Extracellular vesicles mediate the communication between multiple myeloma and bone marrow microenvironment in a NOTCH dependent way

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SUPPLEMENTARY INFORMATION

INVENTORY

Supplemental Information contains the Supplemental Data (six figures, one table and two supplemental videos) and Supplemental experimental Procedures.

Supplemental Data:

Figure S1 is related to Fig. 2

Figures S2 and S3 are related to Fig.3

Figure S4 and S5 are related to Fig.4

Figure S6 is related to Fig. 5

Table S1 is related to Fig. 6

Supplemental videos (video 1 and 2) are related to Fig. 2

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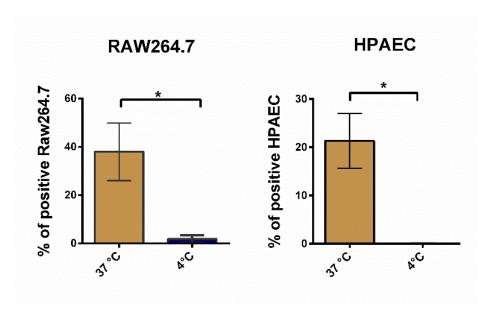


Fig. S1. Flow cytometry analysis of EV uptake in osteoclast progenitors and endothelial cells. Flow cytometry analysis mean percentage values of CM-DIL positive Raw264.7 cells and HPAEC after treatment with CM-DIL labeled RPMI8226-EV at 37°C and 4°C. The data are normalized on the negative control. Data are presented as mean values of three independent experiments. Statistics are by two-tailed Student t-test: *=p<0.05.

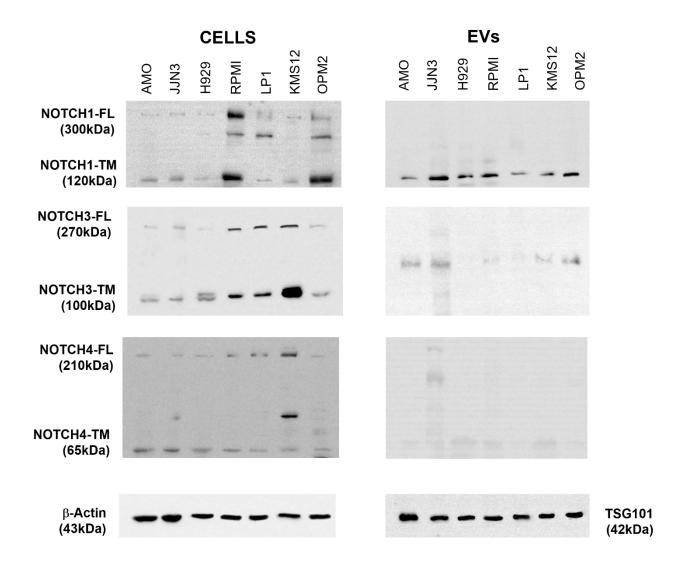


Fig.S2. Western blot analysis for NOTCH1, NOTCH3 and NOTCH4 in HMCL: Western blot analysis for NOTCH1, NOTCH3 and NOTCH4 expressed in 7 different HMCL and the respective shed EV. β -Actin and TSG101 have been used as loading controls for cells and vesicle protein extracts, respectively.

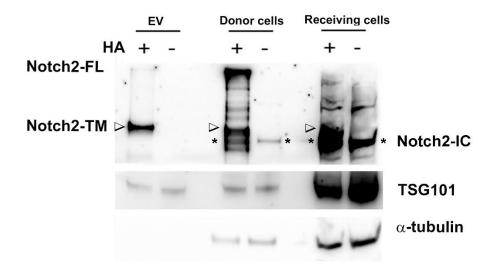


Fig.S3. EV-mediated cell-to-cell transfer of NOTCH2-IC: the membrane of Western blot for NOTCH2-HA shown in Fig.2C was re-hybridized with anti-NOTCH2-IC antibody. Although this antibody cannot distinguish between endogenous NOTCH2-IC and NOTCH2-IC tagged with HA, the signals detected in the negative controls of donor and receiving cells allowed us to identify which among the bands in Fig.2C revealed with the anti-HA antibody represents the NOTCH2-IC form. The identified bands of NOTCH2-IC have been reported here (as well as in Fig.2C) with asterisks. For a better comprehension the bands of NOTCH2-TM revealed by the previous hybridization of this membrane with the anti-HA antibody have been marked with a triangle. By comparing the images in Fig.2C and Fig.S3, we could clarify that in this cell system, NOTCH2-IC is not carried by EV cargo, although it is present in donor cells. On the other side a small amount of transferred NOTCH2-HA seems to be activated when reaching the receiving cells.

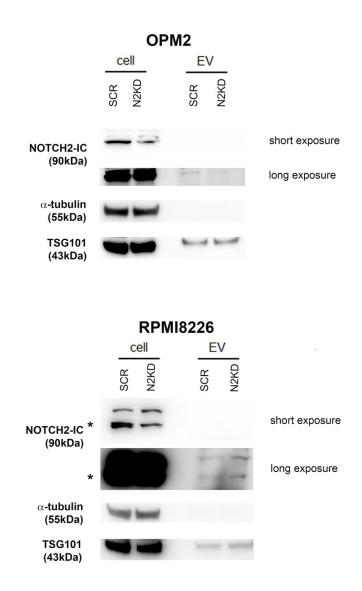


Fig.S4 Immunoblot analysis of NOTCH2-IC on HMCL knockdown for NOTCH2. Western blotting revealed a decrease in NOTCH2-IC in both cell types upon NOTCH2 lentiviral silencing and a clear reduction in OPM2-derived MM-EV, while no significant differences are detectable in RPMI8226-derived EV. EV protein extract loading was half as cell extracts, therefore, to detect NOTCH2-IC in cell lysate a short exposure is shown, while a long exposure is shown to detect it in EV extracts. An asterisk indicates the NOTCH2-IC band in protein extracts from RPMI8226 cells and EV to distinguish it from the aspecific band above. α -Tubulin and TSG101 have been used as loading controls for cell and vesicle protein extracts, respectively.

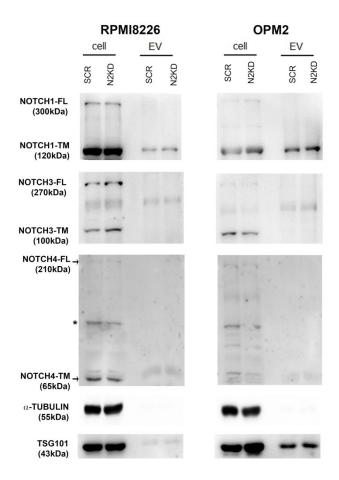


Fig.S5 Immunoblot analysis of NOTCH1, NOTCH3 and NOTCH4 on HMCL knockdown for NOTCH2. Western blotting revealed no differences in the protein levels of NOTCH1, NOTCH3 and NOTCH4 in HMCL and the shed MM-EV upon NOTCH2 lentiviral silencing. Equal amounts of total protein for each sample were loaded. α -Tubulin and TSG101 have been used as loading controls for cell and vesicle protein extracts, respectively. The arrow indicates NOTCH4-FL. Asterisk shows non-specific band detected by the antibody.

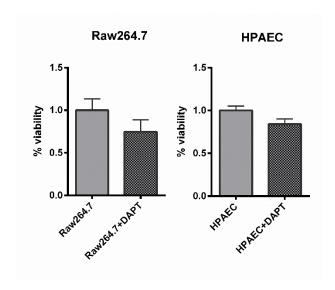


Fig. S6 Viability assay of OCL and EC upon DAPT treatment. MTT assay on Raw264.7 cells and HPAEC treated with DAPT 50μ M. The data were expressed as percentage of mean values +/- SEM of three independent experiments. The statistical analysis carried out with one-tailed t-test did not detect any statistically significant difference.

	All patients		MGUS		MM	
Patients, no. (M/F)	18	(10/8)	6	(3/3)	12	(7/5)
Median age, y (IQR)	74	(16)	74	(16)	77	(15)
Median BM PC, % (IQR)	37.5	(57.0)	8.0	(3.0)	57.5	(30)
Median M-protein, g/dl (IQR)	2.33	(2.99)	0.90	(0.78)	3.57	(2.07)
Median U-protein, g/24h (IQR)	0.35	(0.43)	0.17	(0.25)	0.50	(0.98)
Median sFLC, mg/L (IQR)	397.5	(1083.1)	132.2	(318.2)	605.0	(1244.1)
Median B2-MG, mg/L (IQR)	3.77	(3.80)	2.30	(1.40)	4.30	(3.56)
Median calcemia, mg/dl (IQR)	9.5	(8.0)	9.6	(0.3)	9.0	(1.0)
Median WBC, x 10 ⁹ /L (IQR)	5.00	(2.78)	4.75	(2.78)	5.21	(2.42)
Median Hb, g/dl (IQR)	12.2	(4.2)	13.1	(1.3)	10.3	(3.8)
Median PLT, x 10 ⁹ /L (IQR)	195	(75)	188	(46)	207	(123)

Table S1. Clinical characteristics at presentation of patients. B2-MG: B2-microglobulin; BM PC: bone marrow plasma cells; Hb: hemoglobin; IQR: interquartile range; M-protein: seric monoclonal protein; MGUS: monoclonal gammopathy of undetermined significance; MM: multiple myeloma; PLT: platelets; sFLC: involved seric free light chains; U-protein: urinary monoclonal protein; WBC: white blood cells

Supplemental videos 1 and 2 (provided as separate files). Fluorescence microscopy analysis of EV uptake in bone marrow cells. Video stack analysis of the EV internalization in Raw264.7 cells (video 1) and HPAEC cells (video 2) treated with CM-DIL positive MM-EV performed by z-stack imaging.

Supplemental experimental procedures

Cells and treatments

The human MM cell lines (HMCL), AMO1 (ACC-538), JJN3 (ACC-541), KMS12 (ACC-551), LP1 (ACC-41), OPM2 (ACC-50) were purchased from DSMZ collection of microorganisms whereas RPMI8226 (ATCC® CCL-155) and H929 (ATCC® CRL-906) were purchased from cell cultures and the American Type Culture Collection. HMCL were cultured in RPMI1640 (Euroclone, Italy) supplemented with 10% Fetal bovine serum (FBS) (Euroclone, Italy), 100U/ml penicillin/streptomycin (Microgem, Italy) and 2mM L-glutamine (Microgem, Italy).

The primary human pulmonary artery endothelial cells (HPAEC, ATCC® PCS-100-022) were purchased from the American Type Culture Collection and cultured in Vascular Basal medium (ATCC® PCS-100-030TM) supplemented with Endothelial cells Growth Kit-VEGF (ATCC® PCS-100-041TM) following the manufacturer instruction.

Human embryonic kidney 293 cells, HEK293T (ATCC® CRL-157), HeLa cells (ATCC® CCL-2™), and the murine pre-osteoclasts Raw264.7 (ATCC® TIB-71) were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; Euroclone, Italy) with 10% FBS, 100U/ml penicillin/streptomycin and 2mM L-glutamine.

Recombinant human Receptor Activator of Nuclear Factor κ B (RANKL) (Immunotools, Germany) was resuspended in phosphate buffered saline (PBS) supplemented with 0,1% w/v Bovine Serum Albumin (BSA) and used at 30ng/ml for 7 days as reported. N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl ester (DAPT; Merck, Germany), solubilized in dimethylsulphoxide (DMSO), was used at a final concentration of 50 μ M.

Isolation and treatment with of extracellular vesicles

MM cell lines were seeded at a density of 3x10⁵ cells/ ml in RPMI1640 with 10% EV-depleted FBS previously obtained with 16 h ultracentrifugation at 110,000 g at 4°C. After 48 h, cell debris and aggregates were removed by centrifugation at increasing speeds 1,000 g, 2,000 g, and 3,000 g for

15 min at 4°C and EV were collected by 75 min ultracentrifugation at 110,000 g at 4°C using a Himac CP100NX ultracentrifuge (Himac, Japan)

The obtained pellet was resuspended in 0,1µm filtered PBS for characterization of size and concentration by Nanoparticles Tracking Analysis (NTA) or for the analysis by transition electronic microscopy (TEM). For western blot analyses EV were resuspended in Radioimmunoprecipitation assay (RIPA) buffer and quantified using a Bradford assay (Himedia, Italy). For functional assays, EV were resuspended in serum-free medium RPMI1640. For osteoclastogenesis and angiogenesis, we used an amount of EV isolated from one equivalent volume of the conditioned medium of producing HMCL, or half volume in the case of EV purified by BM aspirates, while for NOTCH reporter assay *in vitro* EV were concentrated 40 times. The ultracentrifuged fresh culture medium + 10% FBS previously depleted of bovine EV was used as negative control.

To isolate different EV subpopulations, firstly CM was ultracentrifuged 30 min at 10,000 g at 4°C to remove cell debris and apoptotic bodies and then the supernatant was subsequentially ultracentrifuged 75 min at 20,000 g to obtain large EV and after at 110,000 g for 75 min at 4°C to collect small vesicles. The obtained pellets were resuspended in RIPA buffer for western blot analysis.

Nanoparticle tracking analysis

Size and concentration of EV were determined by NanoSight NS300 system (Malvern Panalytical Ltd, Malvern, UK). A camera level of 12 and five 30-s recordings were used for acquisition of each sample. Data were analyzed with NTA software (Malvern Panalytical Ltd.).

Transmission electron microscopy

EV resuspended in PBS were adsorbed into 300-mesh carbon-coated copper grids for 5 min, room temperature, then fixed in 2% glutaraldehyde in PBS for 10 min and briefly rinsed in Milli-Q water. After negative staining with 2% phosphotungstic acid, brought to pH 7,0 with NaOH, grids were examined with a Microscope Zeiss STEM GEMINI 500. All materials are from Electron Microscopy Sciences, Hatfield, PA, USA.

In vitro internalization assays of extracellular vesicles

EV were isolated from the 48 h conditioned medium (CM) of RPMI8226 cells as reported above, stained with the $1\mu g/ml$ cell-tracker CM-DIL (Invitrogen, USA) following manufacturer's instructions and washed with $0.1\mu m$ filtered PBS by 75 min ultracentrifugation to remove the non-binding dye. An ultracentrifugation tube containing only PBS and the dye was used as a negative control. $15\mu g$ EV stained with the dye or negative control were used to treat a monolayer of Raw264.7 cells or HPAEC for 4 h at $37^{\circ}C$ or $4^{\circ}C$. The uptake efficiency was assessed by flow cytometry and fluorescence microscopy.

For quantitative assessment of EV uptake, Raw264.7 cells and HPAEC were seeded at the density of 1,5 x 10⁵ cells/ ml in 48-well (Euroclone S.p.A, Italy) with 250µl of DMEM supplemented with 10% FBS or with Vascular Basal medium supplemented with Endothelial cells Growth Kit-VEGF respectively. After 24 h, cells were treated with CM-DIL dye labelled-EV or with negative control at 37°C and 4°C. After 4h, the medium was removed, and the cells were washed with PBS and treated with trypsin. For flow cytometry, cells resuspended in PBS were analyzed in PE channel to assess the CM-DIL positive cells by using the FACS Verse flow cytometry (BD Biosciences, Italy).

For microscopy analysis, Raw264.7 cells or HPAEC cells were stained with $5\mu M$ CFSE (Biolegend, Italy) following manufacturer's instructions and seeded 3.5×10^4 cells/ml in 24 chamber slides in 500 μ l of the appropriated complete medium. After treatment with EV, the slides were washed with PBS and fixed with PFA 2% for 15 min. Fixed cells were washed with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI). The analysis was performed with a DM-IRE 2 Leica microscope equipped with a Retiga Electro CCD camera and Micro-Manager software. Images were acquired in z-stack scan mode with a HCX PL APO 63x objective, by applying the same acquisition setting, excitation intensity, acquisition time, step size.

Western Blot

Whole cells or EV extracts were prepared in RIPA lysis buffer with the proteases and phosphatases inhibitors cocktail (Sigma Aldrich, Italy). Protein samples (5-40µg) were run on 4-12% gradient SDS gel electrophoresis (Genscript, USA), transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Bioscience, Italy), and blocked with 5% nonfat milk in TBS-T (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0,05% Tween 20). Membranes were incubated overnight at 4°C with anti-NOTCH1 (D1E11) antibody (1:1000 dilution, Cell Signaling Technology, USA), anti-NOTCH2 (D76A6) antibody (1:1000 dilution, Cell Signaling Technology, USA), anti-NOTCH2 cleaved from Val1697 (SAB4502022) antibody (1:1000 dilution, Sigma-Aldrich, St. Louis, MI, USA); anti-NOTCH3 (D11B8) antibody (1:1000 dilution, Cell Signaling Technology, USA), anti-NOTCH4 (STJ90070) antibody (1:1000 St John's Laboratory Ltd., London, England, United Kingdom);anti-HA (C29F4) antibody (1:1000 dilution, Cell Signaling Technology, USA), anti-TSG101 (ab125011) antibody (1:500 dilution, AbCam, Cambridge, UK), α-tubulin (sc-12462) antibody (1:1000 dilution, Santa Cruz Biotechnology, USA), β-actin (1:1000, Sigma Aldrich, Italy) followed by incubation with the appropriated HRPconjugated species-specific secondary antibody (Promega, Italy). Chemiluminescence was detected by the Western Bright ECL HRP substrate (Advansta Inc., USA) or by Super Signal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Italy) and using the Alliance HD 6 western blot imaging system (Uvitec, UK).

HEK293-cell based system to track NOTCH2 EV-mediated transfer from donor to receiving cells

For HEK293 cells transfection the cDNA NOTCH2-HA was subcloned from pcDNA5/FRT NOTCH2-HA provided by Groot AJ [1] into pcDNA3.1 2,5 × 10⁶ cells HEK293 cells were transfected in 100mm plate with pcDNA3.1-NOTCH2-HA (N2-HA) plasmid or mock pcDNA3.1 (as a control) plasmid using TurboFect transfection reagent (ThermoScientific) according to the manufacturer's protocol. The medium was replaced after 16 h incubation at 37°C with DMEM 10% FBS and, to obtain EV carrying NOTCH2-HA or control EV, the donor cells were cultured 24 h in DMEM with 10% EV depleted-FBS. EV were purified by 75 min ultracentrifugation at 110,000 g, 4°C. The isolated NOTCH2-HA or control EV were added to the medium of receiving HEK293 cells for 24 h. Cell extracts of donor or receiving cells, and EV extracts were analyzed by western blot using an anti-HA primary antibody and subsequently re-hybridized.

Lentivirus production and transduction of stable HMCLs with shRNAs

Lentiviral vectors pTRIPZ (Horizon Discovery, United Kingdom) expressing scrambled (SCR) or NOTCH2 shRNAs were transfected with Trans-Lentiviral shRNA Packaging mix (Horizon Discovery, United Kingdom) into HEK293 cells using calcium phosphate reagent according to the manufacturer's instructions. Briefly, 5,5x10⁶ cells seeded 24 h prior of transfection were transfected with 42µg pTRIPZ shRNA vector and 30µl Trans-Lentiviral packaging mix using the calcium phosphate reagent. After 16 h calcium phosphate-containing medium was removed from cells and replaced with DMEM supplemented 5% FBS. The viral supernatants were collected at 24 and 48 h post-transfection, filtered, and concentrated using 20% sucrose cushion ultracentrifugation for 4 h at 40,000 g at 4°C to obtain the lentiviral particles.

RPMI8226 and OPM2 cell lines were transduced with the lentiviral particles in presence 20ng/ml IL6 (Peprotech, USA) and 20ng/ml IGF1 (Peprotech, USA) for 48 h by exposing the cells twice to fresh viral supernatant. After 48 h, the infected RPMI8226 and OPM2 cells were selected for stable expression with puromycin at a minimum concentration, as determined by a killing curve, 1μg/mL and 0,5μg/ml, respectively. After 7-10 days under puromycin selection the knockdown efficiency was monitored in transduced cells using 1μg/mL doxycycline to induce TurboRFP and shRNA expression. RFP expression was detected in PE channel by flow cytometry using FACS Verse (BD Biosciences, Italy) whereas the efficiency of NOTCH2 knockdown was evaluated by western blot analysis of cell lysate. Single cell colonies were obtained from a stable SCR and NOTCH2 shRNAs cell pools by limiting dilution. Colonies with maximum NOTCH2 knockdown efficiency were chosen for the further experiments. To produce EV from HMCL^{SCR} and HMCL^{NZKD}, cell lines were maintained for 7 days with Doxycycline 1μg/ml, changing the medium every 2 days; for the last 48 h cells were cultured in complete medium depleted of bovine EV as reported above.

NOTCH reporter assay

HeLa cells were transiently transfected with 240ng of the NOTCH responsive element pNL2.1-6xCSL [2] and with 240ng of the thymidine kinase promoter-driven Firefly luciferase expressing vector (RL-TK pGL4.54) used as a normalizer for the transfection. After 16h, HeLa cells were seeded 12,5 x 10³ cell/well in a 96-well and treated with 100μl of MM-EV resuspended in complete RPMI1640 or the corresponding negative control (see above). After 24 h, luciferase activity was measured using Nano-Glo® Dual-Luciferase® Reporter (NanoDLRTM, N1620, Promega, Italy) and the Glowmax instrument (Promega, Italy).

In vivo experiments

Transgenic zebrafish (*Danio rerio*) embryos obtained by crossing *Tg*(*T2KTp1bglob:hmgb1-mCherry*) with *Tg*(*fli1a:EGFP*) were obtained from the Wilson lab, University College London, London, United Kingdom. Zebrafish embryos were raised and maintained under standard conditions and national guidelines (Italian decree 4th March 2014, n.26).

Embryos were collected by natural spawning, staged according to Kimmel and colleagues [3] and raised at 28°C in fish water (Instant Ocean, 0,1% Methylene Blue) in Petri dishes, according to established techniques. We express the embryonic ages in hours post fertilization (hpf) and days post fertilization (dpf). After 24 hpf, to prevent pigmentation 0,003% 1-phenyl-2-thiourea was added to the fish water.

Before EV microinjections, zebrafish embryos were washed, dechorionated for 5 to 10 min with 1mg/ml pronase 48 hpf and anaesthetized with 0,016% tricaine (Ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich). For each embryo, 10nl of EV produced by 6000 RPMI8226 cells were resuspended in PBS and injected into the duct of Cuvier with a manual microinjector (Eppendorf, Hamburg, Germany) using glass microinjection needles. Following injections, embryos were kept at 28 °C for 30 min and at 32 °C for the duration of the experiments.

To evaluate MM-EV mediated NOTCH activation in zebrafish embryos, the efficiency of MM-EV uptake was evaluated 4 hours post injection (hpi) by fluorescence microscopy using the Leica DM 5500B microscope equipped with the DC480 camera. We measured fluorescence intensity in the trunk region, specifically in the caudal hematopoietic tissue (CHT) area on photomicrographs with ImageJ software. Images were processed using the Adobe Photoshop program.

Representative images were acquired in confocal microscopy using Leica TCS SP2 AOBS equipped with 405 diodes, 488 Ar/ArKr and 543 HeNe lasers and analyzed by Leica Confocal Software (Leica Microsystems, Wetzlar, Germany). 20x images were acquired in xyz scan mode with a 20x objective, by applying comparable arrangement parameters: PMT gain/offset voltages, step size, scan speed, frame, and line average. Sequential scan mode between frames was applied to reduce the fluorophore cross talking between 543 and 488 emission and obtain all scans at the same current z position. 60x images were acquired with the same parameters using a 20x objective with additional 3x electronic zoom.

Osteoclast differentiation assay

Raw264.7 cells were seeded in a 48-well plate in 250µl of RPMI1640 medium supplemented with 10% FBS at a density of 1,25×10⁴ cells/ well with or without 30ng/ml RANKL and treated every 48h with RPMI8226 cell derived-EV or negative control. For experiment with DAPT, cells were treated with MM-EV or the control medium with the drug or the vehicle.

After 7 days, Raw264.7 cells were fixed on the culture plates with citrate-acetone solution and stained for tartrate resistant acid phosphatase (TRAP kit, Sigma-Aldrich). Osteoclasts were identified and enumerated under light microscopy as TRAP positive cells with ≥3 nuclei.

Representative pictures of TRAP positive osteoclasts were acquired with Olympus U-CMAD3 phase-contrast microscope equipped with a Zeiss Axiocam ICc1 camera at 4x magnification.

Angiogenesis assay

For tube formation assay, Growth Factor reduced Matrigel (Corning, NY, USA) was dispensed in a 96-well plate, 50μ L/well, and incubated for 30 min at 37°C. For each well, 8×10^3 HPAEC were seeded and cultured for 13 h in 100 μ l of serum-free RPMI1640 with MM-EV or the control medium. For experiment with DAPT, cells were treated with MM-EV or the control medium with the drug or the vehicle. Pictures of the tube-like structures were acquired with the EVOS-inverted microscope (Euroclone, Italy) at 4x magnification. Numbers of areas and nodes were analyzed using the ImageJ software.

Cell viability assay

Raw264.7 and HPAEC were seeded at the same concentration used for the functional assays with EV and treated with $50\mu\text{M}$ of DAPT for 13 h or 7 days, respectively. After the appropriate experimental time 0,6mg/ml 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) was added to each well for 3 h at 37°C. Formazan crystals were dissolved in 0.01N HCL isopropanol. Specific absorbance was read at 570 nm with subtraction of background at 690 nm. The percentage of cell viability is expressed as: (absorbance treated wells / absorbance of control wells) x 100%.

Ex vivo experiments with EV from patients' BM aspirates

EV were isolated from the BM aspirates of monoclonal gammopathy of undetermined significance (MGUS) (MGUS-BM-EV) and MM patients (MM-BM-EV). The Institutional Review Board of Insubria Italy approved the design of this study (approval n. 1/2018). Written informed consent was obtained in accordance with the Declaration of Helsinki. Clinical information of patients is reported in table S1.

The blood samples were collected at the diagnosis in tubes containing disodium EDTA and processed to obtain plasma through centrifugation at 250 × g for 20 minutes at RT. To isolate EV, the obtained plasma sample was diluted 1:3 with cold PBS and centrifuged three times at increasing speed and ultracentrifuged as reported above. For functional assays, the obtained EV were resuspended in serum-free RPMI1640 in the same original volume and used at a 0,5x final concentration. The functional assays were performed as reported above.

Statistical analyses

Statistical analyses were performed using Student t-test to compare the mean values of two data sets and ANOVA with Tukey post-test for multi-comparison analyses.

The sample minimum size for each *in vivo* experiment on zebrafish embryo was determined based on a priori power analysis for a one-way ANOVA with an alpha level of 0,05 aimed to have power of 0,95, performed on data from a pilot study with 5 embryos for each condition (G-power 3.2 software)[4]. Each *in vivo* experiment involved at least 16 embryos divided in 4 groups. The final analysis was performed by one-way ANOVA with Tukey post-test on data from 4 independent experiments, excluding outliers identified through the ROUT method (Q=1%) [5].

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