Multiple Myeloma-derived MIP1α upregulates RANKL in osteocytes and contributes to bone loss and tumor progression.

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ABSTRACT & BACKGROUND

Multiple myeloma (MM) is a hematologic cancer characterized by the uncontrolled growth of malignant plasma cells in the bone marrow and devastating bone disease. Reciprocal communication between MM cells and cells of the tumor microenvironment (TME) drives bone destruction, which persists in MM patients even during remission. Current therapies, including bisphosphonates and denosumab, reduce skeletal-related events in patients with MM osteolytic disease. Still, long-term use of these drugs has adverse side effects, including complete shutdown of bone resorption. Thus, identifying new mechanisms to restore bone resorption to physiological levels is imperative to find new targets, expand the repertoire of therapeutic options, and reduce the potential negative side effects. We previously showed that MM cells stimulate local bone destruction by increasing osteocyte apoptosis and RANKL production, an essential cytokine for osteoclast formation. Yet, the molecular mechanisms underlying RANKL upregulation in Ot are unknown. MM cells produce abundant MIP1α, a pro-inflammatory chemokine associated with poor outcomes in MM and known to increase RANKL expression in osteoblastic cells. In this study, we studied the contribution of MM-derived MIP1α to the regulation of RANKL in osteocytes in the MM-TME. First, we determined that osteocytes express the well-established MIP1α receptors, CCR1, CCR3, and CCR5 using qPCR. Moreover, we found that treatment with recombinant MIP1α increased by ~2-fold RANKL expression in osteocytes and in ex vivo murine long bones containing primary osteocytes. Next, we showed that treating osteocytes with 25% conditioned media (CM) from human JJN3 MM cells or murine 5TGM1 MM cells resulted in a ~1.5-2-fold increase in RANKL expression. This effect was fully blocked by treatment with an anti-MIP1α antibody. We also observed that siRNA-mediated knockdown of MIP1α in MM cells blocked osteocytic RANKL regulation in vitro. Lastly, we generated a stable MM cell line with MIP1α knocked down ~95% (MM-MIP1α-KD) and inoculated mice intratibially with either saline, MM control cells, or MM-MIP1α-KD cells. Control cells increased the number of osteocytes expressing RANKL in cortical bone, whereas Knockdown of MIP1α in MM cells restored the prevalence of RANKL-positive osteocytes to levels in naïve mice. Consistent with this observation, the knockdown of MIP1α in MM cells mitigated bone loss and reduced tumor growth. These results suggest that MIP1α regulates osteocytic RANKL expression to promote bone destruction and tumor progression.

METHODS

Study population. We obtained gene expression data MM patients from the Multiple Myeloma Research Foundation (MMRF) CoMMpass registry (NCT01454297, version IA13). From an initial 921 patients with accessible gene expression data in the CoMMpass registry, 757 samples at diagnosis (NDMM) were selected. Salmon gene count data were imported into and normalized using the R package DESeq2. To determine patients with high versus low *MIP1α (CCL3)* expression, we compared the top vs. bottom quartiles. DESeq2 was used for differential expression analysis with an adjusted p-value cutoff of 0.05.

Cell Culture. Human JJN3 and murine 5TGM1 MM cells, and murine MLO-A5, MLO-Y4, and Ocy454 osteocyte-like cells were maintained at 37°C in a 5% CO2 incubator. MM cells and osteocytes were maintained in RPMI and alpha-MEM media, respectively. Cells were passaged every 72 hours. Conditioned media (CM) from JJN3/5TGM1 MM cells was collected after 48h of culture. To knockdown MIP1α, JJN3 MM cells were treated with 50uM siRNA-MIP1α (Origene, Technology Inc.; SR304274) for 24 hours prior to generation of CM. To knockdown MIP1α stably, JJN3 MM cells were transfected with CCL3 sgRNA CRISPR/Cas9 lentiviral vector and selected with 3.0ug/mL puromycin over 4 weeks (abm; NM_002983). Osteocytes were treated with 25% CM from 48h-culture of MM cells to osteocytes [**1**]. Gene expression profiles in human MM cell lines were obtained from Affymetrix GeneChip microarrays.

In vivo Myeloma Models. 7-week-old immunodeficient RADL mice were injected intratibially with 1x10^5 5TGM1 MM cells or saline. 7-week-old immunodeficient NSG mice were intratibially injected with saline, 1x10^5 JJN3 control MM cells or JJN3-MIP1α-KD cells in both tibias. Mice were sacrificed after 4 weeks. Tumor progression was monitored via secreted paraprotein analysis via ELISA (Human Kappa or IgG2B) and bone health was monitored by X-ray or in vivo microCT analysis.

RNA Scope analysis was performed with RANKL probe as described by the manufacturers (ACD Biotechne; 410921)

Ex vivo Bone Culture. Tibiae and/or femur were harvested from RADL or NSG mice and cultured in MEM medium with treatments for 24h. Bones were treated with 1ug/mL rMIP1α (R&D Systems Inc.; 270-LD-010/CF) for 24 hours.

Gene Expression. After 4h or 24h of treatment with 200ng/mL or 500ng/mL rMIP1α, MM-CM, or 0.05μg/mL anti-MIP1α antibody (R&D Systems Inc.; MAB270-500), MM cells, osteocyte like cells or long bones underwent total RNA isolation using Trizol. RNA was converted to cDNA and gene expression was quantified by qPCR using Taqman assays [**1**]. Values were normalized by Gapdh or ChoB expression. Gene expression levels were calculated using the comparative threshold (CT) method.

Western Blots. Protein was loaded in 10% SDS-PAGE gels. Proteins were transferred to PVDF membranes. Immunoblots were performed using anti-RANKL followed by goat anti-rabbit secondary antibodies, conjugated to horseradish peroxidase (1:2000). Western blots were developed using the chemiluminescence detection assay.

Statistical Analysis. Statistical analysis was performed using GraphPad. All the results are presented as mean (SD).

ACKNOWLEDGEMENTS

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Figure 1

MM cells increases RANKL expression in Osteocytes

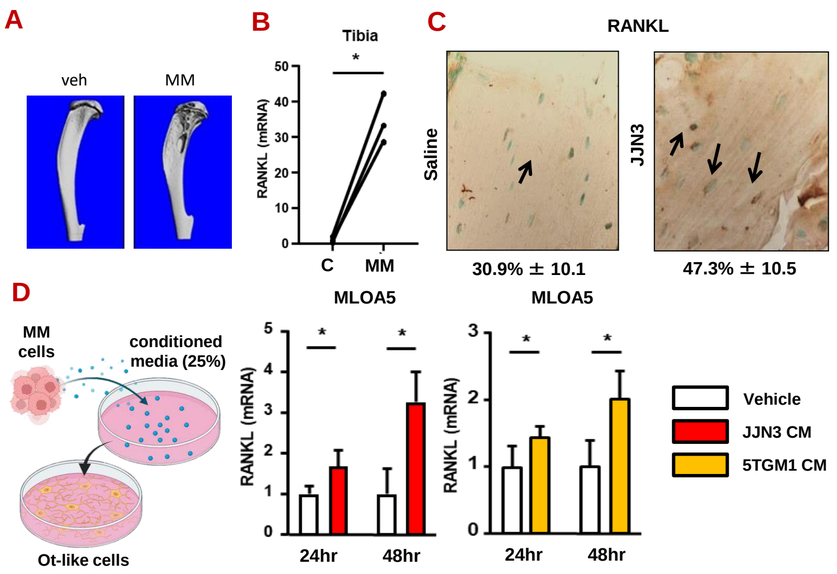

Figure 1. **(A)** uCT of tibia inoculated with saline or JJN3 MM cells. **(B)** RANKL mRNA expression in tibia of mice 4wks after inoculation with 5TGM1 MM cells. **(C)** IHC of RANKL in bones of mice inoculated with saline or JJN3 MM cells. **(D)** RANKL mRNA expression in MLO-A5 cells treated with or without JJN3 CM or 5TGM1 CM, Data are represented as comparative threshold normalized by GAPDH expression with fold change expression normalized to vehicle. Representative experiments out of 3. N=4-6/group. *p<0.05 by T-Test.

Figure 2

MIP-1α is expressed by MM cells and linked with worse survival in newly diagnosed MM patients

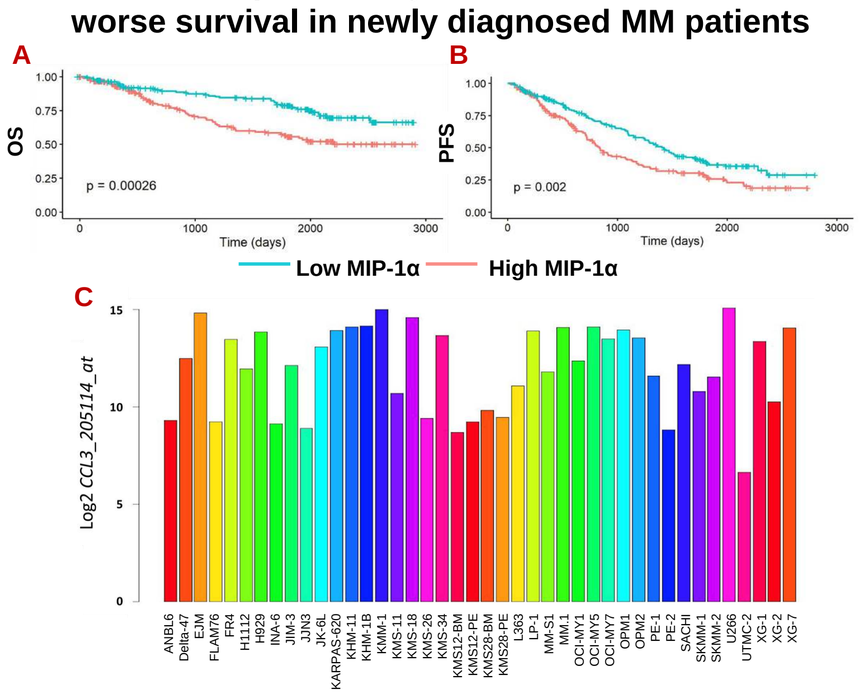

Figure 2. **(A)** overall survival (OS) and **(B)** progression-free survival (PFS) of newly diagnosed MM patients (NDMM) from the CoMMpass cohort (N=757 patients) with high vs low expression of MIP-1α in CD138+ plasma cells. **(C)** mRNA expression of MIP-1α in human MM cell lines.

Figure 3

Osteocytes express MIP-1α receptors and rMIP-1α increases osteocytic RANKL expression

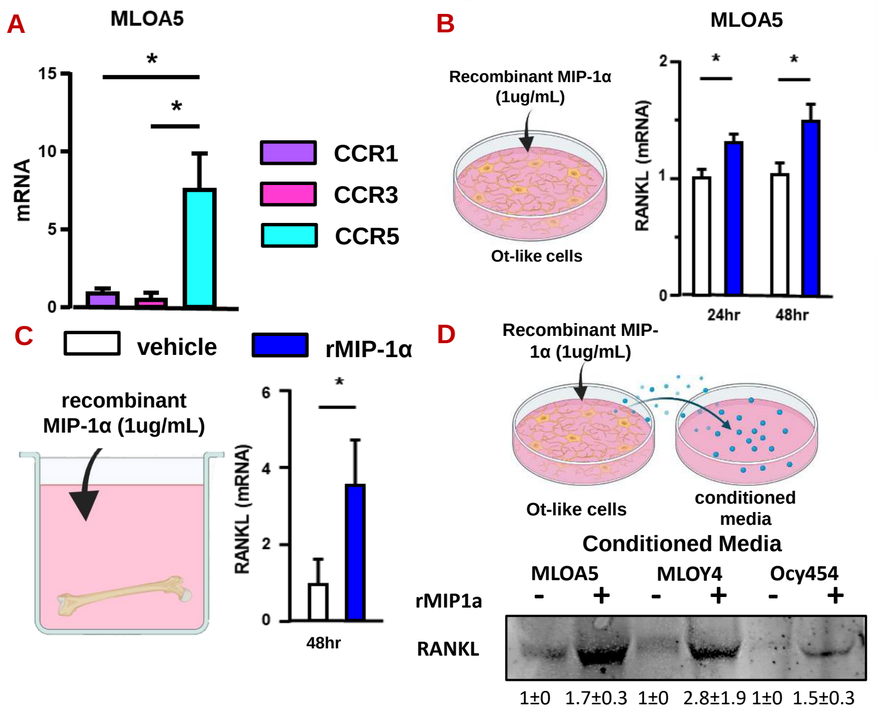

Figure 3. **(A)** mRNA expression of CCR1/3/5 in MLOA5 osteocyte-like cells. RANKL in **(B)** MLOA5 osteocyte-like cells and **(C)** ex vivo long bones treated +/- recombinant MIP-1α (rMIP-1α). **(D)** RANKL protein expression in osteocyte-like cells treated +/- rMIP-1α. Gene expression data is expressed as comparative threshold normalized by GAPDH expression and fold change normalized to CCR1 or vehicle (a-b) (d). Representative experiments out of 3. n=3-4/group; *p<0.05 **p<0.01 vs vehicle/CCR1 by ANOVA or T-Test.

Working Model

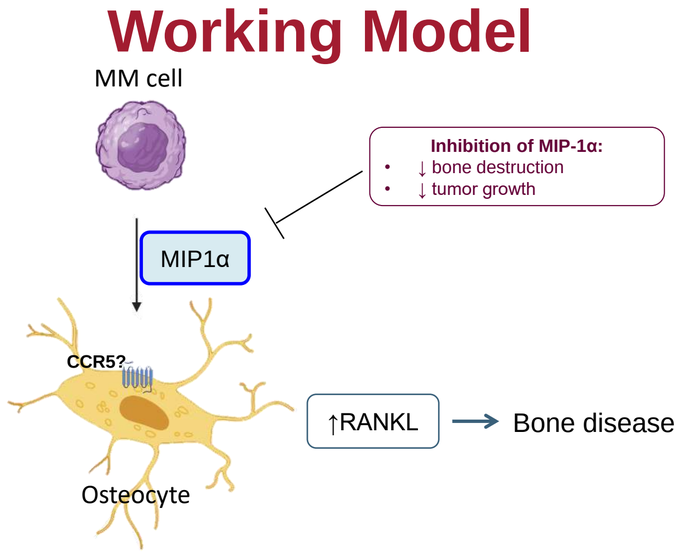

Figure 4

Pharmacological or genetic inhibition of MIP-1α prevents osteocyte RANKL upregulation by MM cells

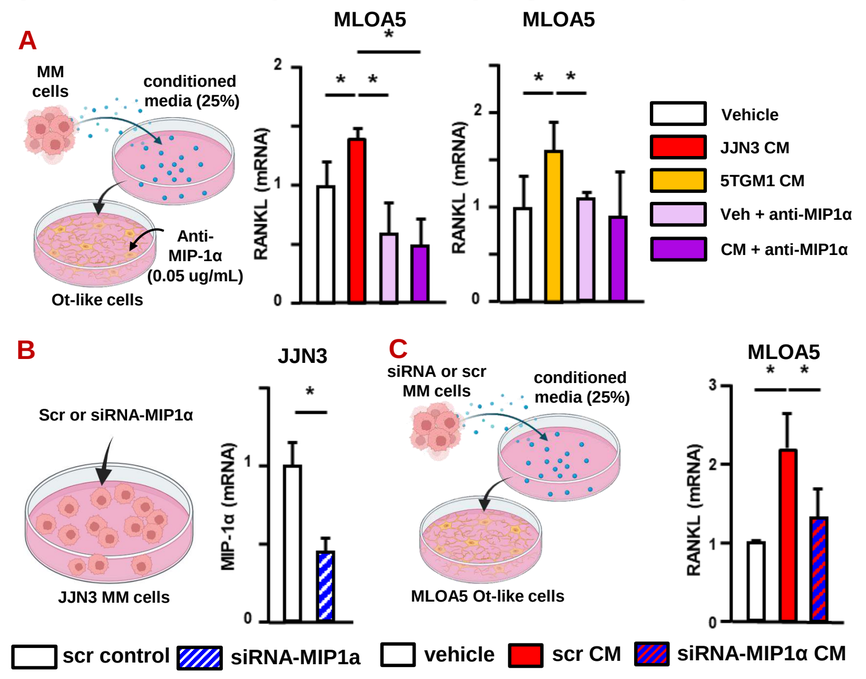

Figure 4. RANKL mRNA expression in MLOA5 cells +/- **(A)** JJN3 CM or 5TGM1 CM and an anti-MIP-1α antibody. **(B)** Knockdown of MIP-1α in JJN3 MM cells with siRNA. **(C)** RANKL mRNA expression in MLOA5 cells treated +/- CM generated from the knockdown cells. Data are represented as comparative threshold normalized by GAPDH expression with fold change expression normalized to vehicle. Representative of 2-3 experiments. N=4-6/group. *p<0.05, **p<0.01 by ANOVA .

Figure 5

MIP-1α Knockdown in MM cells reduces tumor burden and bone destruction in a MM mouse model

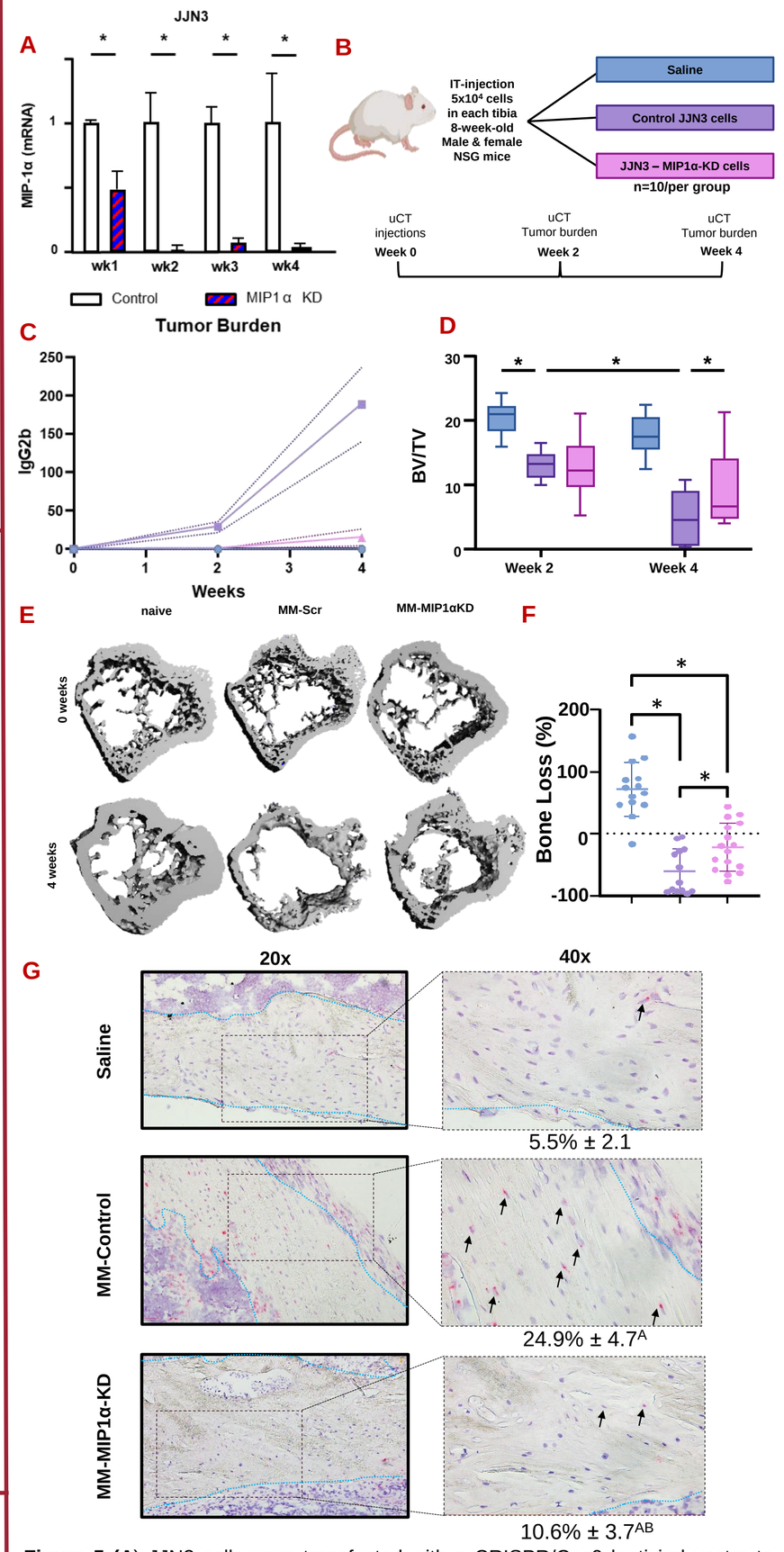

Figure 5 **(A)** JJN3 cells were transfected with a CRISPR/Cas9 lentiviral vector to generate a stable cell line with MIP1α knocked down. **(B)** Mice were inoculated with saline, JJN3 control cells or JJN3 MIP-1α-KD cells. **(C)** tumor burden was monitored by IgG2b serum paraprotein. **(D-F)** progression of bone disease was monitored by in vivo microCT analysis. **(G)** RNAScope images probed for **RANKL** quantified as % RANKL-positive osteocytes in cortical bone. *^{A}p<0.05 by saline control ^{B}p<0.05 by MM-control and by ANOVA .

Conclusions

1. MM cells secrete MIP1α, which regulates RANKL expression in osteocytes.
2. Blockade of MIP1α in MM results in reduced progression of tumor, bone disease, and osteocyte RANKL expression.