Chronic lymphocytic leukemia cells impair osteoblastogenesis and promote osteoclastogenesis: role of TNFα, IL-6 and IL-11 cytokines

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Supplementary Materials and Methods

Patients.

Forty-five patients were enrolled in the present study (Binet stage: 19A, 17B and 9C; 19 female and 26 male with a mean age ± SD of 69 ± 10 years) including subjects already examined in a previous work (Marini C, et al.; Scientific reports. 2017; 7(1):14159). Peripheral blood and sera were collected after informed consent and the study has been approved by the Ethics Committee of IRCCS Policlinic San Martino Hospital. Patients studied in GEP analysis were 217 newly diagnosed CLL cases (early stage Binet A) prospectively enrolled from several Italian institutions in an observational multicenter study (clinictrial.gov#NCT00917540) as previously described (Ronchetti D et al.; BMC MED Genomics 2013; 6:27-37; Ronchetti D et al.; Blood Cancer Journal, 2016; 6(9):e468). The MEC-1 cell line (obtained from DSMZ Cell Line Collection, Germany) was used in some selected experiments. All experiments were performed in accordance with relevant guidelines and regulations.

Preparation of conditioned media from CLL cells cultures and of sera.

Conditioned media were prepared by culturing $1x10^6$ CLL cells/well/ml (24 well plate) for 72h in RPMI 1640 medium with 10% FCS. Spent media were thus collected centrifuged, filtered and frozen at -20°C until further use. Sera from CLL patients were prepared from blood samples following standard procedures and frozen at -20°C until use.

In vitro generation of osteoblasts from BMSC.

Purchased Bone marrow stromal cells (BMSC; Lonza, Walkersville, MD, USA) were seeded at the concentration of $8x10^6$ /plate in petri dishes and cultured in Coon's modified F12 medium (Biochrom A.G., Berlin, Germany) with 10% fetal calf serum (FCS), 1% L-glutamine, 10 ng/mL of Fibroblast Growth Factor (FGF2, Miltenyi Biotech GMbH Friederich-Ebert, Germany). Cells expanded until confluence were washed with PBS (Euroclone S.p.A., Milan, Italy), detached, pooled and plated in 24-well plates. Confluent cells were then osteogenically induced with a medium prepared by adding 200 µM Ascorbic Acid, 10 mM β-Glycerophosphate and $1.0X10^{-8}$ M Dexamethasone to the standard expansion medium for 9 days. $1x10^6$ of CLL B cells (in contact or in transwell), CLL cell conditioned media (CLL-cm: 200 µl) or CLL sera (CLL-sr: 200 µl) were added to differentiating osteoblasts by replacing an equivalent volume of osteogenic medium. Triplicate samples were prepared for each condition and maintained for 5 more days: osteoinduced BMSC were then detached, collected and processed for further RNA analyses (supplementary Figure 1).

Messenger RNA extraction, reverse transcription and quantitative real time RT-PCR.

Messenger RNA was extracted by using the PerfectPure RNA Cultured Cell Kit (5-Prime GmbH, Hamburg, Germany), according to the manufacturer's instructions. Sample pools of cDNA were generated using the SuperScript™ III First-strand synthesis system for RT-PCR Kit (Invitrogen; Milan, Italy). The relative expression of the target genes was assessed by sybr-green real time quantitative RT-PCR by using the RealMasterMix SYBR ROX 2,5X (5'-Prime) in an Eppendorf Mastecycler Realplex^2 apparatus; quadruplicate reactions were performed for each sample, applying the following

settings: a denaturation single step at 95°C for 3 minutes; 35 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 40 sec, and a final step at 72°C for 7 min. The melting curve analysis was used to determine the specificity of reaction. The expression of each gene was normalized to the endogenous control gene GAPDH; primer sets for target genes were derived from published sequences (glyceraldehyde-3-phosphate dehydrogenase, GAPDH; RUNX2; Osteopontin, OP; Osteocalcin, OC (Shyti G., et al.; Int J Artif Organs, 2014, 37-149-154; Narcisi R., et al., J Cell Physiol, 2012, 227:3282-3290); or purposely designed (Dikkopf-1, DKK-1; forward primer: CGGGAATTACTGCAAAAATGGA; reverse primer: GCACAGTCTGATGACCGGAGA; Cathepsin K, Cat-K, forward primer: TCCTTCCAGTTTTACAGCAAAG, reverse primer: GTTTCCCCAGTTTTCTCCCC; NFAT-1, forward primer: TGCAACGGGAAGAGAAAGCG, reverse primer: GTCTTGGGAGAGGACTTAACC; Matrix Metalloproteinase 9, MMP9, forward primer: ACTGAGGAATGATCTAAGCCC; reverse primer: TCGAACTTTGACAGCGACAAG). Expression levels in osteogenically-induced BMSC control cultures were set as reference for further normalization among different samples and/or culture conditions.

Short-interfering RNA-mediated knock-down of specific cytokines in MEC-1 cell line or CLL cells

All short-interfering RNAs (siRNA), whether IL-6-, IL-11- or TNFα-specific (GeneSolution siRNA, Cat. n. 1027416; products: HS_IL6_1,2,5,6; Hs_IL11_4,5,6,7; Hs_TNF_1-2-3-5, respectively) or aspecific (FlexiTube siRNA, AllStars Negative controls, cat. N. 1027415) were purchased from Qiagen (Qiagen s.r.l., Milano, Italy) and used at a final concentration of 80 nM. Aliquots of $1.5X10^6$ cells, either MEC-1 or BA 101, were washed with PBS and cultured in standard RPMI medium in the absence of antibiotics and low serum concentration (5%) for 24 hours, prior to transfection. Transfections were performed by using Lipofectamine 2000 (Invitrogen-Life Technologies; cat. N. 11668-019), diluting siRNAs and Lipofectamine in Opti-MEM Reduced Serum Medium (GIBCO, cat. N. 11058-21) according to the siRNA manufacturer's instructions. Cells were exposed to the siRNA-Lipofectamine complexes in the same medium at 37°C, under gentle rocking, for the subsequent 6 hours. Fresh RPMI medium+5% serum was then replenished and transfected cells maintained in standard culture conditions. After 72 h conditioned medium of cells, knocked-down for each specific cytokine, was collected, aliquoted and stored at -20 C° until further use. TNFα, Il-11 and IL-6 levels in the recovered conditioned media were assessed by ELISA, as subsequently indicated. SiRNAted-cells were also processed to determine the efficacy of the cytokine knock-down procedures through cytofluorographic analysis after intracytoplasmic staining with a PE-conjugated anti-TNFα moAb (Immunotools, Friesoythe, Germany).

Evaluation of matrix deposition by osteo-induced BMSC through Alizarine Red staining.

Osteogenically-induced BMSC were cultured in different conditions as above described. In selected experiments neutralizing anti-TNFα and an anti-IL-6R moAbs (Infliximab, and Tocilizumab both kind gifts from Dr. Puppo, IRCCS- Policlinic San Martino Hospital, Genoa, Italy; 200 μg/mL and 10 μg/mL, respectively), anti-IL-11 (AF218, RD systems, Minneapolis. MN USA; 0.5 μg/mL) or the anti-GP130 moAbs (MAB628 clone 28105, RD Systems; 2.0 μg/mL) or an isotypic control mouse IgG1 K (cat. N. 16-4714-85; eBioscience-Thermo Fisher Scientific, Frederick, MD, USA) were added contemporarily to CLL-cm. Wells were washed in PBS and fixed in para-formaldehyde (4% in PBS) for 15 min. Extensive PBS washings were performed after fixation, followed by a brief rinsing in tap water.

Mineral deposits were then revealed by staining culture wells with Alizarine Red S (2% in water, pH 4.3; Sigma-Aldrich, Milan, Italy) for 10 min. The dye was removed and wells were rinsed twice with absolute ethanol and dried at RT. Images of each well (at least two for each culture condition) were acquired using a Nikon Digital Sight DS-5Mc camera mounted on a Nikon SZM1000 microscope. Quantification of matrix deposition was calculated as the ratio between the Alizarine-positive area of each well, expressed in pixels, vs the total image field area.

Generation of osteoclasts from monocytes purified from healthy donors or CLL patients.

Monocytes ($1x10^5$) purified from healthy or CLL PBMCs by conjugated anti-CD14 micro-beads (Miltenyi) were seeded in 24 well plates in medium (RPMI1640 +FCS10%) and MCSF (25ng/ml; Miltenyi) and RANKL (25ng/ml; Miltenyi) were added 1 day after. Alternatively monocytes were stimulated with only MCSF (25ng/ml) and CLL-cm. Different numbers of monocytes ($1.3x10^5$ or $2.6x10^5$cells/well) were also used in selected experiments performed in the absence of RANKL, where indicated. 500µl of fresh medium together with MCSF+RANKL were replaced at day 4 while at day 7 we added suboptimal concentration of MCSF and RANKL (12,5 ng/ml) together with CLL-cm or CLL-sr (300 µl/1ml total volume) or only medium (supplementary figure 1). 72h-Conditioned media from IL-6, IL-11 and TNFα- silenced MEC-1 cell line was also used in selected experiments. After 7 days, the number of osteoclasts was determined by counting Tartrate-Resistant Alkaline Phosphatase positive ($TRAP^+$) cells on an inverted Olympus CKX-41 microscope. Triplicate samples were prepared for each culture condition and at least 6 image fields were taken for each well. $TRAP^+$ cells showing at least 3 nuclei were considered as osteoclasts. In selected experiments healthy monocytes were co-cultured with CLL cells ($1x10^6$ or $2x10^6$/ml/24well plates) and MCSF (25ng/ml). The percentage of $TRAP^+$ cells exhibiting a small or a large size was also assessed.

Nurse-like cells were generated as previously described (11) to determine TRAP expression and bone resorption activity.

Neutralizing anti-TNFα,-IL-11 moAbs or anti-IL-6R or -GP130 moAbs were used in selected experiments : stimulation of monocytes was performed as above reported (MCSF+RANKL) and at day 7 we added TNFα (30 ng/ml), or CLL-cm together with Infliximab (200 µg/ml), or Tocilizumab (10 µg/ml), or anti- IL-11 (0,5 µg/ml), or anti-GP130 (2 µg/ml) moAbs.) When the experiments were performed in the absence of previous RANKL stimulation, we contemporarily added MCSF+CLL-cm alone with/without neutralizing moAbs. The activity of the BTK inhibitor Ibrutinib (500 nM; D.B.A. Italia S.R.L., Milan, Italy) was further tested by adding it in cultures together with CLL-cm at the time of the last stimulation. Selected experiments were also performed by adding Denosumab (Xgeva, Amgen srl, Milan, Italy). The number of osteoclasts generated for each experimental condition was determined as above described.

TRAP Staining.

Purified monocytes induced to differentiate toward osteoclasts were washed in PBS and fixed for 15 min at RT with a 4% PFA in acetate/citrate buffered solution (pH 6.0). Cells were subsequently

stained according to the protocol provided with the Tartrate-resistant acid phosphatase detection kit (Sigma-Aldrich, kit. N. 387A, Milan, Italy), by exposing fixed cells to a freshly prepared tartrate-containing solution for 60 min at 37°C. The staining solution was then removed and cells were washed in distilled water and photographed. TRAP-positive cells were grouped according to their dimensions. In each image field the area of trinucleated and $TRAP^+$ cells was measured by using the public software NIH Image J (version 1.48v; http://imagej.nih.gov/ij) tools. The cut off size to categorize a cell as small or large was an area of 25000 pixel.

Bone resorption Assay.

Determination of bone resorption activity was performed by seeding $CD14^+$ purified monocytes from healthy donors in 24 well plates coated with an inorganic crystalline material (Corning, Osteo Assay Surface multiwall plates; cat.n. CLS3987-4EA) and inducing their differentiation toward osteoclasts as above described. After 14 days cells were stripped from the plates by treatment with a 10% sodium hypoclorite solution for 10 min; wells were thoroughly washed and dried at RT for 4 hours. Images of triplicate experimental conditions were acquired and the size of resorption pits was evaluated using the public software NIH Image J. The pit size was calculated as the ratio between the area of each pit, expressed in pixels, vs the total image field area.

Analysis of cellular viability and of cell cycle by DiOC6 and Propidium Iodide (PI) staining respectively. Evaluation of Ki67 expression on CLL cells after co-culture with osteoclasts.

Osteoclasts or their progenitors (small trinucleated $TRAP^+$ cells) were derived as above described. After 14 days plates with mature osteoclasts were washed with PBS and further co-cultured with $1x10^6$ CLL cells in RPMI+FCS10%. Percentage of CLL cells in apoptosis was determined at different time points (72h, 96h, 7days) through DiOC6 staining as previously described (De Totero D., et al.; Blood; 2006;107(9):3708-3715). The expression of Ki-67 was evaluated on CLL cells before and after co-culture with osteoclasts through double staining with CD19 and Ki-67 specific moAbs and the samples were analyzed in flow cytometry by FACSCanto (BD Biosciences Pharmigen, San Jose, CA, USA). Multiparameter flow cytometric analysis of cellular viability by propidium iodide (PI) exclusion assays, and cell cycle-phase distribution by DNA content was performed as previously described (Bruno S., et al.; Leukemia. 2012; 26(10):2260-2268). Briefly, CLL cells were incubated with 0.05% Triton X-100, stained with 30 µg/ml PI+0.5 mg/ml RNase (both Sigma Chemical Co.) for 30 min and measured by flow cytometry (FACSCalibur, Becton Dickinson, San José, CA). The resulting DNA content histograms provided information on proliferation (cell-cycle-phase distributions) and apoptotic DNA fragmentation ('sub-G0/G1' region). Proliferation features are herein expressed as % of cells in $S+G_2M$ cell cycle phases (hyper diploid fraction).

The expression of Ki-67 was evaluated on CLL cells before and after co-culture with osteoclasts through double staining with CD19 and Ki-67 specific moAbs and processing samples for flow cytometry by a FACSCanto apparatus (BD Biosciences Pharmigen). Briefly, samples were stained with the anti-human CD19 fluorescein-conjugated antibody (Immunotools, Friesoythe, Germany) at 4°C for 30 min. After 3 washes with PBS cells were incubated with PFA1% for 15 min at 4°C, washed again and then incubated with Methanol for 10 min; during the last 4 min 0.1% of saponin was

further added. Following 3 washes 20 µl of FITC mouse anti-Ki-67 (Ki-67 Set BD Biosciences Europe, Erembodegem, Belgium) or its specific negative control were added together with 0.1% saponin and incubated at 4°C for 45 min. Thereafter cells were washed and analyzed.

Evaluation of pSTAT3 and AKT expression in osteo-induced BMSC cultured with CLL-cm in the presence/absence of neutralizing TNF alpha moAb or in osteogenic-medium only.

Osteo-induced BMSC cultured with only medium or with CLL-cm (TM145) in the presence or absence of Infliximab for 48h were first detached, washed with PBS and incubated with PFA 1% for 15 min at 4°C, washed again and then incubated with Methanol for 10 min; during the last 4 min 0.1% of saponin was further added. Following 3 washes, 20 µl of anti-phospho-STAT3 (ser727) (Invitrogen) or of anti-AKT (Cell Signaling Technologies, Danvers, MA, USA) polyclonal antibody or a specific control were added together with 0,1% of saponin and incubated at 4°C. After 45 min of incubation and 3 washes in PBS, samples were further stained with a PE-conjugated anti-rabbit secondary antibody + 30ul of 0.1% of saponin. Thereafter cells were washed and analyzed.

Immuno-histochemical analyses of bone biopsies from CLL patients

Tissue was fixed in B5 (Sigma Aldrich) for 2.5h and decalcified for 3h before processing. B5-fixed, paraffin-embedded blocks were sectioned at 2 mm, deparaffinized and rehydrated. Endogenous peroxidase was blocked with 5% H_2O_2 for 10 min. Immunoreactions for TRAP staining (clone 26E5 Life Technologies, Monza, Italy) and CD79α staining (clone SP18, Ventana Medical Systems), were performed using the automated BenchMark Ultra XT immunostainer (Ventana Medical Systems, Arizona, USA). Standard heat-based antigen retrieval (pH 6.0) was performed for TRAP and CD79α (64 min and 36 min, respectively). The Ultraview DAB detection kit, a streptavidin–biotin-based indirect method (Ventana Medical System), was used for immunostaing at 37°C for 40 min. Therefore slides were then processed for standard mounting. Images acquired from each section were scored for TRAP+ and/or CD79a+ cells. The number of multinucleated TRAP+ cells was counted in 2 different sections from bone biopsies of 3 CLL patients (LSA01, DA06, BS13), one multiple myeloma patient and one normal control. Data are mean of the number of osteoclasts and of trabeculae of the 2 sections for each patient.

Detection of TNFα, IL11 or IL-6 in sera from mice grafted with CLL cells and/or in media recovered from co-cultures of osteo-induced BMSCs and CLL cells.

An in vivo xenograft model of CLL growth has been developed in our Laboratory and loss in trabecular bone structure in the femurs of mice injected with leukemic cells has been documented (Marini C., et al.; Scientific reports. 2017;7(1):141592). Human and mouse TNFα levels present in sera collected from the same mice previously studied were measured using Millipore's Milliplex MAP human Bone Magnetic Bead Kit (HBNMAG-51k, Millipore Sigma, Burlington, MA, USA) and acquired by MAGPIX instrumentation. The levels of human TNFα present in media from CLL cells cultured alone or co-cultured with osteo-induced BMSC for 5 days were also determined by using the same kit. Levels of TNFα, IL-11 or IL-6 in conditioned media of CLL cells, *per se* or upon cytokine-knock down, were assessed by standard ELISA procedures, by using EH3TNFA (Thermo Fisher

Scientific), RAB0250A-EA Human IL-11 and RABIL6A-EA Human Il-6 detection kits (SIGMA Life Sciences), respectively.

Comparison of the levels of TNFα, present in co-cultures of monocytes stimulated with MCSF+RANKL with/without conditioned media from different CLL patients.

Comparison of the levels of TNFα present in co-cultures of monocytes differentiating toward osteoclasts under different conditions was further performed through a qualitative Elisa assay (Quiagen Sciences Maryland, USA) following manufacturer's instructions.

GEP analysis.

$CD19^+$ purified CLL samples and normal peripheral blood (PB) B cells were profiled on GeneChip Gene 1.0 ST Array (Affymetrix, Santa Clara, CA), as previously described (Morabito F., e al.; Clin Cancer Res. 2013;19(21):5890-5900). Normalized expression levels were processed by the Robust Multi-array Average procedure (Irizarry RA, et al.; Biostatistics 2003; 4:249–64.) with the re-annotated Chip Definition Files from BrainArray libraries version 19 (Dai M, et al.; Nucleic Acids Res 2005; 33:e175), available at http://brainarray.mbni.med.umich.edu.

In silico interrogation of public data on human MSC co-cultured with CLL cells.

Expression levels of selected genes (RUNX2, Collagen 1A, BMP-4, BMP-6, DKK-1, OP, Osterix, RANK, RANKL, β-CAT, NOTCH1, NOTCH2, OC, BMP-2, Wnt3a, Wnt5a) were extracted from GSE129108 dataset profiled on Illumina HumanHT-12 v4 Expression BeadChip (Schulze-Edinghausen L et al., Cancers 2019), comparing untreated human primary mesenchymal stromal cells (MSCs) from two different donors each of which co-cultured for 5 days with CLL cells derived from two different patients.

Statistic.

Two-sided Student *t*-test, U-Mann-Whitney test, or χ^2 test with Fisher's correction were used. All data sets used displayed a normal distribution; p values are depicted as *, ** or *** for $p \leq 0.05$, ≤ 0.01 or <0.001, respectively.

Legends to SUPPLEMENTARY FIGURES

Supplementary Figure 1: Experimental models. Here are depicted different passages employed to derive osteoblasts or osteoclasts and to assess their differentiation under the various culture conditions adopted.

Supplementary Figure 2: Effects of the addition of neutralizing anti-cytokines moAbs to BMSC cultured with CLL-cm (TM145). A and B: The addition of anti-TNFα or the IL6R moAbs, but not of an isotypic control moAb, induced a decrease in matrix mineralization**. C** and **D:** RUNX2 and Osteocalcin mRNAs levels were rescued by the addition of neutralizing moAbs to osteo-induced BMSC cultured with CLL cm (TM145). **E** and **F**: pSTAT3^{ser727} and AKT were downmodulated in osteoinduced-BMSC cultured for 48h with CLL cm (TM145). The addition of anti-TNFα moAb however re-induced higher levels of expression similar to those of BMSC cultured in osteogenic medium only (BMSC).

Supplementary Figure 3: Evaluation of TNFα, IL-11 and IL-6 levels in RNA-silenced MEC-1 cell line or leukemic CLL cells or in conditioned media from different CLL patients through immunofluorescence or Elisa assays. A: Downmodulated expression of TNFα in siRNA-transfected MEC-1 or CLL cells (BA101), as detected by immuno-fluorescence and cytofluorographic analysis. **B, D, E, G:** Histograms evidence reduced levels of the secreted cytokines in cm from siRNA transfected cells. **C, F, H:** Discrete amounts of TNFα, IL-11 and IL-6 are present in CLL-cm from different CLL patients.

Supplementary Figure 4: Gene expression profiles of IL-11 and IL-11RA in CLL cells and normal B controls. IL-11 and IL-11RA are significantly more expressed in CLL B cells than in normal B cells of controls, as determined through GEP analysis (*n*=217 early stage Binet A CLL cases, and *n*=6 B control cases). Statistical significance was evaluated by the U-Mann Whitney test.

Supplementary Figure 5: Evaluation of TNFα levels in media derived from CLL cells cultured alone or with osteo-induced BMSC. Higher TNFα levels were detected in media derived from the 5-days cultures of CLL cells alone than after their co-culture with BMSC differentiating towards osteoblasts. BMSC: bone marrow stromal cells. Six patients were assessed (FW16, BE08, CRO7, JF12, MS15, MM13)

Supplementary Figure 6: The addition of exogenous RANKL exploits the full maturation of committed osteoclasts precursors. Monocytes stimulated with MCSF+CLL-cm, without RANKL, proceed toward the first step of osteoclasts differentiation (**A, B)** but do not reach terminal differentiation (**C, D**). In agreement to these observations, the anti-RANKL antibody Denosumab (1 ng/ml or 10 ng/ml, Dns) affects osteoclastogenesis only if RANKL is present (**C, D**). Red arrows indicate tri-(or more)-nucleated TRAP^{+} cells. Bar sizes are 25μm and 12.5μm for 10X and 20X respectively.

Supplementary Figure 7: Effects of cytokine interference on osteoclastogenesis. Conditioned medium derived from TNFα siRNA knocked-down MEC-1 cells induced a weak decrease in large fully differentiated osteoclasts and an increase of small TRAP+ cells, similarly to the effect caused by supplementation of an anti-TNFα moAb.

Supplementary Figure 8: Increased levels of human TNFα were detected in mouse serum after human CLL cells engraftment. A: In the CLL mouse model the levels of human TNFα were progressively increased from the 3rd to the 6th week, after human CLL cells injection and engraftment**. B.** Human TNFα concentration in NSG (Nod SCID γ-null) mice engrafted with CLL cells as compared to controls, at the time of sacrifice.[(2)]

Supplementary Figure 9: Ibrutinib treatment inhibited increased-osteoclasts maturation due to the addition of CLL-cm. A: Ibrutinib strongly affects osteoclastogenesis also when this process is enhanced by addition of CLL-cm in culture. Red arrows indicate TRAP^{+} osteoclasts at least tri-nucleated. Size bar: 63μm (4X) and 25μm (10X). **B**: A statistically significant reduction of osteoclasts generation was evident under Ibrutinib treatment. Ibrutinib affected both large fully mature osteoclasts and small precursors. Histograms depict mean values ± SD of 6 CLL cases, evaluated in 3 different experiments.

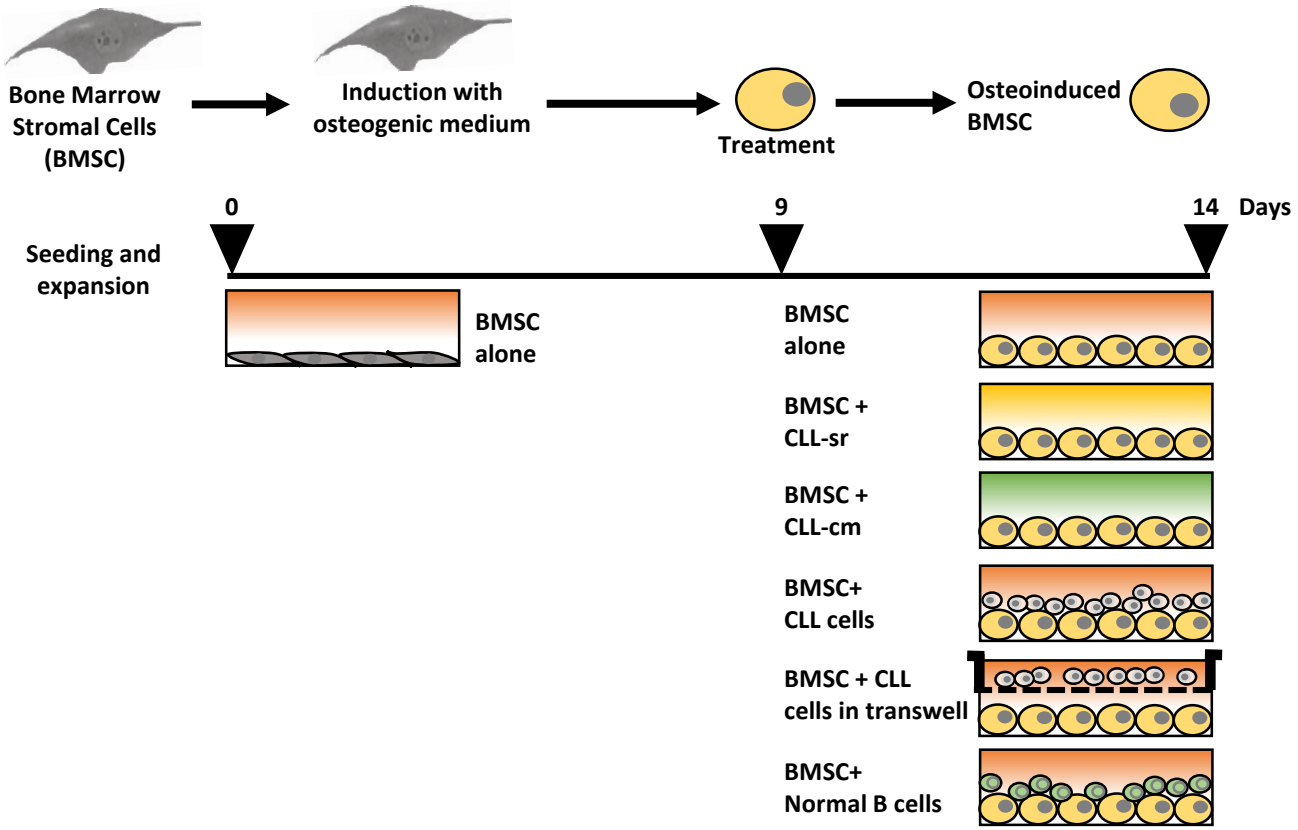
Supplementary Figure 10: **GEP of different molecules correlated to osteoblastogenesis regulation as derived from *in silico* available data.** Histograms depict significant alterations in the mean values of normalized expression levels of selected genes (RUNX2, Collagen 1A, BMP-4, BMP-6, DKK-1 and OP) in 2 MSC samples untreated or co-cultured with 2 different CLL patients for 5 days. Other analyzed targets (Osterix, RANK, RANKL, β-CAT, NOTCH1, NOTCH2, OC, BMP-2, Wnt3a, Wnt5a;dat not presented) did not show significant modulations.

Supplementary Figure 11: **Correlation between IGVH status of CLL patients and CLL-cm-driven formation of trinucleated TRAP+ cells.** Histograms depict, as fold increase, the number of trinucleated TRAP+ cells obtained when pre-activated monocytes are cultured with CLL-derived cm, with respect to non-cm exposed controls; values higher than 1.25-fold significantly correlate with cm derived from unmutated IGVH patients (χ^2 test, p=0.0147).

Supplementary Figure 1

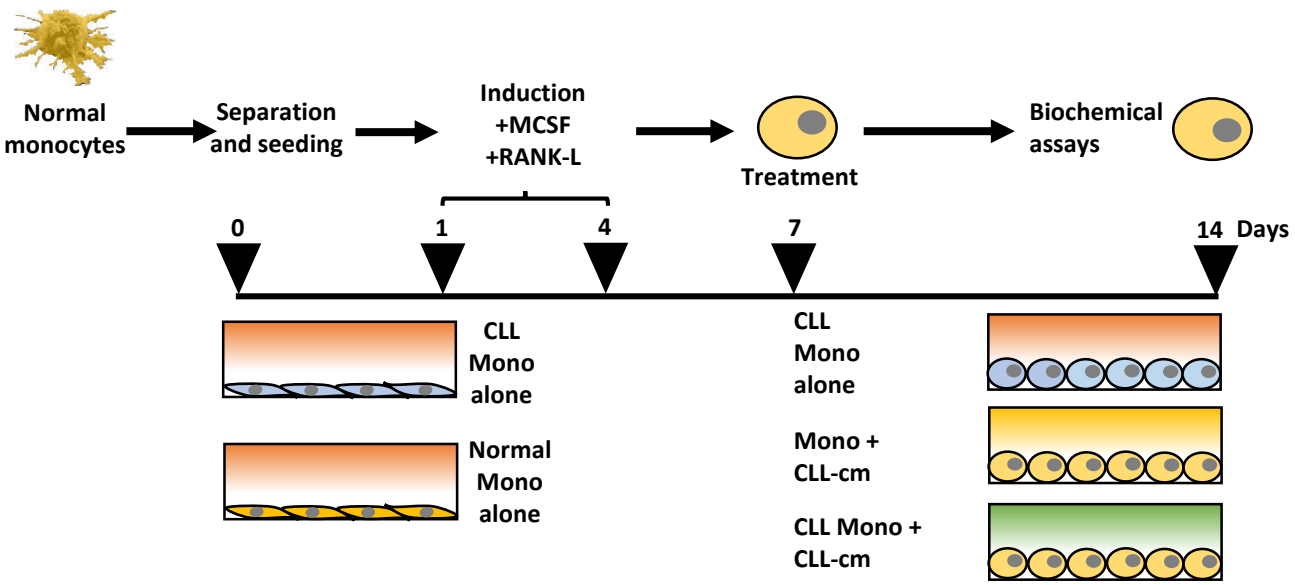
A

Evaluation of potential inhibition of osteoblast differentiation by CLL cells conditioned medium or serum or CLL cells co-culture

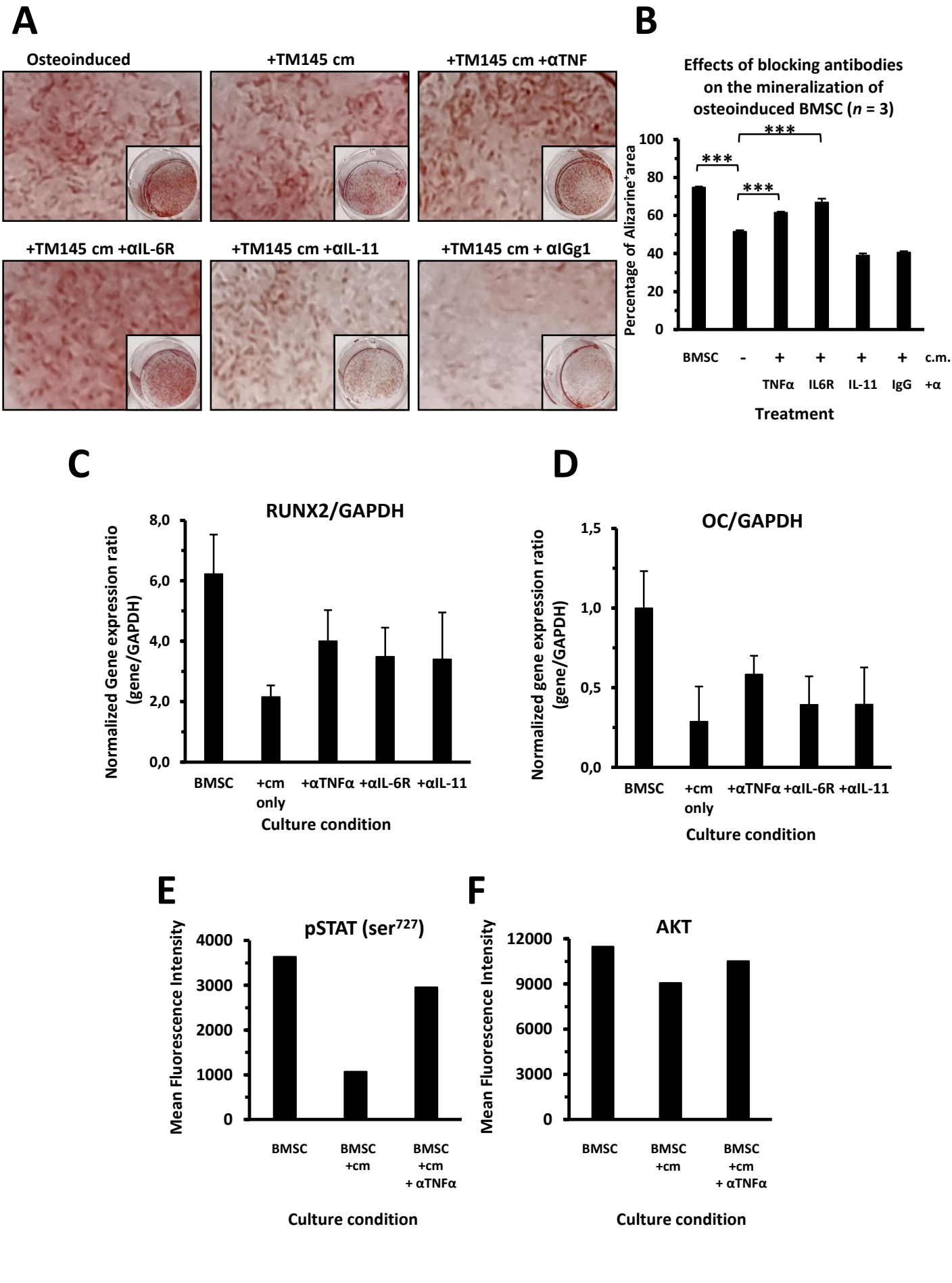


B

Evaluation of osteoclastogenesis of normal or CLL monocytes in the presence of CLL cells conditioned medium



Supplementary Figure 2



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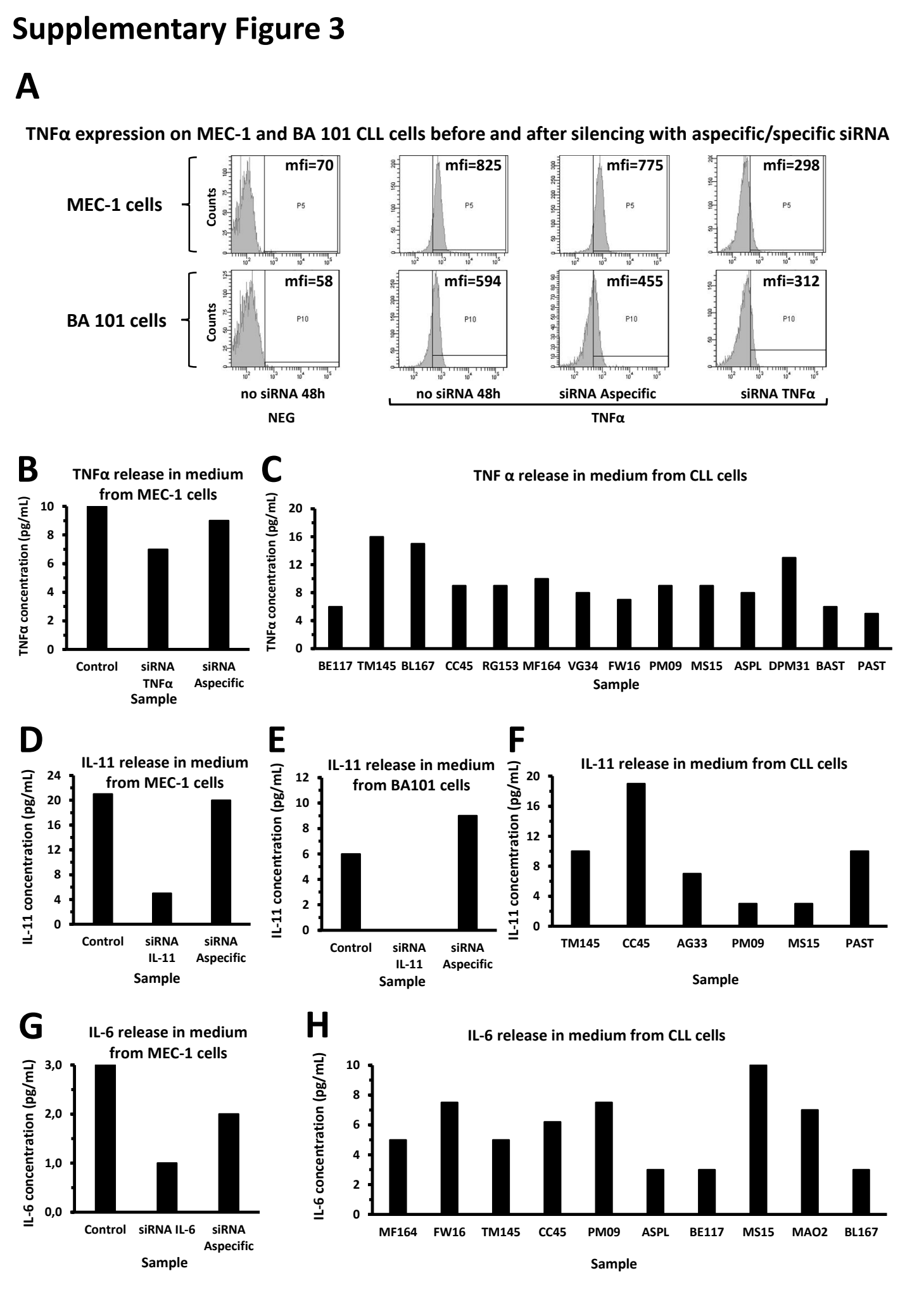
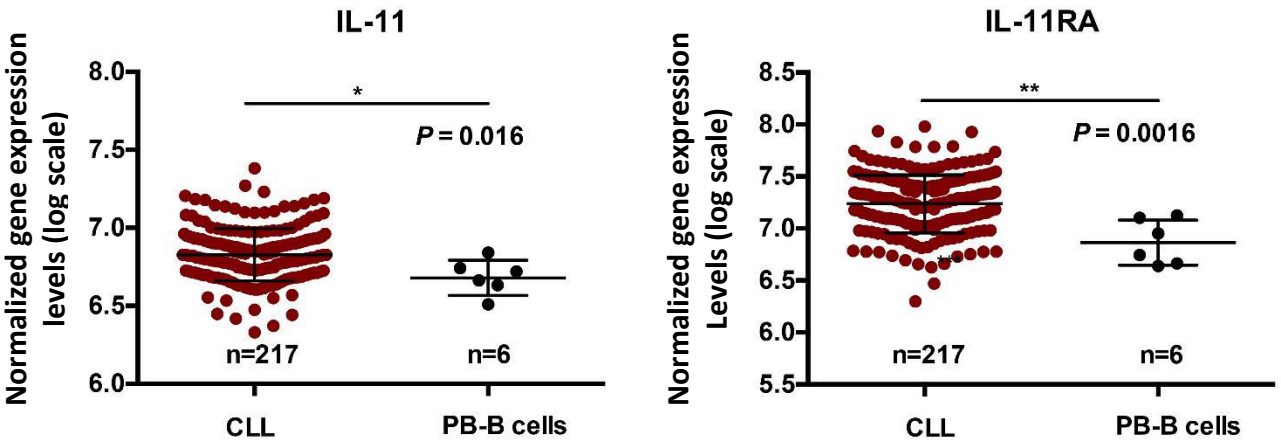


Supplementary Figure 4



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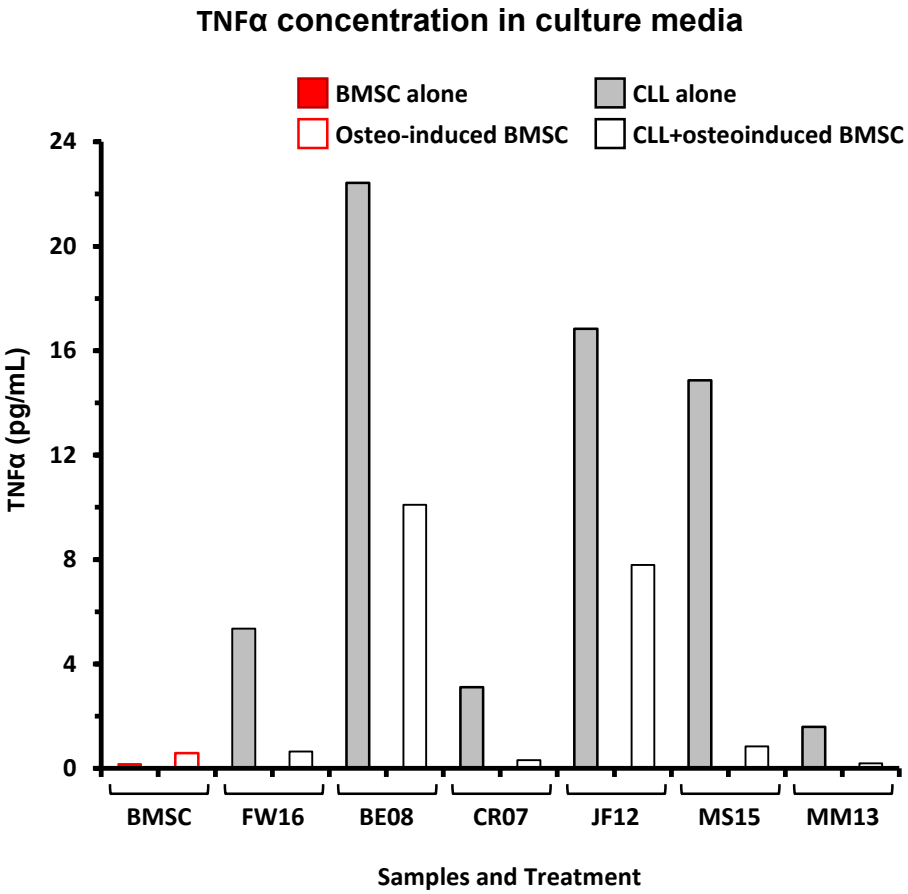
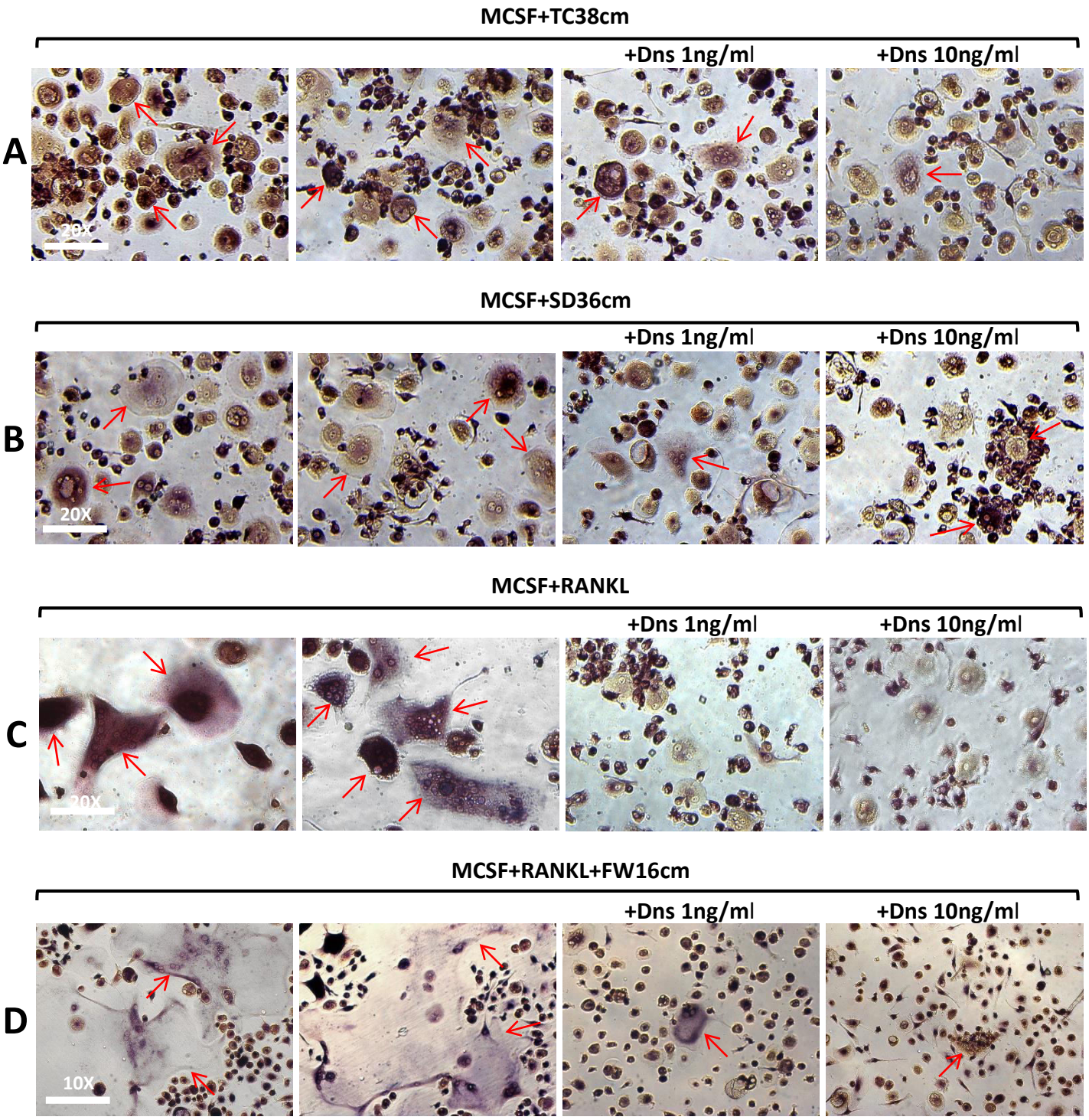


Supplementary Figure 6



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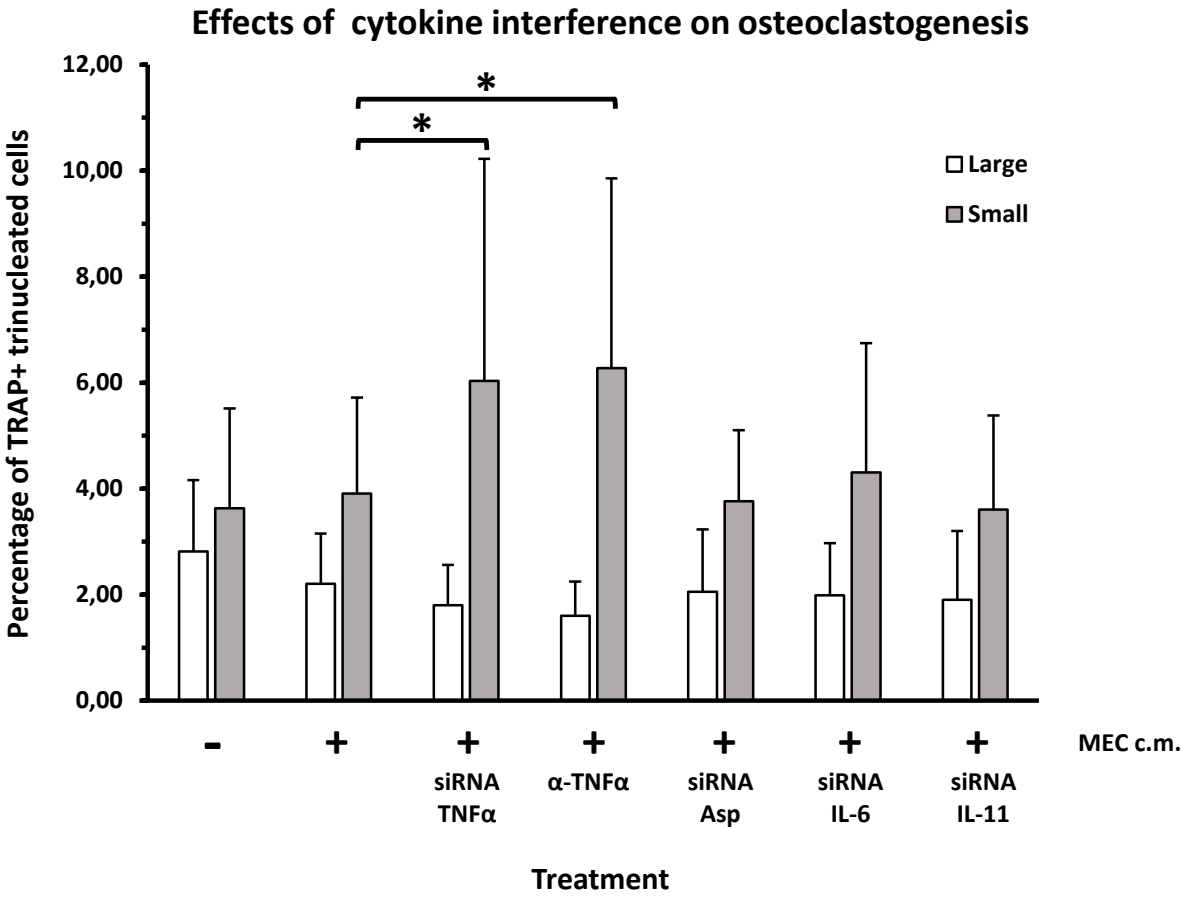
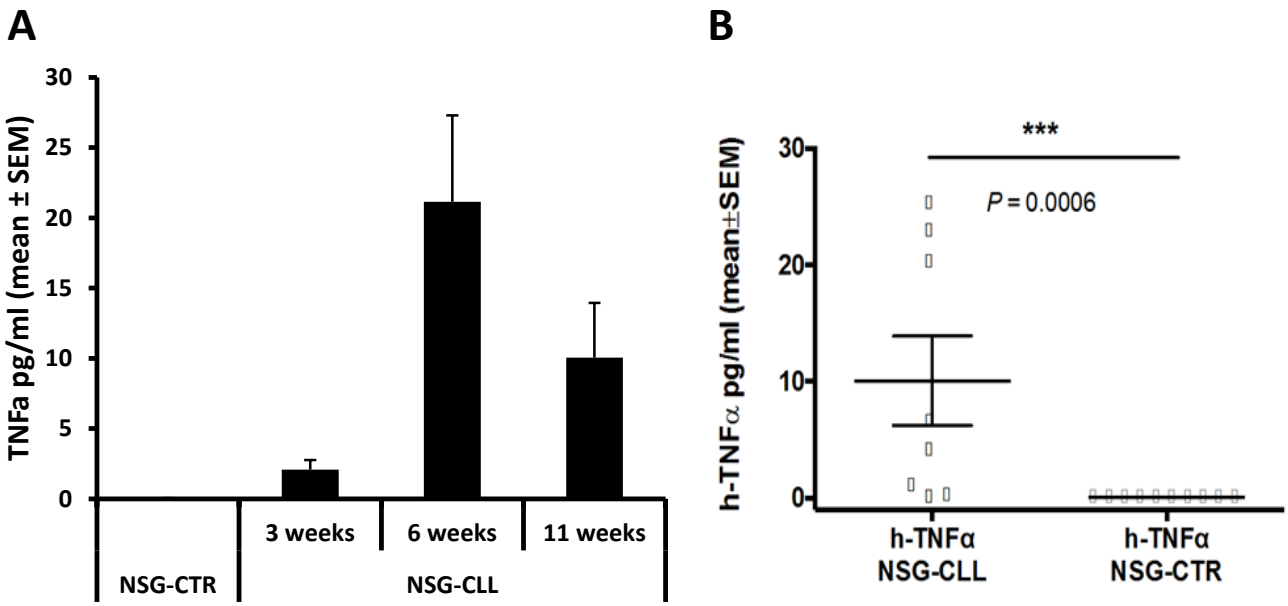
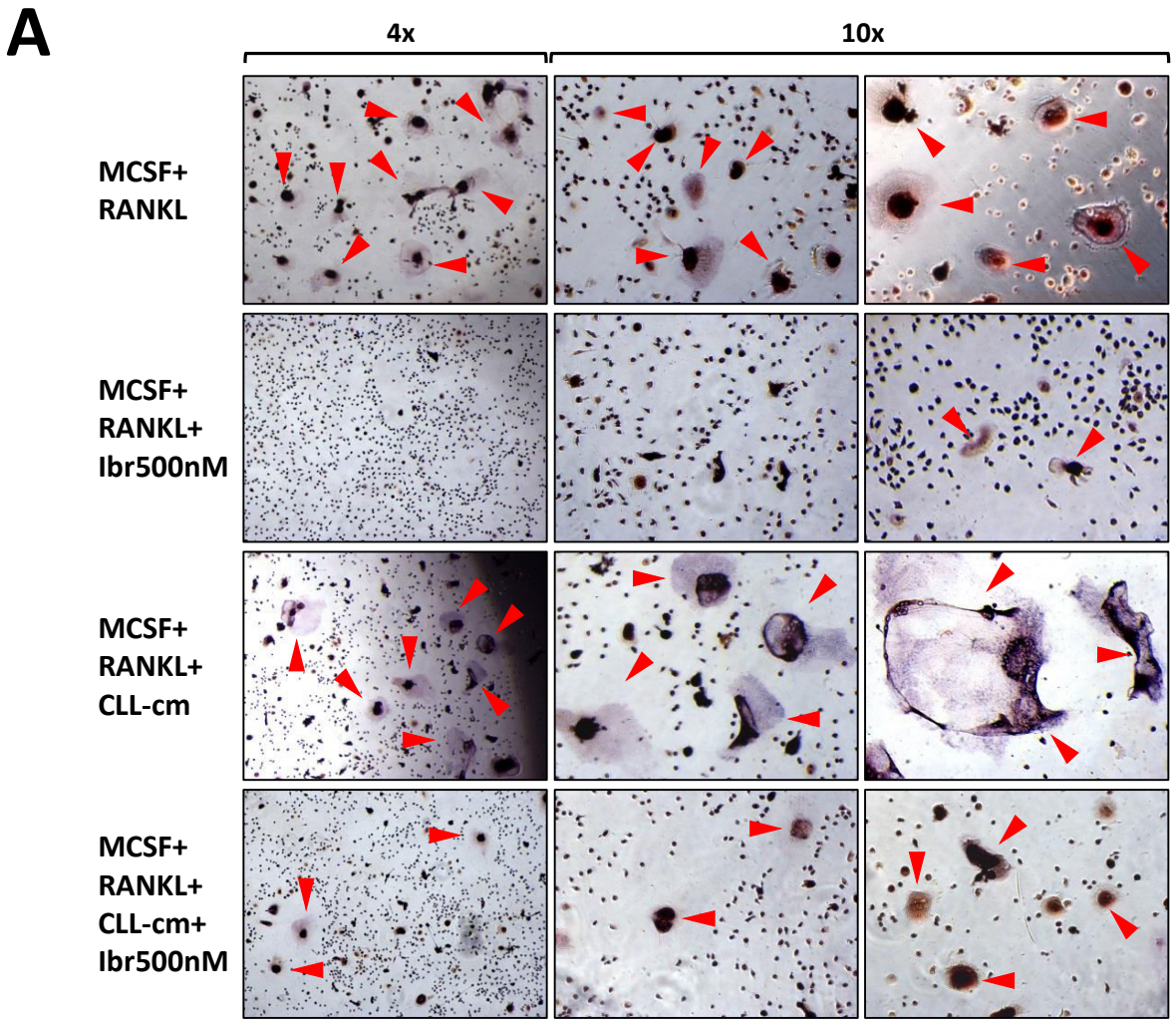
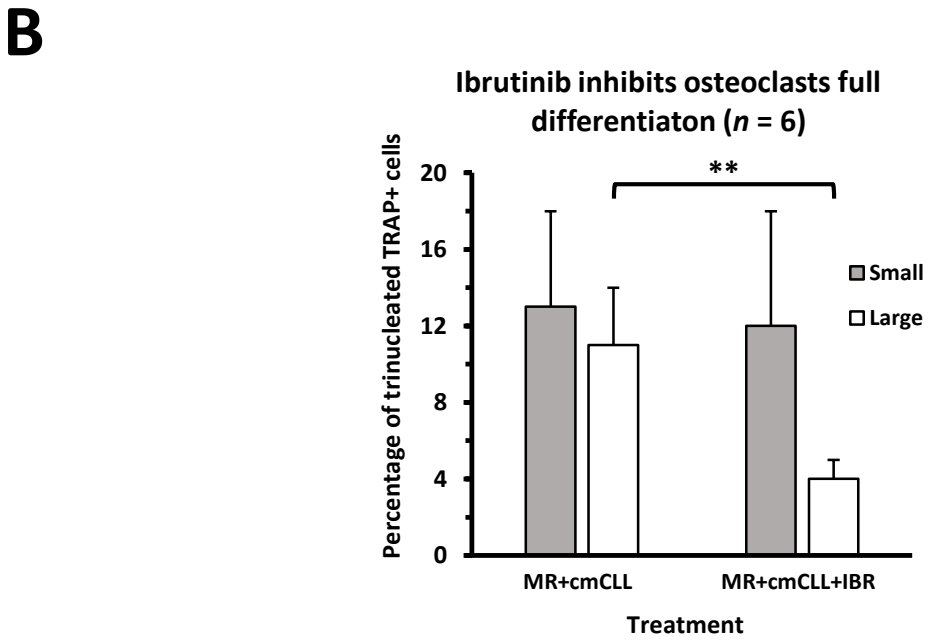


Supplementary Figure 8

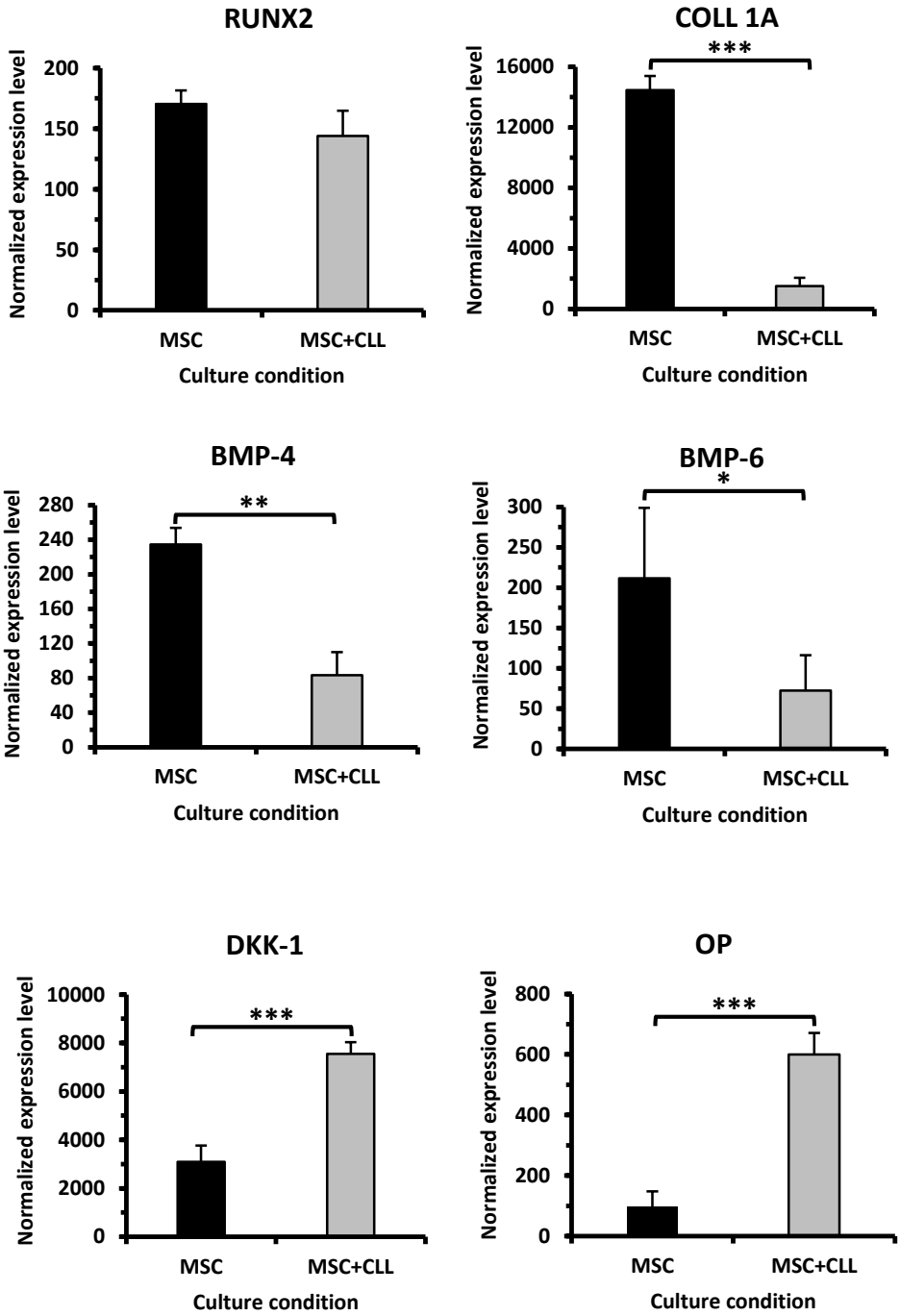


Supplementary Figure 9





Supplementary Figure 10



Supplementary Figure 11