM of L-mevalonate (solubilized in ethanol 1:1000), the metabolic product of HMG-CoA reductase, was added at the onset of the treatment with mevastatin. Control cells were incubated with solvents of the drugs. Imgaes of LNMSC were taken on the inverted microscope IX71 with the ORCA camera (Hamamatsu, Japan) Perimeter and area of LNMSC were evaluated using the image analysis program Analysis SIS FIVE associated to the CELLr image system (Olympus, Gernmany) and results are reported as μm or μm^2 respectively. For analysis of actin rearrangement, $2.5x10^4$ LNMSC were seeded on 0.2mm thin round glass cover slides and incubated for 48h with one or another combination of drugs. Then, cells were fixed with formaldehyde 1% and permeabilized with 0,1% Triton-X-100. Each slide was stained with AlexaFluor488-conjugated phalloidin to stain actin for 30min at 4°C, washed and analyzed by FV500 (Fluoview confocal Laser Scanning Microscope System, Olympus Europe GMBH, Hamburg, Germany) equipped with an Argon laser to excite AlexaFluor488, associated to a IX81 motorized microscope (Olympus). Samples were observed with PlanApo 40x NA1.35 oil objectives and data analyzed with FluoView 4.3b computer program (Olympus). Results are shown in pseudocolor.

RNA extraction. RNA was extracted either from cultured LNMSC or Vδ2 T cells or from paraffin-embedded sections (8μm thick) of LN obtained from NHL patients (18 DLCL and 30 FL) and 15 normal LN.[31] Tissue sections were fixed on PEN membrane glass slides (MDS Analytical Technologies, Germany). Additional 4μm thick sections were stained with hematoxiline-eosine to

appreciate morphology. Then tissue sections were dried at RT under a chemical safety hood for 5min, dipped in xylene for 10min twice for each sample, followed by a three-step immersion in 100%-95%-75% ethanol solution. Samples were then washed in DEPC RNAse-free water for 1 min, treated with a staining solution (Histogene Arcturus, Life Technologies, Carlsbad, CA) and dipped in 100%-95%-75% ethanol solution for 30sec each passage followed by xylene for 5min. Tissue sections were then dried at RT. Some samples were processed as whole slides and total RNA was extracted with the Paradise TM Reagent System (Arcturus, Life Technologies) after incubation with proteinase K for 4-6h at 56°C. A DNAse treatment step was included. RNA was diluted in 50µl elution buffer, according to the manufacturer's protocol and quantified by NanoDrop Spectrophotometer (ND-1000 Celbio, Milan, Italy) and by Qubit TM fluorometer using the Quant-it TM Assay Kit (Life Technologies).

cDNA Reverse Transcription and Quantitative Real-Time PCR (Q-RT-PCR). RNA was extracted as described.[31] cDNA synthesis was performed with random hexamers by the use of the High Capacity Archive Kit (Life Technologies). The following primers and probes were purchased from Applied Biosystems (Foster City, CA, USA): IL4 Hs00174122_m1, IL10 Hs00961619_m1, IL12 Hs01011519_m1, IL15 Hs01003716_m1, TGFβ Hs00998130_m1, interferon (IFN)γ, Hs00989291_m1, tumor necrosis factor (TNF)α Hs00236874_m1, GATA3 Hs00231122_m1, TXB21 Hs00203436_m1, STAT1, STAT5 Hs00559643_m1, STAT6 Hs00598528_m1□. To verify RT-Q-PCR efficiency, decreasing amounts (50ng, 10ng, 0.1ng) of normal RNA were used for threshold cycle (C_T) titration. Q-RT-PCR was performed on the 7900HT FastRT-PCR system (Applied Biosystems) with the fluorescent Taqman method. mRNAs were normalized to RPLP0 as a control gene and referred to a standard curve (Ipsogen, Marseille, France). After subtracting the C_T value for RPLP0 from the C_T values of the target genes, results were expressed as ΔC_T. [34,35]

Statistical analysis. Data are presented as mean±SD. Statistical analysis was performed using two-tails student's *t* test. The cut-off value of significance is indicated in each legend to figure.

SUPPLEMENTAL RESULTS

Effects of N-BPs on LNMSC. N-BPs doses and time points were selected on the basis of preliminary experiments, showing that N-BPs did not change LNMSC phenotype (Suppl. Fig.1) and were not toxic for these cells as demonstrated by the MTT assay (Suppl.Fig.2). In addition, we found that N-BPs-treated LNMSC were not lysed by ex-vivo isolated Vδ2 T cells (Suppl.Fig.2D), as assessed in a standard 4h chromium release assay; although not shown, no evident LNMSC damage effect was detected in the following 36-48, in terms of morphological changes, and detachment from the substrate evaluated by colorimetric assay. On the other hand, IL2-cultured Vδ2 T cells could exert a slight cytolytic effect on LNMSC (15% or 10% at E:T ratio of 20.1 or 10:1 respectively); this cytolytic effect increased when N-BPs-treated LNMSC were used as target cells (20% vs 10% at 10:1 E:T ratio for LNMSC incubated with PAM or ZOL compared to untreated cells, Suppl.Fig.2E).

Suppl.Table 1. Characteristics of NHL patients.

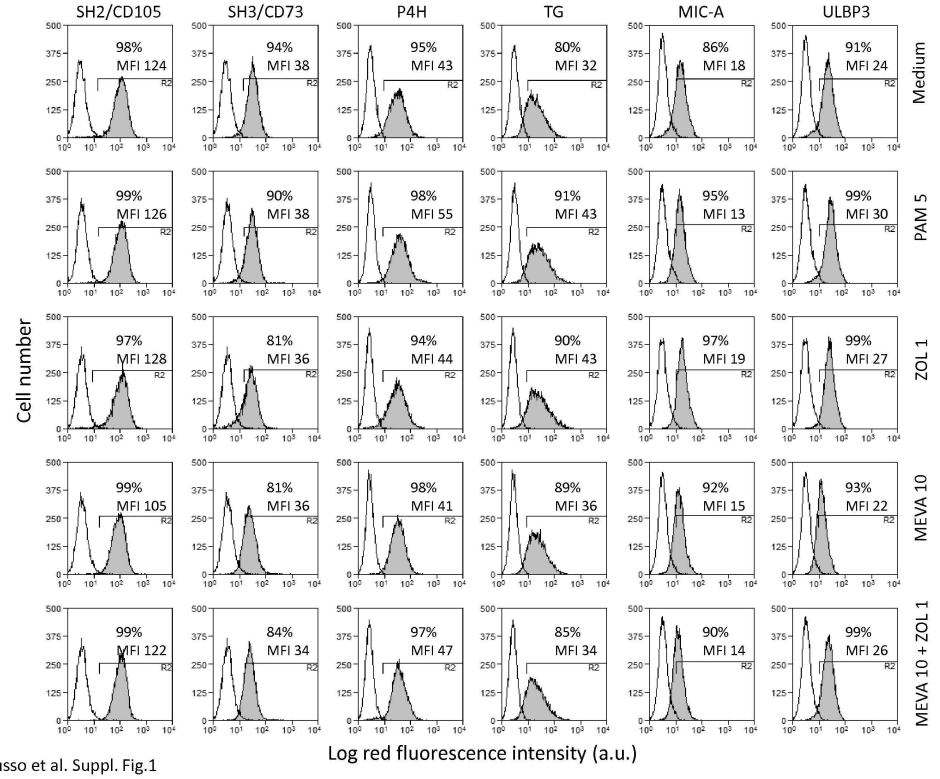
Age Median (range)	49 (20-69)
Sex Males/Females	23/25
Histology	
Follicular (FL)	30
Diffuse Large B Cell Lymphomas (DLCL)	18
Site	
Cervical	15
Axillary	15
Inguinal	18

Forty-eight patients with NHL classified according to the WHO classification[32] were analyzed. The diagnosis was assessed by immunostaining fixed sections of diagnostic node.

LEGEND TO SUPPLEMENTAL FIGURES

Supplemental Figure 1. N-BPs do not alter the phenotype of LNMSC.

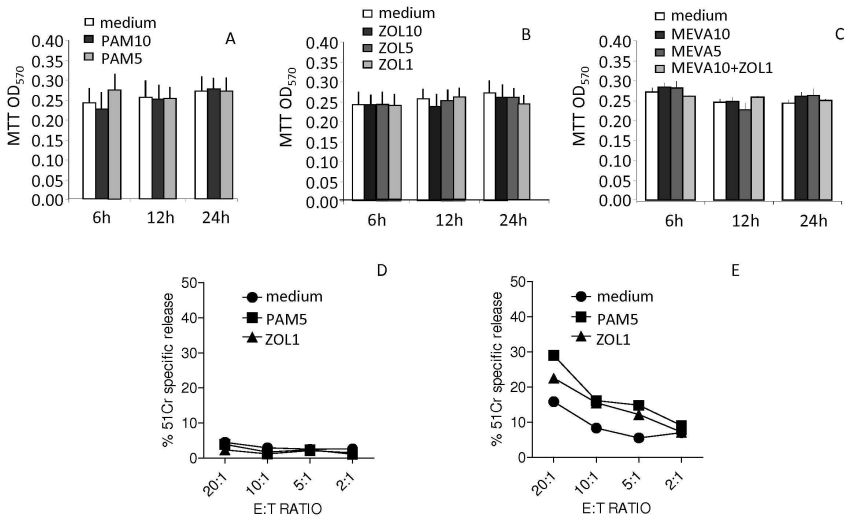
Phenotype was assessed on LNMSC, either untreated (medium) or pre-treated for 12h with Pam (5μM) or Zol (1μM) or Meva (10μM) or Meva 10μM plus Zol 1μM, as indicated, by indirect immunofluorescence using the anti-SH2/CD105, SH3/CD73a, P4H, TG, MIC-A or ULBP3 specific mAbs followed by PE-GAM and FACS analysis. Empty histograms: cells labelled with an unrelated mAb matched for isotype; grey histograms: cells stained with the indicated mAbs. In each subpanel are shown the % and MFI of positive cells. Results are shown as Log red fluorescence intensity (a.u.) vs number of cells. One representative experiment out of six.



Musso et al. Suppl. Fig.1

Supplemental Figure 2. Effects of N-BPs on LNMSC viability and susceptibility to Vδ2 effector T cells.

Panels A-C: MTT assay of LNMSC, either untreated (white columns, medium) or pre-treated for 6, 12 or 24h with Pam (10 or 5μM, A) or Zol (10, 5, 1μM, B), or Meva (10, 5μM, C) or Meva 10μM plus Zol 1μM (C) for 6h, 12h or 24h as indicated. Results are expressed as OD_{570}. Mean±SD from 6 experiments. Panels D-E: untreated (medium) or N-BPs-pre-treated (12h) LNMSC (PAM5, ZOL1) were labelled with ^{51}Cr and used as targets with ex-vivo isolated (D, in the presence of 10ng/ml of IL2 as in co-culture experiments) or IL2 cultured (15 days, E) Vδ2 T cells as effectors, at the indicated E:T ratios. Results are shown as % ^{51}Cr specific release and are representative of results from 4 independent experiments.

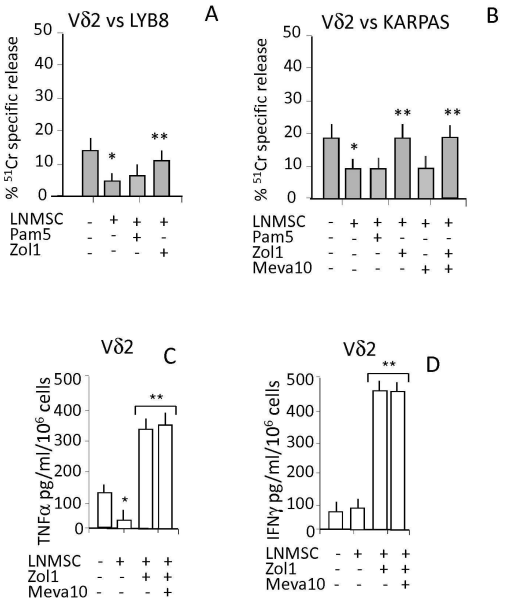


Musso et al. Suppl. Fig.2

Supplemental Figure 3. Zoledronate prevents the inhibition of Vδ2 T cell-mediated lymphoma cell killing induced by LNMSC.

Vδ2 T cells isolated from PBMC were cultured with IL2 alone or with IL2 and either untreated LNMSC or LNMSC pre-treated for 12h with Pam (5μg/ml) or Zol (1μg/ml), or Meva (10μM) or Zol+Meva, as indicated, at the 1:10 LNMSC:Vδ2 T ratio. On day 5, Vδ2 T cells were harvested and used in a standard 4h cytolytic assay against the LYB8 (panel A) or the KARPAS (panel B) lymphoma cell lines, at the effector-target (E:T) ratio of 10:1. Results are shown as % ^{51}Cr specific release and are the mean±SD from 3 experiments; in each panel: * $p<0.01$ vs. Vδ2 T cells cultured without LNMSC; ** $p<0.01$ vs Vδ2 T cells co-cultured with untreated LNMSC.

Panels C and D. TNFα (panel C) or IFNγ (panel D) measured by ELISA in the SN (harvested on 48h) of Vδ2 T cells cultured alone (first column) or co-cultured with untreated LNMSC (second column) or LNMSC pre-treated with Zol (1μg/ml, third column) or with mevastatin for 48h (10μM) and incubated with Zol during the last 12h (1μM) (fourth column). Results are expressed as pg/ml/10^6 cells and are the mean±SD from 4 experiments. *$p<0.001$ vs. Vδ2 T cells alone (first column); **$p<0.001$ vs. untreated LNMSC-Vδ2 T cell co-cultures (second column). Statistical analysis: two-tails student's *t* test.

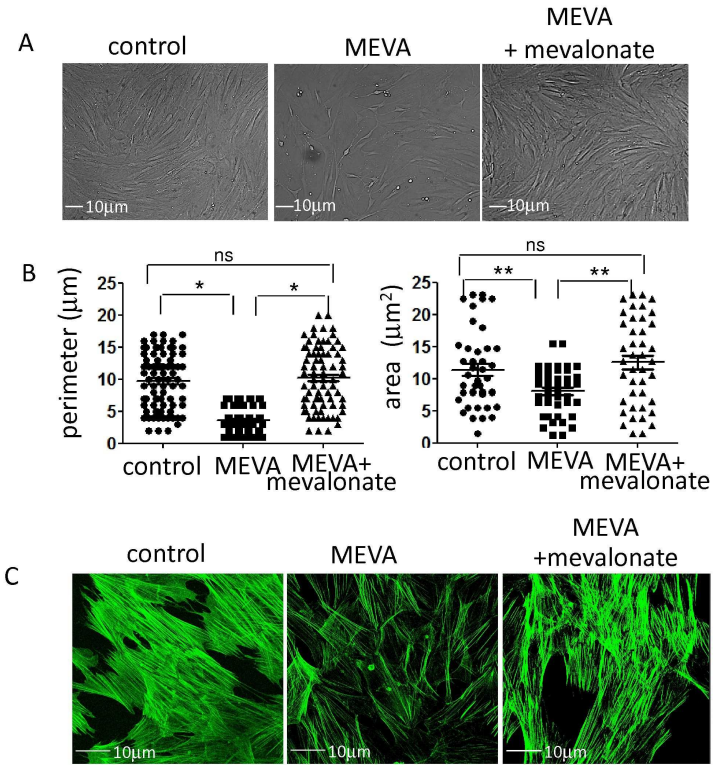


Musso et al. Suppl. Fig.3

Supplemental Figure 4. Effect of mevastatin on LNMSC morphology and actin distribution.

Panel A: LNMSC were cultured in solvent of drugs (control) or with 10μM mevastatin alone (MEVA) or with 1mM L-mevalonate (MEVA+mevalonate) for 48hr and analyzed under the microscope in bright field with Olympus IX71 microscope (200x magnification). Panels C: Perimeter (left) and area (right) of LNMS shown in panel A; data are expressed as μm (left subpanel) or μm^2 (right supanel) using the Analysis SIS FIVE computer program. Each symbol indicate the value of at leat 40 cells analyzed. Horizontal bars indicate the mean of each culture condition. Results are representative of three independent experiments. *$p<0.001$ MEVA vs control or MEVA vs MEVA+mevalonate; ** $p<0.05$ MEVA vs control or MEVA vs MEVA+mevalonate; ns: not significant. Statistical analysis: two-tails student's *t* test.

Panel C: LNMSC incubated with mevastatin or mevastatin and L-mevalonate as in panel A were analyzed by confocal microscopy (400x magnification, Olympus IX81 microscope, objective 40xoil, NA1.40) for the expression and distribution of actin labelled with phallotoxin Alexa488 shown in green pseudocolor.



Musso et al. Suppl. Fig.4