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Fcγ Receptor Targeting Reduces Bone Disease in a Pre-clinical Model Of Multiple Myeloma.

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Introduction

Multiple Myeloma (MM) is currently incurable, with MM-associated bone disease (MM-OBD), representing a major cause of morbidity and mortality in MM patients. Despite therapies for MM-OBD exhibiting significant potential (e.g. Zoledronate), their clinical use has been restricted due to severe treatment-associated toxicities. Safe novel therapies for MM-OBD are therefore required. Cross talk between receptor activator of nuclear factor Kappa B ligand (RANKL), present on and secreted by MM plasma cells (PCs), and its corresponding receptor (RANK) on osteoclast (OC) precursors (OCPs) is a key mechanism driving osteoclastogenesis and subsequent bone pathology in MM. Our previous studies have demonstrated that Fcγ receptor (FcγR)-mediated signals via *Staphylococcus Aureus; Sp*A-IgG complexes (SIC), inhibit RANKL induced osteoclastogenesis *in vitro*[1]. Additionally, preliminary study findings show that FcγR-mediated signalling in pre-OCs reduce MM PC driven osteoclastogenesis *in vitro*. Interrogation of the underlying molecular mechanisms, show that FcγR-mediated signals profoundly reduce RANK transcript and protein expression, in pre-OCs. However, the effects of FcγR engagement on MM-OBD *in vivo*, and the FcγR induced mechanisms responsible for RANK inhibition have still to be elucidated.

Aims

1. Determine the *in vivo* potential of targeting FcγRs to treat Multiple Myeloma Bone disease.
2. Elucidate the mechanisms underlying FcγR-mediated down-regulation of RANK expression in osteoclast precursors.

Results

Multiple Myeloma (MM) induced osteoclastogenesis is inhibited by Small Immune Complexes (SIC) in a pre-clinical murine model of MM.

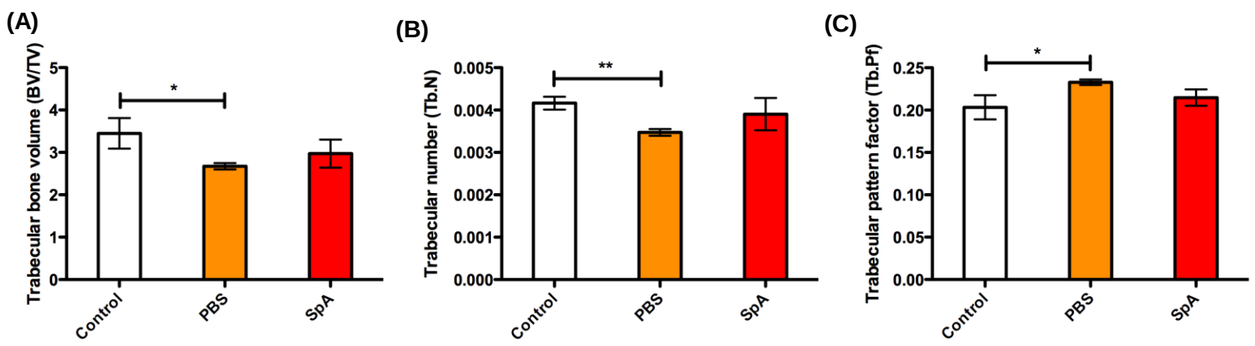

***Fig 1* : μ-CT analysis of bone disease in the 5T-GM1 murine model of MM following SIC treatment**
(A-C) Micro-CT analysis of femurs from control (non-myeloma injected mice) and 5TGM1-GFP myeloma bearing mice (26 days post myeloma cell injection), treated with PBS (vehicle control) or SpA. (A) Data represents mean ± SEM of 3 control mice, 5 PBS and 5 SpA treated mice. Significance was determined by an unpaired student t test. **, p < 0.05; **, p < 0.01.*

Exposure of pre-OCs to SIC leads to differential expression of genes implicated in bone homeostasis.

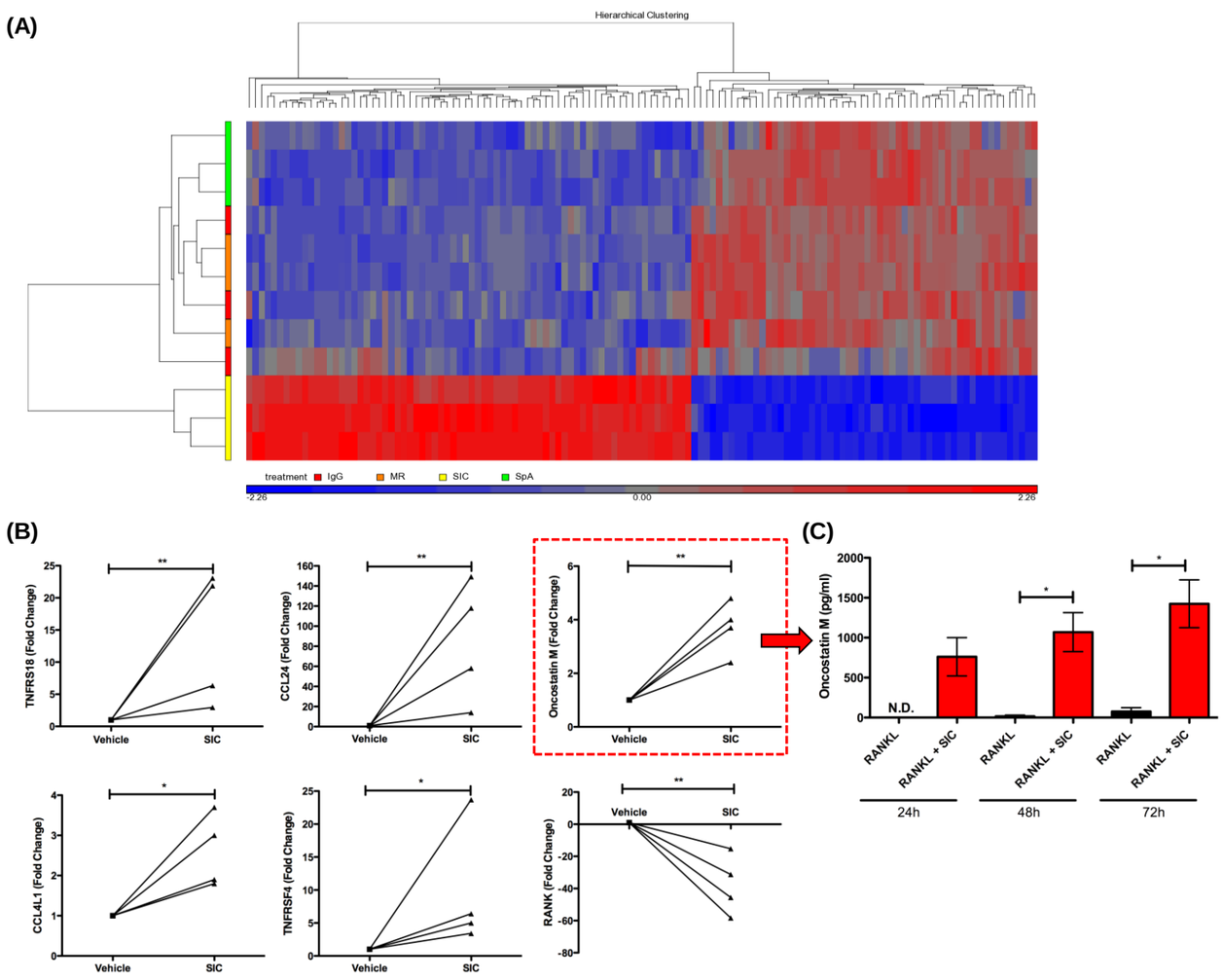

***Fig 2* : Identification of genes differentially regulated by SIC in pre-OCs via microarray analysis.**
(A) CD14$^+$monocytes derived from healthy individuals were cultured with M-CSF for 24h followed by exposure to RANKL for a further 24h in the presence or absence of SIC or the appropriate controls (IgG or SpA). RNA was extracted and then assessed for quality and integrity via the Agilent Bioanalyzer 6000 Nano LabChip platform. The total RNAs were amplified and biotinylated using Illumina TotalPrep RNA kit following hybridization by Whole-Genome Gene Expression Direct Hybridization Assay to Illumina HumanHT-12 V4.0 beadchip. The hybridized arrays were scanned on the Illumina BeadArray Reader and raw data was quantile normalized with Illumina GenomeStudio (V2011.1, Illumina Inc., San Diego, CA, USA). Control (M-CSF/RANKL) and treatment/Isotype Control (SIC, SpA or IgG) groups were generated with three independent biological replicates per group (paired samples from three different donors). (B) Validation of hits from panel A by qPCR of CD14$^+$monocytes exposed to conditions outlined in (a). QPCR confirmed significant upregulation of *Oncostatin M, TNFRS4, TNFRS18, CCL4L1, CCL24 and* a decrease in *RANK* gene expression by SIC. Log transformed (Y=Log(Y)) data (n=4 independent experiments) are shown, which were analysed using a ratio t test. (C) CD14+ monocytes were set up as in (a) and cultured with M-CSF for 24h, cells were then cultured with RANKL for 24, 48 and 72 hrs in the presence or absence of SIC. Supernatants were then collected and Oncostatin M protein levels determined by ELISA. Data from 4 independent experiments are shown, which were analysed using a paired t test. **, p < 0.05; **, p < 0.01.* **N.D**. = Not detectable.

SIC reduces RANK gene and protein expression and suppresses RANKL induced signaling in pre-OCs.

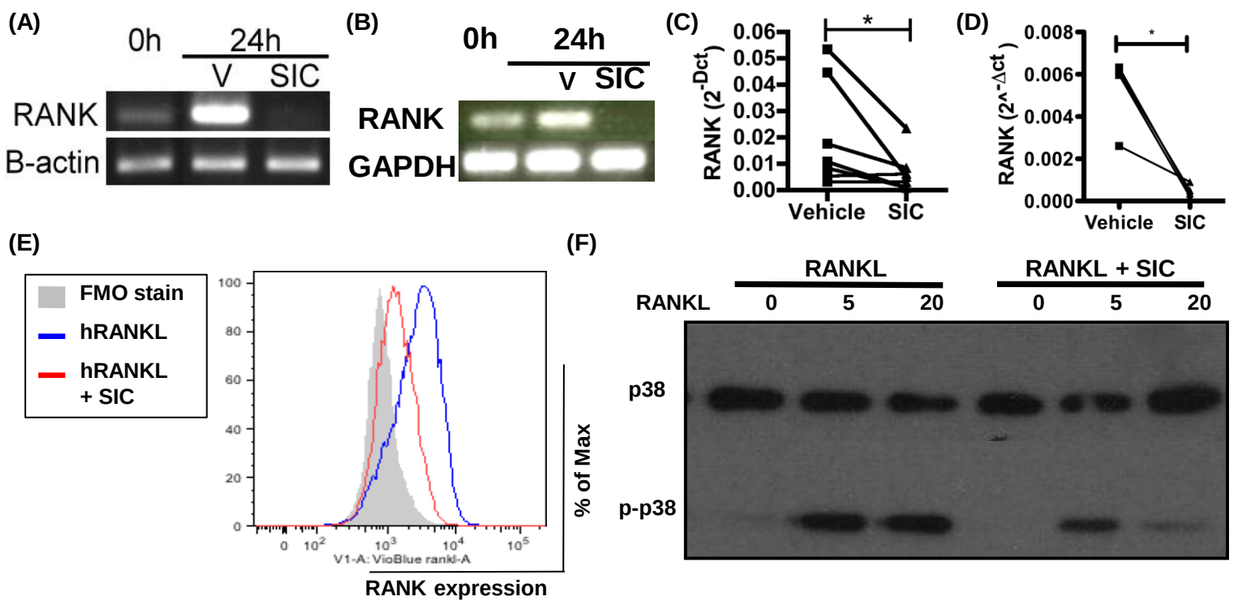

***Fig 3* : SIC downregulates RANK expression at the transcript, protein and functional level.**
(A) CD14$^+$monocytes derived from healthy (A) and Multiple Myeloma individuals (B) were cultured with M-CSF for 24h, followed by exposure to RANKL for a further 24h in the presence (SIC) or absence of SIC (V = vehicle control) and PCR performed to assess the levels of RANK mRNA by PCR. (C) Monocytes obtained from healthy donors (C) and MM patients (D) were exposed to conditions stated in A, and RANK mRNA expression determined by qPCR. Data shown are representative of 7 (healthy) and 4 (Multiple Myeloma) independent experiments. (E) Monocytes derived from healthy individuals were cultured similar to conditions stated in A, and in the presence (red line) or absence of SIC (blue line). Cultures were performed in duplicates and after 2 days in culture cells were stained for human RANK expression (binding of pacific blue labeled RANKL). (F) CD14+ monocytes derived from healthy donors were treated with M-CSF and RANKL for 3 days in the presence or absence of SIC and serum starved for 6 hr. After re-incubation with RANKL (100ng/ml) for 5 or 20 mins, whole cell lysates were created and probed with antibodies against total p38 and phosphorylated p38. Data represent 3 independent experiments.

SIC treatment leads to a loss in a specific chromosome loop at the RANK promoter.

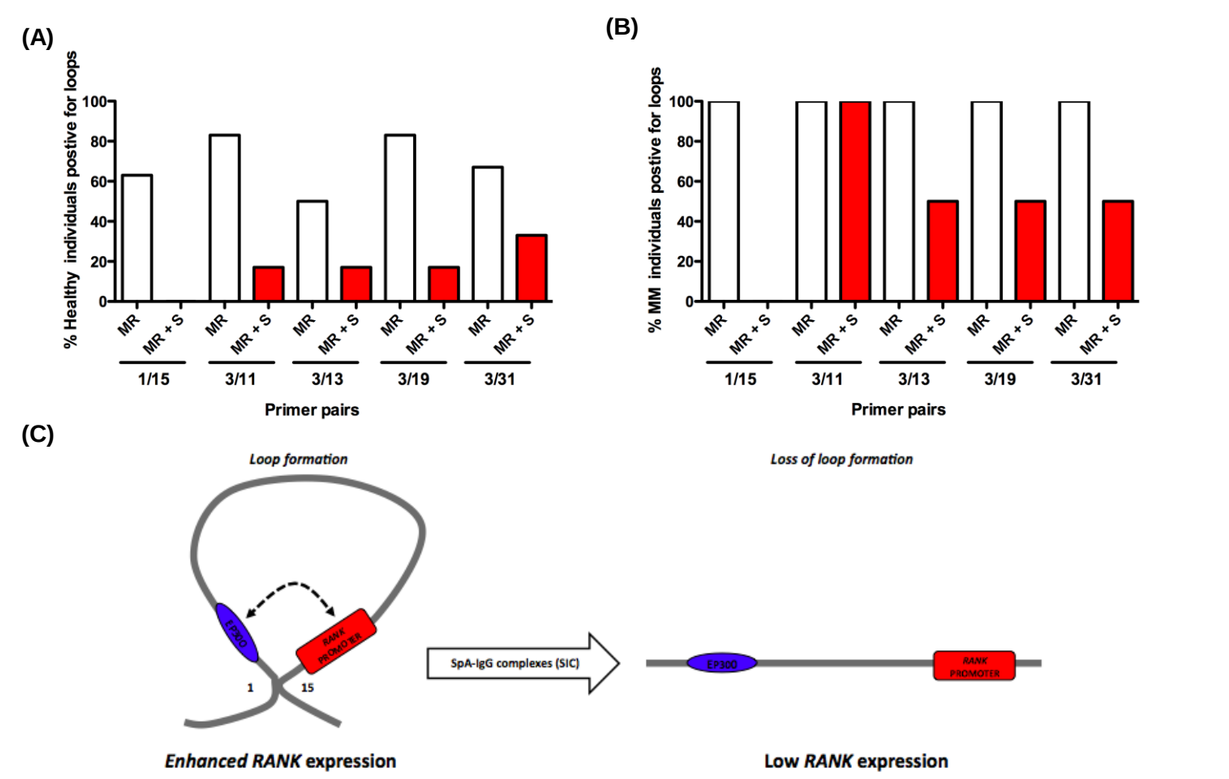

***Fig 4* : 3C analysis shows loss of a specific chromosome loop in pre-OCs after SIC treatment.**
CD14+ monocytes isolated from healthy donors (A) and MM patients (B) were cultured with M-CSF for 24h, followed by treatment with RANKL for a further 24h in the absence (MR) or presence of SIC (MR+S). Cells were processed and analyzed via chromosome conformation capture (3C) assay. In brief, EpiSwitch^{TM} sites within and around the RANK promoter were identified and loop status determined by the 3C assay using specific primer pairs. Analysis of both healthy (n = 6-8) and MM (n = 2) samples show that SIC treatment leads to a failure in chromosome loop formation between sites identified by primers 1 and 15. (C) A schematic illustrates of the impact of SIC treatment on a specific chromosome loop at the RANK promoter. Importantly, genome analysis revealed that formation of a loop between site 1 and site 15 results in the presence of a p300 super-enhancer regions in close proximity to the RANK promoter.

Conclusions

- SIC suppresses MM driven bone disease *in vivo*.
- FcγR engagement produces bone protective effects via differential gene expression.
- Exposure to SIC leads to the loss of a specific chromosome loop at the RANK promoter in healthy and MM pre-OCs.
- FcγR targeting represents a potential novel therapeutic strategy for treating bone disease in MM.

worldwide cancer research formerly known as AICR

1. MacLellan, L. M. et al. Co-opting endogenous immunoglobulin for the regulation of inflammation and osteoclastogenesis in humans and mice. Arthritis Rheum. **63,** 3897–3907 (2011).