Title

Ionizing Radiation Stimulates Expression of Pro-Osteoclastogenic Genes in Marrow and Skeletal Tissue

Running Title: Ionizing Radiation and Skeletal Cytokine Gene Expression

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Abstract

Exposure to ionizing radiation can cause rapid mineral loss and increase bone-resorbing osteoclasts within metabolically-active, cancellous-bone tissue leading to structural deficits. To better understand mechanisms involved in rapid, radiation-induced bone loss, we determined the influence of total-body irradiation on expression of select cytokines known both to stimulate osteoclastogenesis and contribute to inflammatory bone disease. Adult (16wk), male C57BL/6J mice were exposed to either 2Gy gamma rays (^{137}Cs, 0.8Gy/min) or heavy ions (^{56}Fe, 600MeV, 0.50-1.1Gy/min); this dose corresponds to either a single fraction of radiotherapy (typical total dose is ≥10Gy) or accumulates over long-duration, interplanetary missions. Serum, marrow, and mineralized tissue were harvested 4hrs-7d later. Gamma irradiation caused a prompt (2.6-fold within 4hrs) and persistent (peaking at 4.1-fold within 1d) rise in the expression of the obligate osteoclastogenic cytokine, receptor activator of nuclear factor kappaB-ligand (*Rankl*) within marrow cells over controls. Similarly, *Rankl* expression peaked in marrow cells within 3d of iron exposure (9.2-fold). Changes in *Rankl* expression induced by gamma irradiation preceded and overlapped with a rise in expression of other pro-osteoclastic cytokines in marrow (e.g., monocyte chemotactic protein-1 increased 11.9-fold, tumor necrosis factor-alpha increased 1.7-fold over controls). Marrow expression of the RANKL decoy receptor, osteoprotegerin (*Opg*), also rose after irradiation (11.3-fold). The ratio *Rankl/Opg* in marrow was increased 1.8-fold, a net pro-resorption balance. As expected, radiation increased a serum marker of resorption (tartrate resistant acid phosphatase) and led to cancellous bone loss (16% decrease in bone volume/total volume) through reduced trabecular struts. We conclude that total-body irradiation (gamma or heavy-ion) caused temporal, concerted regulation of gene expression within marrow and mineralized tissue for select cytokines which are responsible for osteoclastogenesis and

elevated resorption; this is likely to account for rapid and progressive deterioration of cancellous microarchitecture following exposure to ionizing radiation.

Introduction

During spaceflight beyond the Earth's protective magnetosphere, astronauts are exposed to a complex mixture of ionizing radiation (Durante and Cucinotta 2011), including low linear-energy-transfer (LET) gamma-rays and protons, as well as more damaging high-LET radiation. Exposure to simulated space radiation is characterized by relatively low doses (≤2Gy) of ion species due to Solar Particle Events (SPEs) (Parsons and Townsend 2000), (Shurshakov and others 1999) or galactic cosmic rays (Hassler and others 2014; Zeitlin and others 2013). Simulated space radiation at these doses can cause acute and adverse effects within the skeletal tissue of the rodent (Hamilton and others 2006), (Kondo and others 2010), (Yumoto and others 2010), (Alwood and others 2010), (Lloyd and others 2012). Doses in the range of 1-2 Gy also are relevant to radiotherapy; total therapeutic doses can vary, but total body doses of 10-15 Gy typically are fractionated into single doses of ~2Gy which ultimately can lead to increased fracture incidence (Baxter and others 2005). Radiation exposure, in particular, to high-LET particles, may exacerbate the deleterious effects of musculoskeletal disuse (Keyak and others 2009; Lang and others 2004; Lang and others 2006; LeBlanc and others 2000), which occurs during prolonged bed rest or spaceflight.

Bone-resorbing osteoclasts are thought to cause the rapid (Kondo and others 2009), (Willey and others 2010), (Turner and others 2013), (Alwood and others 2012) cancellous strut losses following simulated space-irradiation (≤ 2Gy). Radiation increases the numbers of osteoclasts and the extent of cancellous surfaces covered by osteoclasts. However, the role that receptor activator of nuclear factor kappa-B ligand (RANKL), the principal osteoclastogenic cytokine, plays in concert with other pro-osteoclastic inflammatory cytokines (Takayanagi 2007), (Boyce and Xing 2008), (Kim and others 2006) is not fully understood with respect to the

rapidity of cancellous bone loss (Kondo and others 2009). Therefore, we examined the temporal expression within both marrow and mineralized tissue of *Rankl* and select pro-osteoclastogenic cytokines implicated in various models of inflammatory bone loss (Braun and Schett 2012), following low- and high-LET space-like radiation exposure.

We hypothesized that radiation exposure induces expression of pro-osteoclastogenic genes related to inflammation within both marrow and mineralized tissue compartments, increases markers of bone resorption, and is likely to contribute to later cancellous bone loss. This work shows an acute and time-dependent elevation of *Rankl,* osteoprotegerin *(Opg),* monocyte chemotactic protein-1 *(Mcp1),* and tumor necrosis factor alpha (*Tnf)* gene expression in the marrow and skeletal compartments due to low- or high-LET irradiation; these changes precede (before 3 days) manifestation of bone loss (3-7 days) following iron irradiation at a dose relevant to fractionated radiotherapy or space missions.

Materials and Methods

Animals

Post-pubescent (16 weeks ± 4 days at time of irradiation), male, C57BL/6J mice (Jackson Labs) were individually housed and provided food (LabDiet 5001, St. Louis, MO) and water *ad libitum*, as described elsewhere (Yumoto and others 2010). Animals were euthanized by CO_2 inhalation or anesthetized with isoflurane followed by blood draw via cardiac puncture. The Institutional Animal Care and Use Committees for NASA Ames Research Center and Brookhaven National Lab approved all procedures.

Experiment Design and Radiation Exposure

Experiments were conducted to determine the temporal changes in the levels of key genes and circulating proteins related to bone resorption in the latency period prior to the onset of overt structural loss. To evaluate heavy-ion effects, conscious mice were exposed to high-LET iron ions (^{56}Fe, 600 MeV/ion, 5cGy or 2Gy, 5 or 0.50 - 1.10 Gy/min, respectively) at the NASA Space Radiation Lab, Brookhaven National Lab or were sham-irradiated as previously described (n=6-8/group). Mice were euthanized and tissues harvested 3 or 7 days after exposure. To evaluate gamma radiation effects, conscious mice were irradiated with low-LET ^{137}Cs gamma rays (2Gy, 0.80 Gy/min, as described in detail in (Kondo and others 2010)), or were sham-irradiated. Euthanasia and tissue harvest took place 4 hours or 1 day (± 2 hours), 3 days, or 7 days after exposure (n=5-7/group).

Bone Microarchitecture

Bone volume and microarchitecture of the proximal tibial metaphysis were quantified by microcomputed tomography (6.8 μm pixel size, 3500 ms integration time, 50 kV, Skyscan 1174, Bruker microCT, Kontich, Belgium), similar to (Kondo and others 2009). Briefly, a 1.0 mm–thick region located 0.24 mm distal to the proximal growth plate of the tibia was selected and semi-autonomously contoured to include cancellous tissue. To assess bone loss, the bone volume to total volume fraction (BV/TV, %), trabecular thickness (mm), and trabecular number (TbN, 1/mm) were calculated and reported following the convention of Bouxsein, et al (Bouxsein and others 2010).

qRT-PCR for Gene Expression within Marrow and Skeletal Tissue

Femora and tibiae were dissected, cleaned of soft tissues, flushed of bone marrow with PBS, and stored in RNALater (Qiagen, Valencia, CA) at -80C. Bone marrow cells were lysed and preserved with guanidine-thiocyanate-containing RLT buffer (Qiagen, Valencia, CA) with 1% beta-mercaptoethanol at -80C. RNA was extracted from homogenized bone or marrow lysates using Trizol (Ambion, Carlsbad, CA, USA), QIAshredder, and RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA). For each tissue, RNA was treated with RNase-free DNase Set (Qiagen, Inc., Valencia, CA, USA) in accordance to manufacturer's instructions. RNA quality and quantity were determined using a spectrophotometer (NanoDrop, Wilmington, DE, USA). The RNA quality was confirmed by electrophoresis using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Following manufacturer's recommendations, RNA was reversed transcribed and simultaneously used for qPCR using GoTaq® Probe 1-Step RT-qPCR System (Promega, Madison, WI, USA). Portions of the following mouse gene sequences were amplified using Taqman gene expression assays (Applied Biosystems, Inc., Foster City, CA, USA): receptor activator of nuclear factor kappa-B ligand (*Rankl*, assay ID: Mm00441906_m1), Osteoprotegerin (*Opg*, assay ID: Mm01205928_m1), Tumor necrosis factor alpha (*Tnf*, assay ID: Mm00443260_g1), monocyte chemotactic protein-1 (*Mcp1,* assay ID: Mm00441242_m1), interleukin-6 (*Il6,* assay ID Mm00446190_m1), tartrate-resistant acid phosphatase (*Acp5,* assay ID: Mm00475698_m1), cathepsin-K (*Ctk,* assay ID: Mm00484039_m1), nuclear factor of activated T-cells, cytoplasmic 1 (*Nfatc1,* assay ID: Mm00479445_m1), and colony stimulating factor 1 (*Csf1,* assay ID: Mm00432686_m1). We standardized expression levels to mitochondrial ribosomal protein L19 (L19, assay ID: Mm02601633_g1) to facilitate comparison among samples. Hypoxanthine-guanine phosphoribosyltransferase (*Hprt1*, assay ID: Mm01545399_m1)

and transmembrane protein 40 (*Tmem40*, assay ID: Mm00460636_m1) were analyzed as alternate housekeeping genes. The reactions were performed in the 7300 RT-PCR System (Applied Biosystems, Foster City, CA) or SmartCycler Real-Time PCR System (Cepheid, Sunnyvale, CA, USA).

We analyzed multiple candidate housekeeping genes for normalization, including *L19*, *Hprt1*, and *Tmem40*. Gamma radiation exposure did not modulate levels of the gene *L19* at the various time points, but transiently and modestly increased gene expression of *Hprt1* (-0.4 cycles, 1.4-fold) and *Tmem40* (-1 cycle, 2.3 fold). Following iron irradiation, *L19* (as well as *Hprt1*) showed small increases in cycle number due to treatment (-0.4 cycles, 1.4 fold for *L19*). As these differences housekeeping genes were small relative to those of cytokine and resorption marker levels, gene expression results reported were normalized relative to *L19* for both gamma and iron irradiation experiments.

Serum TRACP 5b

Blood was collected from the heart at the time of euthanasia and serum was separated and stored at -80°C until processed. Enzyme immunoassays were performed for measurement of tartrate-resistant acid phosphatase 5b (TRACP 5b), a biomarker for osteoclast-mediated bone resorption, using a commercial kit (Immunodiagnostic Systems, Fountain Hills, AZ) and according to the manufacturer protocol.

Statistics

All data are reported as mean ± standard deviation. To determine significant differences compared to sham-irradiated controls, a one-way analysis of variance (or t-test when only two

groups), or Welch's test with heteroscedastic data, was used, followed by Dunnett's post-hoc test, with $p \leq 0.05$ accepted as significant.

Results

Cancellous Microarchitecture Following Iron Irradiation

To determine the extent of bone loss over the short term, mice were irradiated with ^{56}Fe ions (600 MeV) or were sham irradiated (0 Gy controls), then 7 days later bones were harvested and cancellous microarchitecture in the proximal tibia quantified *ex vivo* using 3D microcomputed tomography. Body weights of irradiated and control animals at the time of tissue harvest did not differ (data not shown). Consistent with previous results, irradiation with 2Gy reduced the ratio of bone volume to total volume (BV/TV) by 16% and trabecular number (TbN) by 15% compared to controls (Figure 1), but did not affect trabecular thickness (not shown). A lower dose of 5 cGy iron failed to elicit changes in bone structural parameters compared to control and is therefore below the threshold dose for causing bone loss (data not shown). These results validate the model of ionizing radiation-induced cancellous bone loss.

Marrow Gene Expression Following Iron Irradiation

To determine if irradiation regulates expression of various pro-osteoclastogenic genes, mRNA levels in bone marrow cell lysates were measured using quantitative real-time RT-PCR three days after high-LET iron irradiation and in sham-irradiated controls. Within 3 days of exposure, iron irradiation increased expression of the *Rankl* gene 9.2-fold compared to sham-irradiated controls (Figure 2). At this time point, transcripts of *Opg* and *Mcp1* were not detected in the marrow (data not shown). Radiation exposure did not alter *Tnf* expression (Figure 2). In

contrast, a lower dose of iron (5 cGy) did not elicit changes in gene expression (data not shown). These data demonstrated that high-LET particulate irradiation with 2 Gy elicited a pro-osteoclastogenic cytokine expression in the bone marrow.

Gene Expression of Skeletal Tissue Following Iron Irradiation

To determine if ionizing radiation regulated expression of select genes related to osteoclastogenesis (*Rankl, Opg*) and osteoclast-mediated bone resorption (*Ctk, Acp5*) in cells of within the mineralized compartment of skeletal tissue, RNA was purified from bone after removal of the marrow (leaving predominantly osteocytes). Within 3 days, iron irradiation increased expression of *Rankl* by 1.9-fold, *Acp5* by 1.5-fold, and *Ctk* by 2.1–fold over sham controls, as shown in Figure 3. Expression levels of *Opg* did not change. The ratio of *Rankl/Opg* expression increased 2.8-fold, which provides a relative index that, on balance, cytokine levels favored increased bone resorption.

Marrow Gene Expression Following Gamma Irradiation

To determine whether irradiation up-regulated osteoclastogenic and inflammatory genes, mRNA expression was measured in bone marrow at 4 hours or 1, 3, or 7 days after low-LET gamma irradiation and in sham-irradiated controls. Within 4 hours, 2Gy gamma-irradiation elevated *Rankl* in bone marrow 2.6-fold over the sham, whereas gene expression of *Opg* was undetectable regardless of treatment (data not shown). Subsequently, the expression of each gene of interest measured in bone marrow (*Rankl*, *Opg*, *Csf1*, *Nfatc1*, *Tnf*, *Mcp1*, and *Il6*) transiently increased within 1 day and subsequently declined towards sham-levels, see Figure 4. Expression of *Csf1*, and *Tnf* remained elevated through day 3 post-irradiation, while high *Rankl* expression

persisted through day 7 post-irradiation. At their peak, pro-osteoclastogenic genes *Rankl* and *Csf1,* and the osteoclast-related transcription factor *Nfatc1,* increased by 4.1-fold, 4.2-fold, and 2.0-fold, respectively (Fig 4a, 4d, 4e); the *Rankl*-decoy receptor *Opg* increased by 11.3-fold (Fig 4b); pro-inflammatory genes *Tnf*, *Mcp1*, and *Il6* increased by 1.7-fold, 11.9-fold, and 1.6-fold, respectively (Fig 4f, 4g, 4h) relative to controls. The ratio of *Rankl* / *Opg* increased by 1.8-fold at day 7 after irradiation (Fig 4c). These data show the temporal nature of cytokine regulation in the marrow following radiation exposure.

Gene Expression of Skeletal Tissue Following Gamma Irradiation

Following gamma irradiation, mRNA levels of flushed femora was quantified at 3 and 7 days after irradiation or sham using qRT-PCR normalized to the housekeeping gene, *L19*, as shown in Figure 5. Within 3 days, gamma radiation exposure up-regulated the expression of *Rankl* (2.3-fold), *Acp5* (2.2-fold) and *Ctk* (2.3-fold). Expression of *Tnf*, *Opg*, and the *Rankl/Opg* ratio was not changed (Figure 5). These data suggest embedded and/or lining cells contribute to osteoclast stimulation and provide gene expression evidence of increased resorption in the skeletal tissue.

Serum TRACP 5b Following Gamma Irradiation

To determine if temporal changes in skeletal gene expression (Figure 3,5) coincide with changes in a protein biomarker of resorption, the circulating levels of osteoclast-specific TRACP 5b were measured at 1, 3, and 7 days after gamma irradiation and in sham-controls. Gamma irradiation increased TRACP 5b serum levels by 34% on day 1 and 17% on day 3, compared to sham, with a subsequent gradual decline towards control levels by day 7 (data not shown). Thus,

239 circulating TRACP 5b levels showed a similar time course, though lower in magnitude,
240 compared to skeletal gene expression for *Rankl* and other pro-osteoclastogenic cytokines.
241
242 Discussion
243 To better understand the mechanisms underlying radiation-induced stimulation of bone
244 resorption, we investigated molecular signals within the latency period between radiation
245 exposure and the manifestation of cancellous tissue loss. In summary, the results show that
246 exposure to either gamma or heavy ion radiation up-regulates gene expression for the canonical,
247 osteoclastogenic factor RANKL, as well as other pro-osteoclast cytokines (*Mcp1, Tnf, Il6*); this
248 occurs in cells that reside in the marrow cavity and within mineralized tissue. Further, radiation-
249 induced changes in cytokine expression are temporally related to changes in several indices of
250 bone resorption, including gene expression (*Ctk, Acp5, Nfatc1*) and a serum biomarker
251 (TRACP5b levels). Gene expression changes at the molecular scale (as early as 4 hrs or 1 day)
252 precede measurable structural losses, which manifest here by day 7 after the 2 Gy iron exposure,
253 although in some cases such as gamma irradiation and lower doses of iron, decrements in
254 cancellous bone volume can be observed as early as 3 days after exposure (Kondo and others
255 2009), (Yumoto and others 2010). A dose threshold was observed, as 5 cGy iron exposure failed
256 to elicit changes in cytokine gene expression or cancellous bone structure (data not shown),
257 providing additional indirect evidence in support of the hypothesis that early induction of
258 osteoclastogenic cytokine gene expression by biologically effective doses of radiation leads to
259 cancellous bone loss.
260 Together, the results demonstrate radiation-induced structural changes are associated with a
261 marrow environment favoring osteoclast differentiation and stimulation by both RANKL and

other inflammation-related cytokines as follows. Radiation increased gene expression levels for pro-osteoclastogenic signaling molecules (*Csf1*, *Rankl*, *Tnf*), as well as anti-osteoclastogenic molecules (*Opg*), in the marrow and mineralized tissue of irradiated mice compared with sham-controls. Iron irradiation elevated the ratio of *Rankl/Opg* in both the marrow and skeletal tissue by day 3, while after gamma irradiation, the ratio was elevated in the marrow at 4 hours and then again at day 7. A rapid stimulation of RANKL in the marrow and bone compartment, potentially from haematopoietic-lineage cells (Pacifici 2012), (Fumoto and others 2014), (Pacifici 2010) or stromal-lineage cells (Suda and others 1999),(Boyle and others 2003), after irradiation is consistent with studies that used radio-therapeutic doses and regimens. In young mice, a single dose of 5 or 10 Gy increased *Rankl/Opg* in whole femora within 3 days (Han and others 2014). Using mice deficient in the global anti-oxidant transcription factor Nrf2, radiation exposure at high dose (20Gy) increased RNA expression for *Rankl* in cultured osteoblasts grown *ex vivo,* but this was not observed in cells from wild-type mice (Rana and others 2012). These findings suggest Nrf2-mediated regulation of antioxidant expression may dampen bone resorption responses to radiation. Further, when a macrophage cell line (RAW264.7) capable of differentiating into osteoclast-like cells after RANKL treatment is exposed to ionizing radiation (2 Gy gamma), gene expression levels rise for *β3 integrin*, an adhesion receptor that is important for osteoclast differentiation, as well as receptor activator of nuclear factor kappa-B (RANK), the receptor for RANKL on osteoclasts (Yang and others 2012). In other *in vitro* work, irradiation at 2 or 4 Gy up-regulates *Rankl* in differentiated, MC3T3-E1 osteoblast-like cells (Yang and others 2013). Our work is the first to demonstrate the time course of changes in osteoclastogenic gene expression following 2Gy exposure (both low- and high-LET) with subsequent bone loss, and within the upper range of space-relevant doses and types of radiation.

Ionizing radiation also increased expression of pro-inflammatory, osteoclastogenic ligands *Mcp1*, *Tnf* and *Il6*, which are all factors generally thought to stimulate osteoclast activity (Takayanagi 2007) in the presence of RANKL (Kostenuik and Shalhoub 2001), (Yu and others 2004), (Kim and others 2005), (Sul and others 2012), (Kim and others 2006), (Liu and others 2013). In some reports, TNF may act independently of RANKL to stimulate osteoclastogenesis (Kobayashi and others 2000). Our results are consistent with other work showing that *in vivo* exposure to ionizing radiation leads to rapid, complex, and interrelated sequence of signals constituting an immune-related, cytokine response in bone marrow (Willey and others 2011),(Schaue and others 2012; Schaue and McBride 2010), (Buchwald and Aurora 2013). At very high dose (10Gy), which is sufficient to ablate the bone marrow of haematopoietic cells, radiation causes bone loss related to elevated fractalkine expression by vascular endothelial cells, inflammatory cytokines *Tnf*, interleukin 1 beta, and interferon gamma, and recruitment of pre-osteoclasts (CD11b) (Han and others 2014). Additionally, the time course of cytokine and resorption-related gene expression shown in this study coincides with that of marrow cell death and repopulation (Kondo and others 2010), (Otsuka and others 2008), suggesting a possible relationship between an expanded population of marrow macrophages clearing apoptotic cells and debris after irradiation, the differentiation of macrophages into osteoclasts, and increased resorption activity.

Taken together, these data lead us to propose a three-stage process for radiation-induced osteoclastogenesis: first, radiation-induced gene expression of inflammatory cytokines lead to enrichment of the marrow with osteoclast precursors (monocyte-macrophage, myeloid lineage cells); second, the marrow and skeletal microenvironment drives osteoclast differentiation; and, third, inflammatory signals co-stimulate differentiating and mature osteoclasts.

As a functional measure of active osteoclasts, we provide evidence that 2Gy gamma irradiation elevates circulating levels of osteoclast-specific TRACP 5b protein, indicative of increased bone resorption. In our work, the radiation-induced elevation in serum TRACP 5b returned to control levels by day 7 following exposure, These results are consistent with those reported by Willey et al. showing that X-irradiation (2Gy) of female mice increases circulating TRACP 5b levels 1, 3, and 7 days after irradiation (Willey and others 2011), (Willey and others 2010).

Radiation-induced decrements in cancellous tissue observed in these experiments were consistent with our previous results. Acute cancellous bone loss temporally manifests on day 3 at a dose as low as 10 cGy iron (Yumoto and others 2010). Persistent structural decrements (lasting >1 week) manifest at doses above ~50 cGy iron (Yumoto and others 2010) and 1 Gy gamma exposure (Hamilton and others 2006), (Kondo and others 2010) before being overtaken, in the case of gamma irradiation, by age-related bone loss (Alwood and others 2012). Taken together, given the observed time course of skeletal gene expression and serum resorption markers, we conclude the structural deficits arose from a spike in osteoclastogenic cytokine expression that follows exposure to ionizing radiation (Kondo and others 2009),(Willey and others 2010).

Limitations of this work include the dose rate used to model space radiation. It is an open question whether lower dose rate exposures that constitute true space radiation stimulate osteoclasts and bone loss to the same extent as the exposures used here. In addition, other signaling molecules, including various other cytokines not studied here, are likely also to play a role in regulating bone resorption after challenge with ionizing radiation.

In conclusion, an improved understanding of the molecular response to radiation exposure may aid the development of biological treatments to mitigate potentially deleterious skeletal consequences during space flight.

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Figure Legends

Figure 1

2 Gy iron irradiation caused acute bone loss in the tibial metaphysis by 7 days via removal of trabecular struts. A) Bone volume fraction (BV/TV) and B) trabecular number (TbN). Data are mean ± SD, with * denoting $p<0.05$ vs. sham.

Figure 2

2 Gy iron irradiation effects on cytokine gene expression in tibial marrow cells on day 3. Radiation exposure increased the gene expression levels of A) *Rankl.* Gene expression levels of B) *Tnf* were unchanged. Data are mean ± SD, with ** denoting $p<0.01$ vs. sham.

Figure 3

2 Gy iron irradiation effects on gene expression in mineralized tibial tissue (marrow flushed) by day 3. Comparison of expression levels of A) *Rankl*, B) *Opg,* C) *Rankl / Opg,* D) *Acp5,* and E) *Ctk* genes after iron irradiation compared to controls. Data are mean ± SD, with ** denoting $p<0.01$ vs. sham.

Figure 4

2 Gy gamma radiation regulated expression of pro-osteoclastic and resorption-related genes in pooled tibial and femoral marrow and lining cells. Time course (+1, +3, and +7 days post-irradiation) for the following genes compared to sham control: A) *Rankl*, B) *Opg*, C) *Rankl/Opg*, D) *Csf1*, E) *Nfatc1*, F) *TNF*, G) *Mcp1*, and H) *Il6*. Data are mean ± SD, with * denoting $p<0.05$ and ** $p<0.01$ vs. sham.

Figure 5

2 Gy gamma irradiation regulated gene expression in flushed femoral and tibial tissue by day 3. Comparison of expression levels of A) *Rankl,* B) *Opg,* C) *Rankl*/*Opg,* D) *Acp5,* E) *Ctk,* and F) *Tnf,* genes after iron irradiation compared to controls. Data are mean ± SD, with * denoting $p<0.05$ and ** denoting $p<0.01$ vs. sham.

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Figure 1

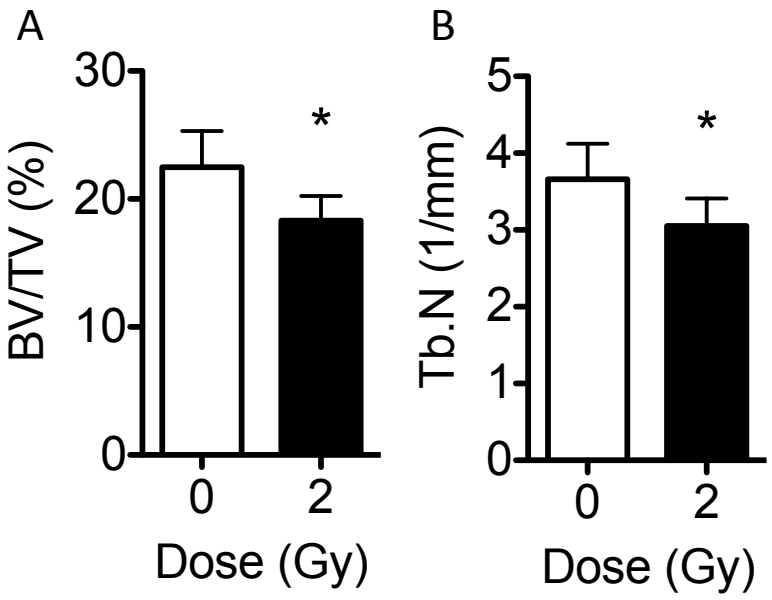
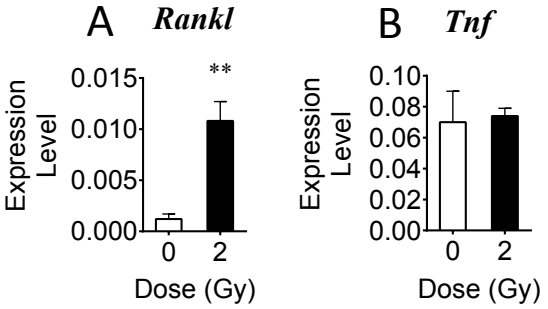


Figure 2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0.005
0.000
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2
Dose (Gy)
B
Tnf
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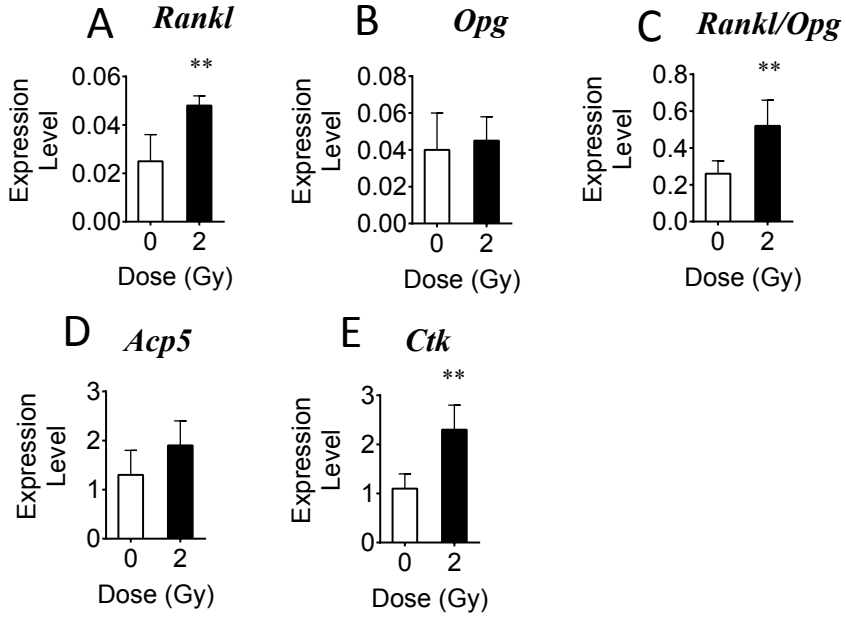
0.08
0.06
0.04
0.02
0.00
Expression Level
0
2
Dose (Gy)

Figure 3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0.04
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0.00
0
2
Dose (Gy)
C
Rankl/Opg
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0.4
0.2
0.0
0
2
Dose (Gy)
D
Acp5
Expression Level
3
2
1
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2
Dose (Gy)
E
Ctk
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Expression Level
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2
1
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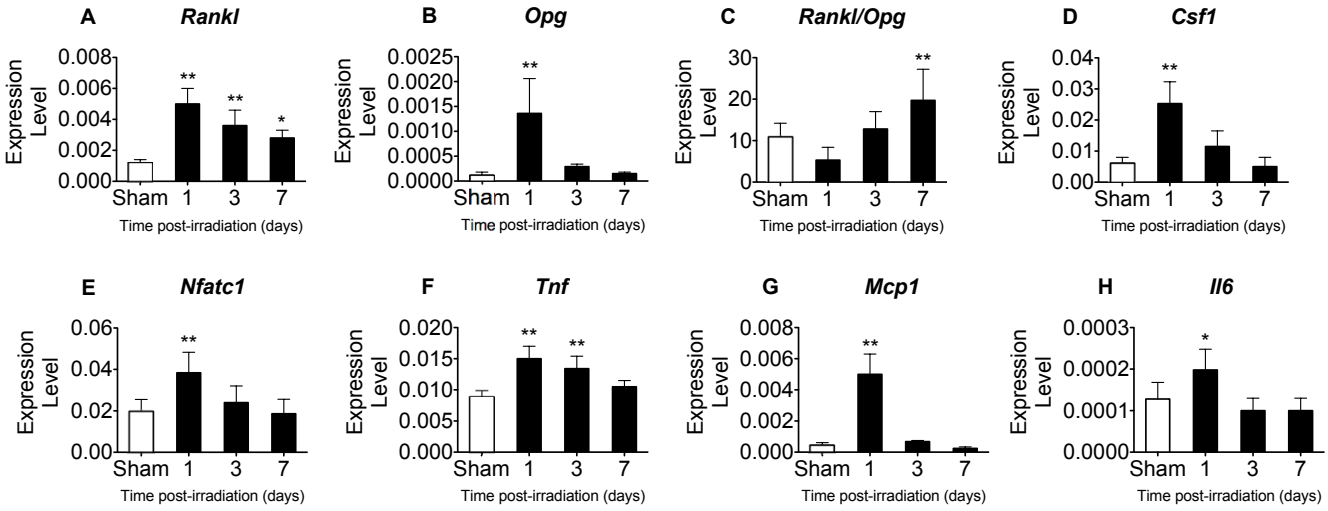
2
Dose (Gy)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B
Opg
Expression Level
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0.0010
0.0005
0.0000
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Sham 1 3 7
Time post-irradiation (days)
C
Rankl/Opg
Expression Level
30
20
10
0
**
Sham 1 3 7
Time post-irradiation (days)
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Expression Level
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Time post-irradiation (days)
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Expression Level
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**
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Time post-irradiation (days)
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Mcp1
Expression Level
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0.006
0.004
0.002
0.000
**
Sham 1 3 7
Time post-irradiation (days)
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Il6
Expression Level
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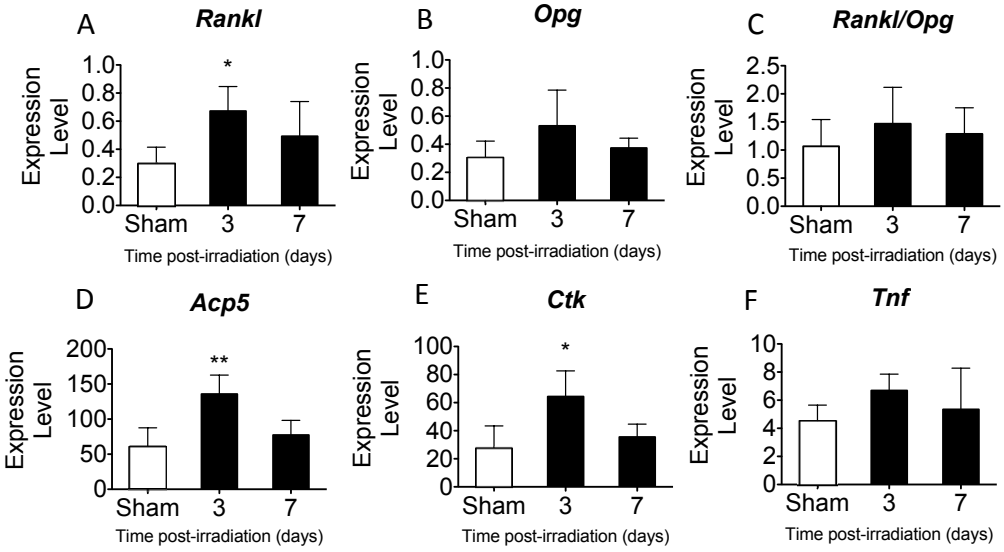
0.0001
0.0000
*
Sham 1 3 7
Time post-irradiation (days)

Figure 4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Rankl
Expression Level
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Sham
3
7
Time post-irradiation (days)
B
Opg
Expression Level
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0.8
0.6
0.4
0.2
0.0
Sham
3
7
Time post-irradiation (days)
C
Rankl/Opg
Expression Level
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2.0
1.5
1.0
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Sham
3
7
Time post-irradiation (days)
D
Acp5
Expression Level
200
150
100
50
0
**
Sham
3
7
Time post-irradiation (days)
E
Ctk
Expression Level
100
80
60
40
20
0
*
Sham
3
7
Time post-irradiation (days)
F
Tnf
Expression Level
10
8
6
4
2
0
Sham
3
7
Time post-irradiation (days)

Figure 5