A novel CCL3-HMGB1 signaling axis regulating osteocyte RANKL expression in multiple myeloma

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Supplementary Methods.

Reagents. RPMI 1640 media, Minimum Essential Media (MEM) α, Opti-MEM, fetal bovine serum, bovine calf serum, Normocin, antibiotics (penicillin/streptomycin), and TriZol were purchased from Invitrogen Life Technologies (Grand Island, NY, USA). Trypan Blue and the anti-HMGB1 neutralizing antibody (Cat. #H9537, RRID:AB 260090) were purchased from Millipore Sigma (Burlington, MA, USA). Recombinant CCL3 (Cat. #270-LD-010/CF) and the anti- CCL3 neutralizing antibody (Cat. #MAB270-500, RRID:AB_2070775) were purchased from R&D Systems (Minneapolis, MN, USA). The *CCL3* sgRNA CRISPR/spCas9 lentiviral particles were purchased from Applied Biological Materials Inc. (Richmond, BC, CA, Cat. #sc-417845) and the *Hmgb1* mouse siRNA Oligo Duplex (Locus ID: 15289; Cat. #SR426038) were purchased from Origene Technology Inc. (Richmond, BC, Canada). Puromycin (Cat#58-58-2) was purchased from InvivoGen (San Diego, CA, USA).

Human samples methods and histological analysis. Bone biopsies of 3-mm diameter were fixed in 4% paraformaldehyde and decalcified in 0.5M EDTA prior to paraffin embedding. Formalin-fixed paraffin-embedded (FFPE) tissues were sectioned into 3.5-µm thickness and deparaffinized in a xylene-ethanol gradient. Slides were incubated in TE buffer overnight at 60°C, blocked with 5% casein/Tris-buffered saline (TBS), and incubated for 2 hours with a monoclonal mouse antibody against HMGB1 (Thermo Fisher Scientific, Cat # MA5-17278; RRID: AB_2538744). The signal was amplified with horse radish peroxidase-conjugated anti-mouse IgG polymers (BrightVision, Duiven, Holland) for 30 minutes at room temperature and revealed with opal 620 dye (Akoya Biosciences, Marlborough, MA, USA). Thereafter, sections were incubated in Custom Reagent (Advanced Cell Diagnostics, Hayward, CA, USA) for 20 min at 40°C and hybridized overnight at 40°C with a 20-basepair probe targeting *RANKL* mRNA within the target region

between base pairs 366 and 1507 (NM_003701.3; Advanced Cell Diagnostics, Hayward, CA, USA). The day after, signal amplification was performed following the manufacturer´s recommendations and visualized with opal 570 dye (Akoya Biosciences, Marlborough, MA, USA). Sections were Hoechst counterstained, mounted in Prolong Gold (Fisher Scientific, Roskilde, Denmark), and scanned in a VS200 Olympus Slidescanner (Tokyo, Japan). Scans were obtained at 40X with 40 ms exposure in the DAPI channel and 100 ms exposure in the Cy3 (565 nm) and Texas Red Kromigon (615 nm) channels); visualization settings were similar across all images during quantification, and differentially adjusted to capture representative images. Negative controls were performed by omission of the primary antibody/hybridization probe. Analyses were performed by a researcher blinded to study groups using artificial intelligence-assisted histology in the IF-FISH v2.1.5 HALO module (v3.6.4134, Indica Labs). Briefly, trabecular bone was manually delimited, and cellular *RANKL* and HMGB1 expression were automatically quantified; $RANKL^{+}$ osteocytes presenting >4 copies/cell were excluded from analyses.

Genetic inhibition in MM cells and osteocyte-like cells.

To generate stable *CCL3* knockdown cells ($CCL3^{KD}$), JJN3 MM cells were transduced with *CCL3* sgRNA CRISPR/Cas9 lentiviral particles (MOI=10) and selected with 3.0ug/mL Puromycin over 4 weeks. To transiently knockdown *CCL3* in MM cells or *Hmgb1* in Ocy454s, cells were incubated with 50μM of *CCL3* human or *Hmgb1* mouse siRNA Oligo Duplex in alpha-MEM complete media with15% Opti-MEM and 0.4% lipofectamine for 48hrs.

***Ex vivo* Bone Organ Cultures.** Murine *ex vivo* bone organ cultures were established with tibiae and/or femurs from C57BL/KaLwRijHsd (murine MM cells) or NSG (human MM cells) mice, as described before.[1-4] Murine bones were treated with 1ug/mL or recombinant CCL3 in the

presence/absence of 10µg/mL of anti-HMGB1 for 24 hours. Human *ex vivo* bone organ cultures were established using human cancellous bone fragments similar in size obtained from the femoral head of patients with no pathologies or medications that could affect bone mass or architecture discarded after hip arthroplasty.[5] For these *ex vivo* cultures, $2x10^5$ Ctl or *CCL3*^{KD} JJN3 MM cells were plated on human bones or bones were treated with 48h-CM from Ctl or *CCL3*^{KD} JJN3 MM cells for 3 days. Treatments were refreshed daily.

Gene mRNA expression. Total RNA was isolated from MM cells, osteocyte-like cells, and bone tissues using Trizol and converted to cDNA (Invitrogen Life Technologies), following the manufacturer's directions. RNA was converted to cDNA, and gene expression was quantified by qPCR using Taqman assays from Applied Biosystems (Foster City, CA, USA). Values were normalized by *Gapdh* or *ChoB* expression. Gene expression levels were calculated using the comparative threshold (CT) method.

Enzyme-linked immunosorbent assays (ELISA). ELISAs for RANKL (R&D Systems, Cat. #MTR00) and HMGB1 (Fischer Scientific, Cat. #EEL102) were performed according to the manufacturer's instructions. Conditioned media was concentrated using Amicon® ultra-centrifugal Filter (10kDa; Cat. #UFC20124) according to the manufacturer's instructions. Cell protein lysates were obtained using Pierce™ RIPA buffer (Fisher Scientific, Cat. #89900) with a protease inhibitor cocktail (Fisher Scientific, Cat. #4906837001 and phosphatase inhibitor (Millipore Sigma, Cat. #4906837001), then quantified using the Pierce™ BCA assay (MilliporeSigma, Cat. #23228/23224). Cell lysates were loaded onto the ELISAs using 100µg of protein/well.

Western Blots. Protein was loaded in 10% SDS-PAGE gels. Proteins were transferred to PVDF membranes. Immunoblots were performed using anti-HMGB1(Cat. #3935s, Cell Signaling Technology Inc., Danvers, MA, USA) and anti-albumin (Cat. #NBP2-26510, Novus Biologicals

Inc., Centennial, CO, USA) followed by goat anti-rabbit secondary antibodies, conjugated to horseradish peroxidase (1:2000). Western blots were developed using the chemiluminescence detection assay.

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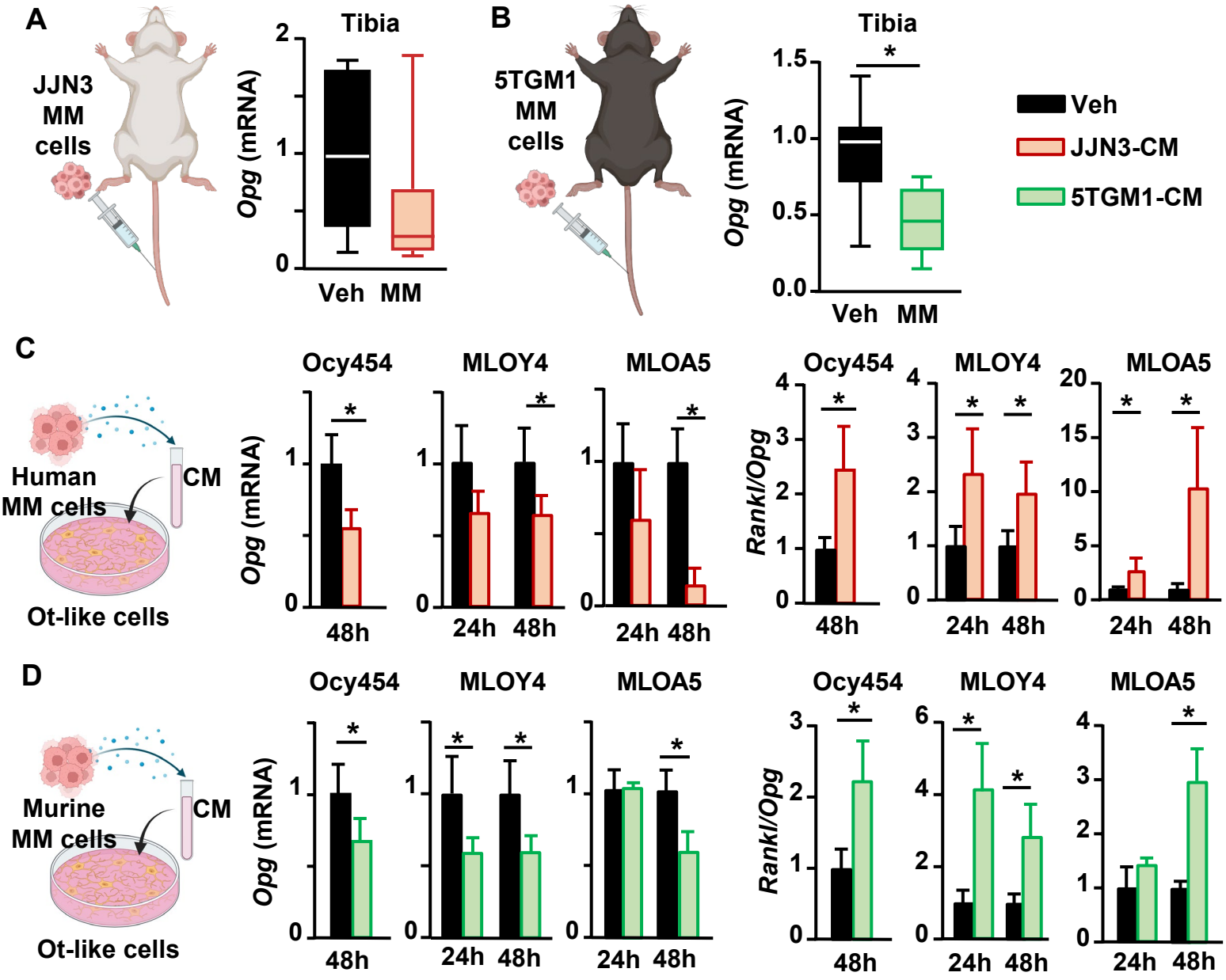
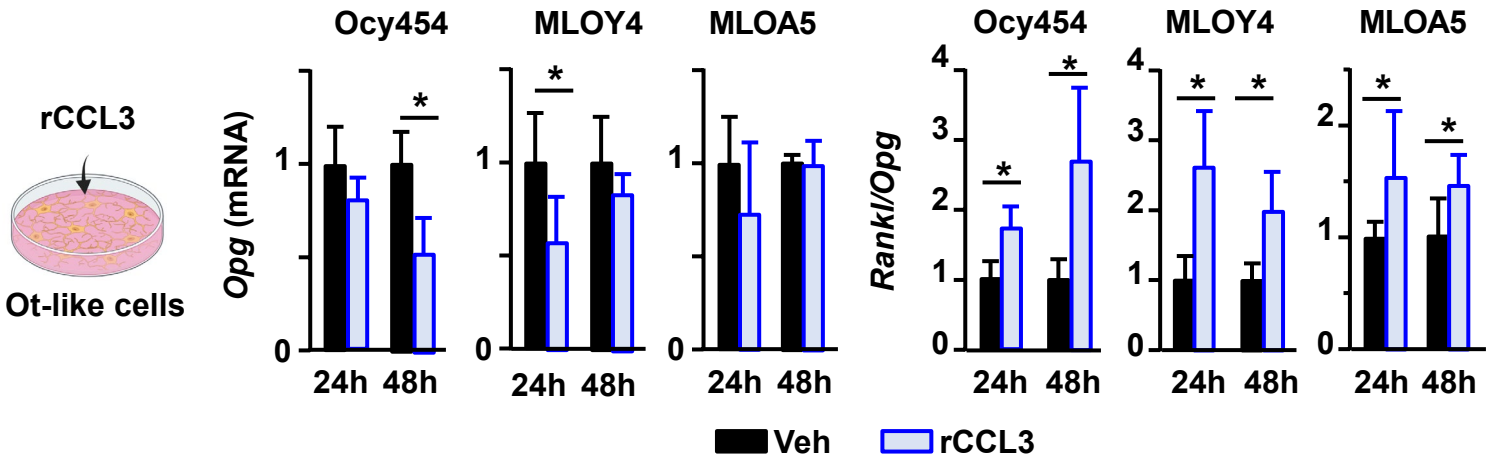
Suppl. Fig 1



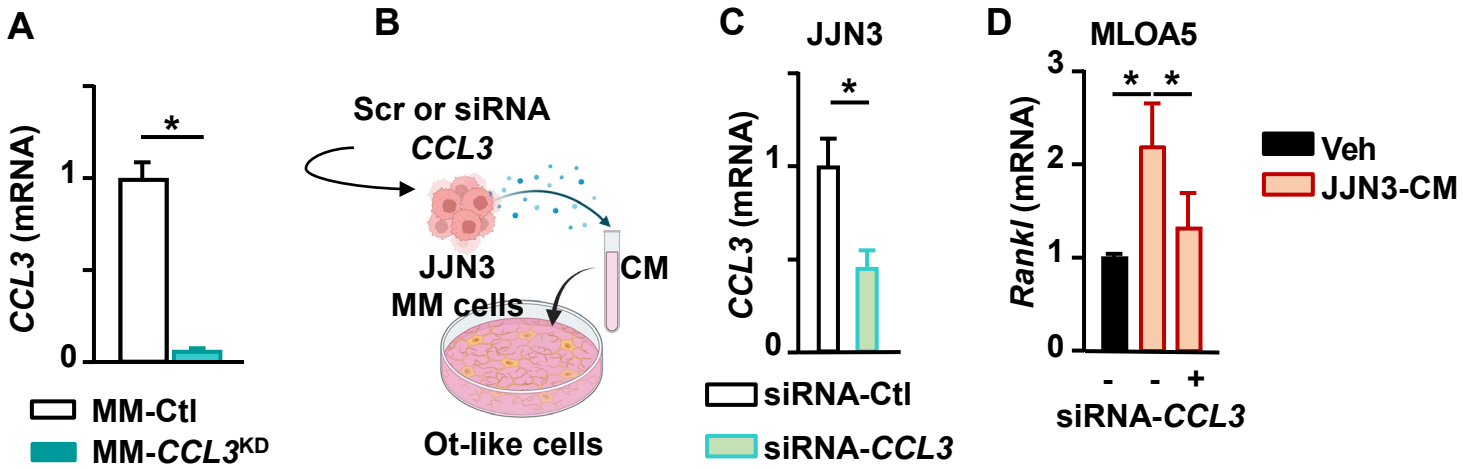
Suppl. Figure 1. Multiple myeloma cells increase the *Rankl/Opg ratio* in osteocytes. *Opg* expression and *Rankl/Opg* ratio in murine Ocy-454 (mature), MLO-Y4 (mature), and MLO-A5 (early) osteocyte (Ot)-like cell lines treated with 48h-conditioned media (CM) from human JJN3 cells (A) or murine 5TGM1 (B) multiple myeloma (MM) cells. N=4/group. *p<0.05 vs. cell treated with Veh by Student's *t*-test for each time point. Data are shown as mean ± SD. Representative experiments out of two are shown.

Suppl. Fig 2



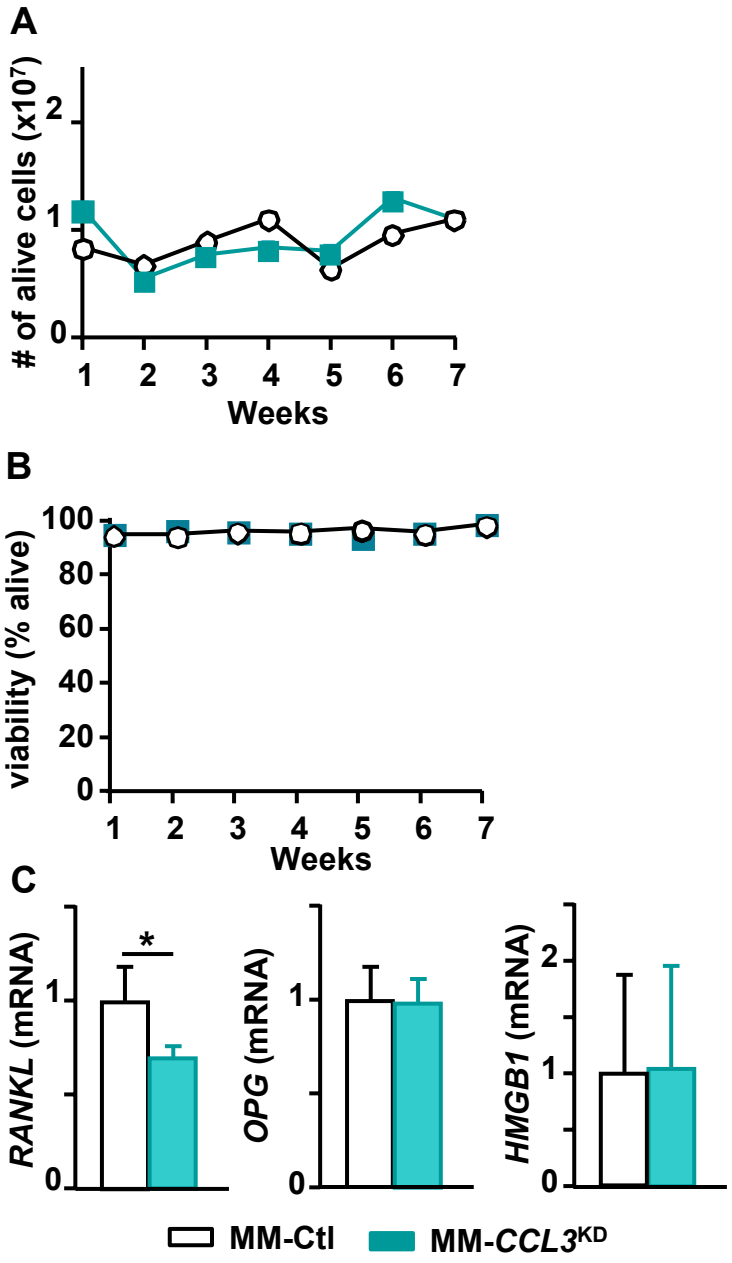
Suppl. Figure 2. Recombinant CCL3 increases the Rankl/Opg ratio in osteocytes. *Opg* expression and *Rankl/Opg* ratio in murine Ocy-454 (mature), MLO-Y4 (mature), and MLO-A5 (early) osteocyte (Ot)-like cell lines treated with recombinant CCL3 (1µg/mL). N=4-6/group. *p<0.05 vs. cell treated with Veh by Student's *t*-test for each time point. Data are shown as mean ± SD. Representative experiments out of two are shown.

Suppl. Fig 3

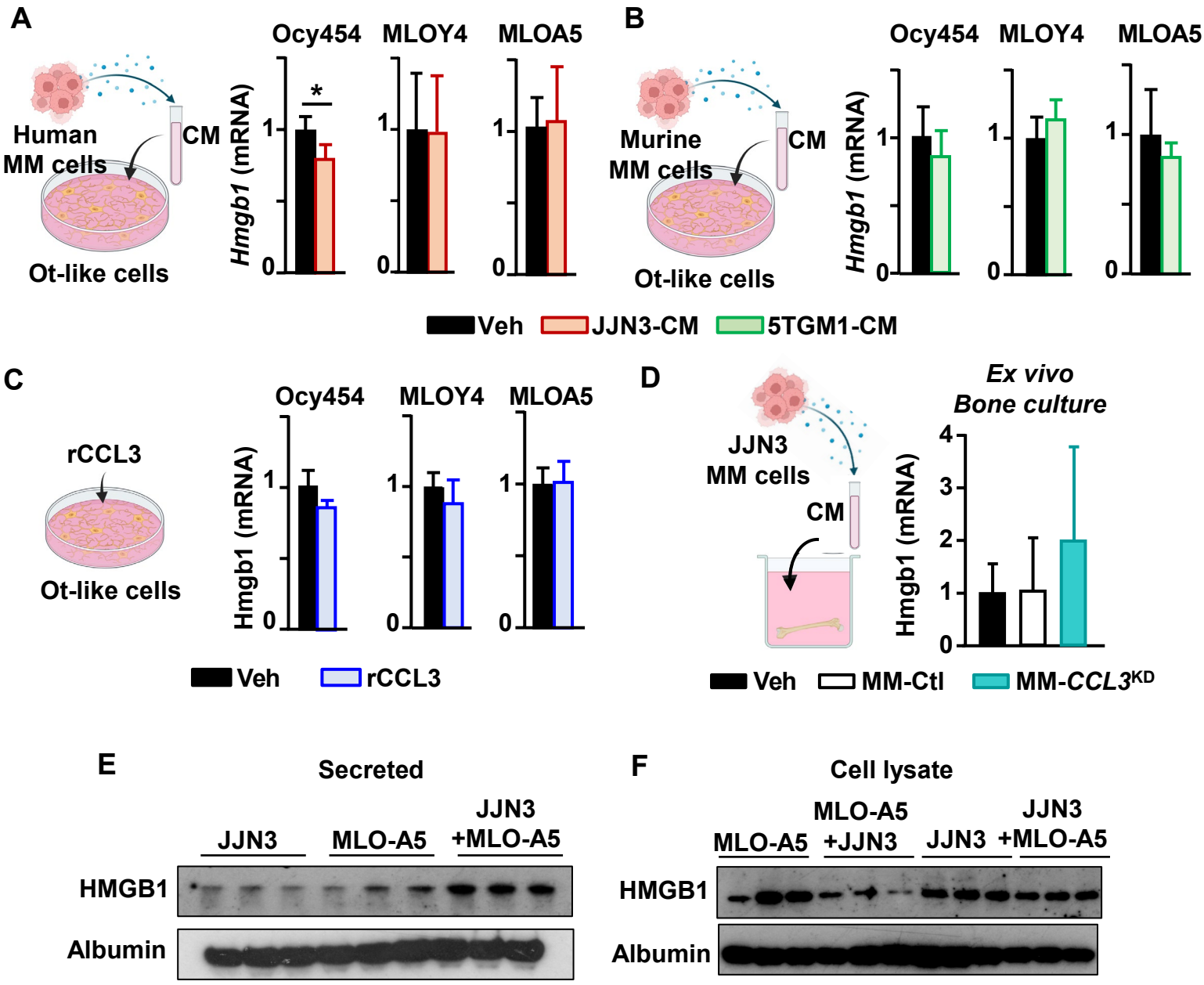


Suppl. Figure 3. siRNA-mediated inhibition of *CCL3* in multiple myeloma cells prevents *Rankl* upregulation in osteocytes by multiple myeloma cells. (A) CCL3 expression in control (Ctl) and CCL3 knockdown human JJN3 multiple myeloma (MM) cells. N=4/group. *p<0.05 vs. cells treated with Veh by Student's t-test. (B) Experimental design. (C) *CCL3* expression in human JJN3 MM cells treated with control (Ctl)- or *CCL3*-siRNAs for 48h. N=4-6/group. *p<0.05 vs. cell treated with Veh by Student's *t*-test. (D) *Rankl* expression in murine MLO-A5 osteocyte-like cells treated with Ctl- or *CCL3*-siRNA and cultured with/without 48h-CM from human JJN3 for 1 day. N=4-6/group. *p<0.05 as indicated by the lines by One-way ANOVA, followed by a Tukey post hoc test. Data are shown as mean ± SD. Representative experiments out of two are shown.

Suppl. Fig 4

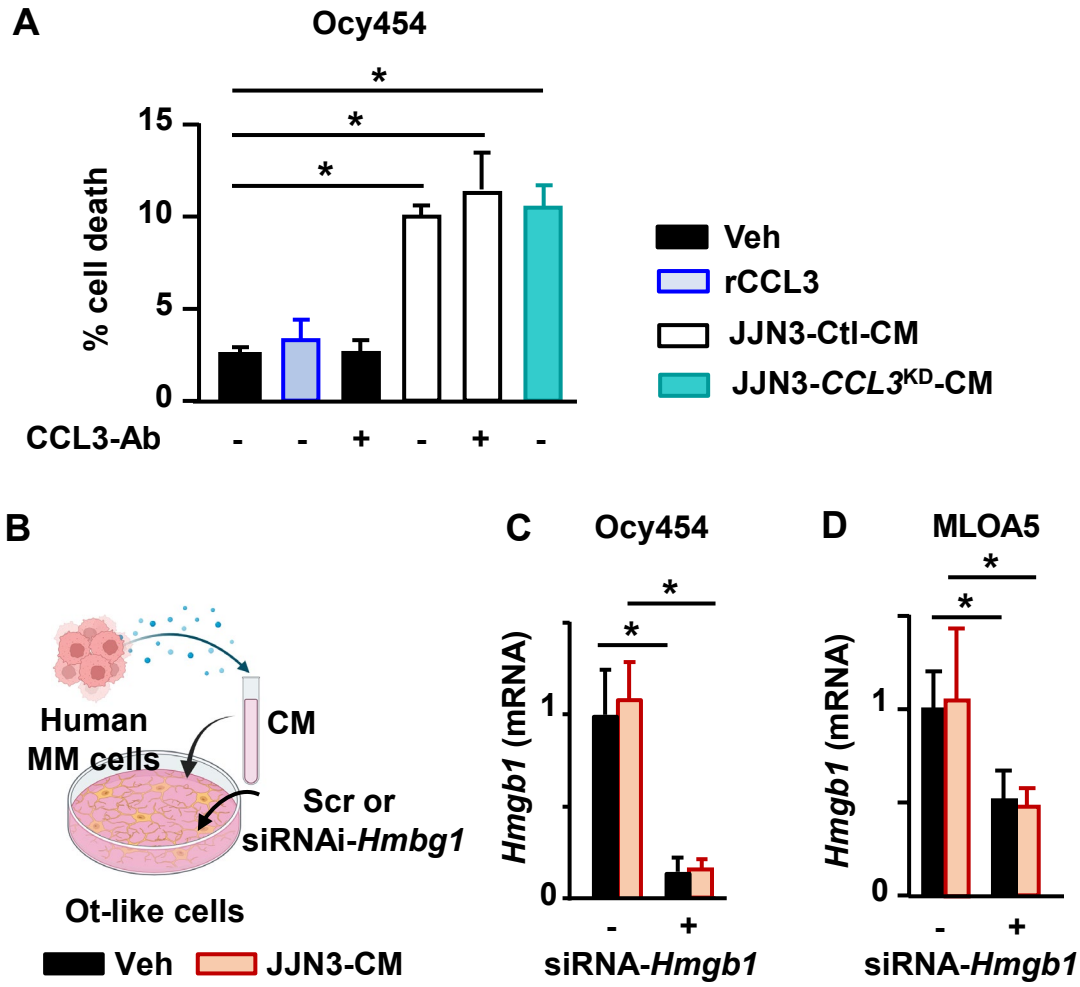


Suppl. Figure 4. Cellular and molecular characterization of human JJN3 multiple myeloma cells with *CCL3* knockdown. Cell number (A), cell viability (B), and (C) *RANKL*, *OPG*, and *HMGB1* expression in control (Ctl) and *CCL3* knockdown human JJN3 multiple myeloma (MM) cells. N=4/group. *p<0.05 vs. cells treated with Veh by Student's *t*-test. Data are shown as mean ± SD. Representative experiments out of two are shown.



Suppl. Figure 5. Multiple myeloma cells stimulate HMGB1 release by osteocytes. *Hmgb1* expression in murine Ocy-454 (mature), MLO-Y4 (mature), and MLO-A5 (early) osteocyte (Ot)-like cell lines treated with (A) 48h-conditioned media (CM) from human JJN3 multiple myeloma (MM) cells, (B) CM from murine 5TGM1-MM cells (B), or (C) recombinant (r) CCL3 (1μg/mL) for 1 day. N=4-6/group. *$p<0.05$ vs. cell treated with Veh by Student's *t*-test for each time point. (D) *Hmgb1* expression in murine bones cultured *ex vivo* and treated CM from control (Ctl) or *CCL3* knocked down (*CCL3*^{KD}) JJN3 cells for 2 days. N=4-6/group. (E) Secreted HMGB1 detected in the conditioned media of human JJN3 MM cells or MLO-A5 osteocyte-like cell cultures alone or co-cultured for 48h. (F) HMGB1 detected in cell lysates from human JJN3 MM cells or murine MLO-A5 osteocyte-like cells cultured alone or co-cultured for 48h. Data are shown as mean ± SD. Representative experiments out of two are shown (A, B, C, D).

Suppl. Fig 6



Suppl. Figure 6. Osteocytic HMGB1 release mediates the *Rankl* upregulation in osteocytes induced by MM cells. (A) Cell death in Ocy-454 osteocyte (ot)-like cells treated with recombinant CCL3, anti-CCL3 antibody (CCL3-Ab), or 48h-conditioned media (CM) from control (Ctl)- or *CCL3* knockdown human JJN3 MM cells for 1 day. N=4/group. *p<0.05 as indicated by the lines by One-way ANOVA, followed by a Tukey post hoc test. (B) Experimental design. *Hmgb1* expression in murine Ocy-454 (C) or MLO-A5 (D) Ot-like cell lines treated with/without control (Ctl)- or *Hmgb1*-siRNA and cultured in the presence/absence of 48h-CM from human JJN3 MM cells for 1 days. N=4/group. *p<0.05 as indicated by the lines by Two-way ANOVA, followed by a Tukey post hoc test. Data are shown as mean ± SD. Representative experiments out of two are shown.