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Supplemental Information

Inhibition of C5AR1 impairs osteoclast mobilization and prevents bone loss

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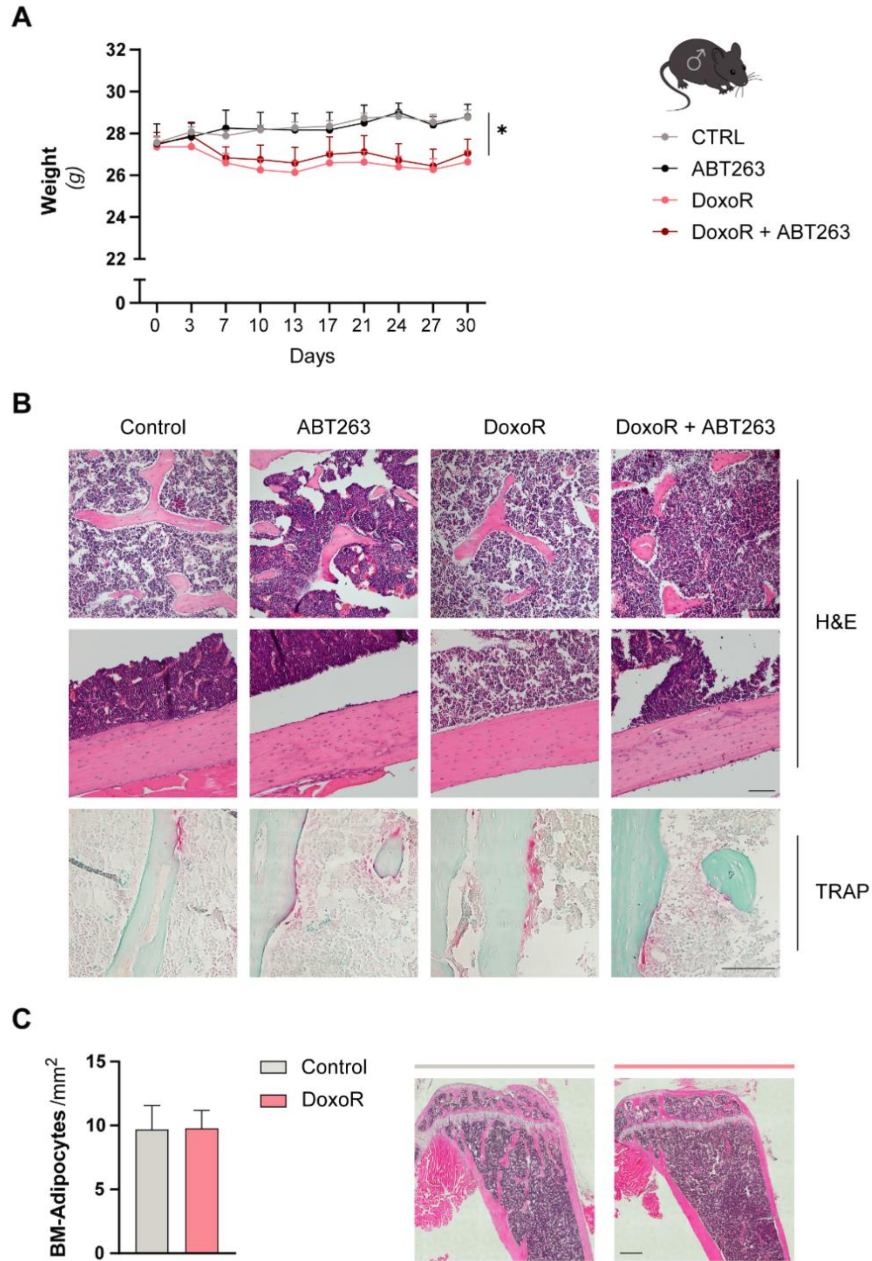


Figure S1: Mouse model of doxorubicin-induced senescence. (A) Body weight curves of vehicle- (Control), ABT263-, doxorubicin- and doxorubicin + ABT263-treated mice. n = 6-10. Data are shown as mean ± SEM *$p < 0.05$. Statistics were calculated considering the overall area under the curve. **(B)** Histological images of Control, ABT263, DoxoR and DoxoR + ABT263 bone samples stained with hematoxylin and eosin (H&E), and tartrate-resistant acid phosphatase (TRAP) staining. Trabecular and cortical compartments are shown for osteoblasts and osteocytes identification (H&E) and TRAP-stained osteoclasts appear in red attached to bluish-trabecular bone (TRAP). Scale bars = 100 µm. **(C)** Quantification of the number of bone-marrow (BM) adipocytes per marrow area (BM-Adipocytes/mm^2) from H&E-stained sections of tibiae. Representative H&E sections from Control and DoxoR-treated mice. n = 4. Scale bar = 500 µm.

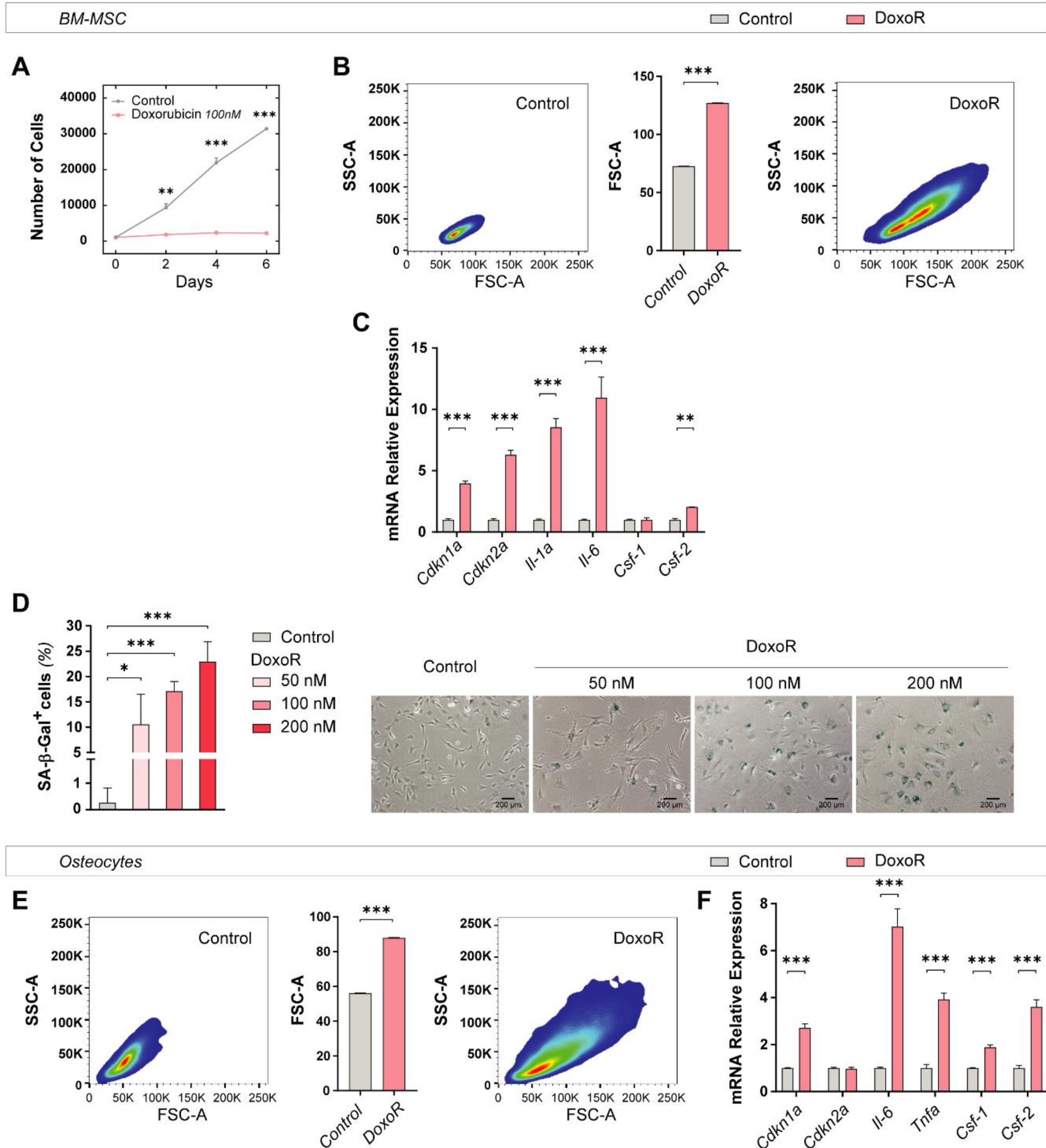


Figure S2: **Doxorubicin-induced senescence in BM-MSCs and Osteocytes *in vitro*.** (**A**) Number of BM-MSC untreated (control) or treated with 100 nM of doxorubicin for 24 h. Cells were counted at days 0, 2, 4 and 6. n = 3. **(B)** Flow cytometry side vs. forward scatter plots of BMMSC untreated (control) and treated with 100 nM of doxorubicin for 24 h. Flow cytometry was performed at day 7. **(C)** RT-qPCR of senescence markers (*Cdkn1a, Cdkn2a, Il1a, Il6, Csf1 and Csf2*). RNA isolated from senescent (DoxoR) and control BM-MSC. n = 4-9. **(D)** Images of senescent cells shown by SA-β-Gal staining and quantification of SA-β-Gal+ BMMSC at day 7 after treatment with 50 nM, 100 nM or 200 nM of doxorubicin. n = 4-8. Scale bars: 200 µm **(E)** Flow cytometry side vs. forward scatter plots of osteocytes untreated (control) and treated with 100 nM of doxorubicin for 24 h. Flow cytometry was performed at day 7. **(F)** RT-qPCR of senescence markers from senescent (DoxoR) and control osteocytes. n = 6-11. Data shown as mean ± SEM *p < 0.05, **p < 0.01 and *** p < 0.001. D, one-way ANOVA. For the other, student's t-test.

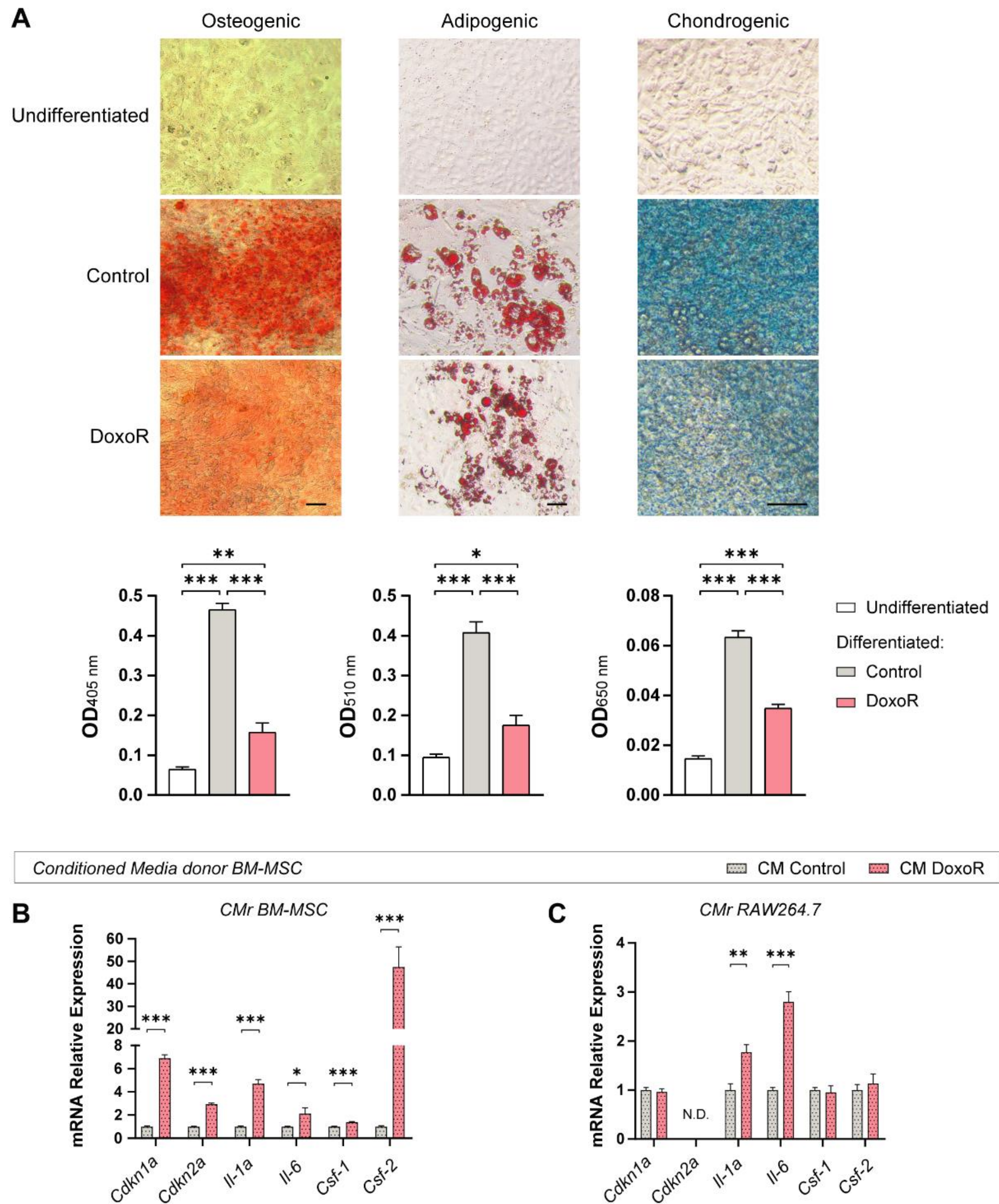


Figure S3: Autocrine and paracrine impact of doxorubicin-induced cellular senescence. (A) Trilineage differentiation of undifferentiated, control and BM-MSC treated with 100 nM DoxoR for 24 hours. Staining and quantification of osteogenic (day 14, Alizarin Red S at 450 nm), adipogenic (day 14, Oil Red O at 510 nm) and chondrogenic (day 21, Alcian Blue at 650 nm) differentiation. n = 4. **(B)** Change in senescent gene markers in conditioned media experiments. BM-MSC were exposed to control and senescent conditioned media (CM) from BM-MSC for 5 days. n = 6-12. **(C)** RAW264.7, were exposed to control and senescent CM from BM-MSC for 5 days. n = 7. N.D. undetermined. Data shown as mean ± SEM $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. Student's t-test.

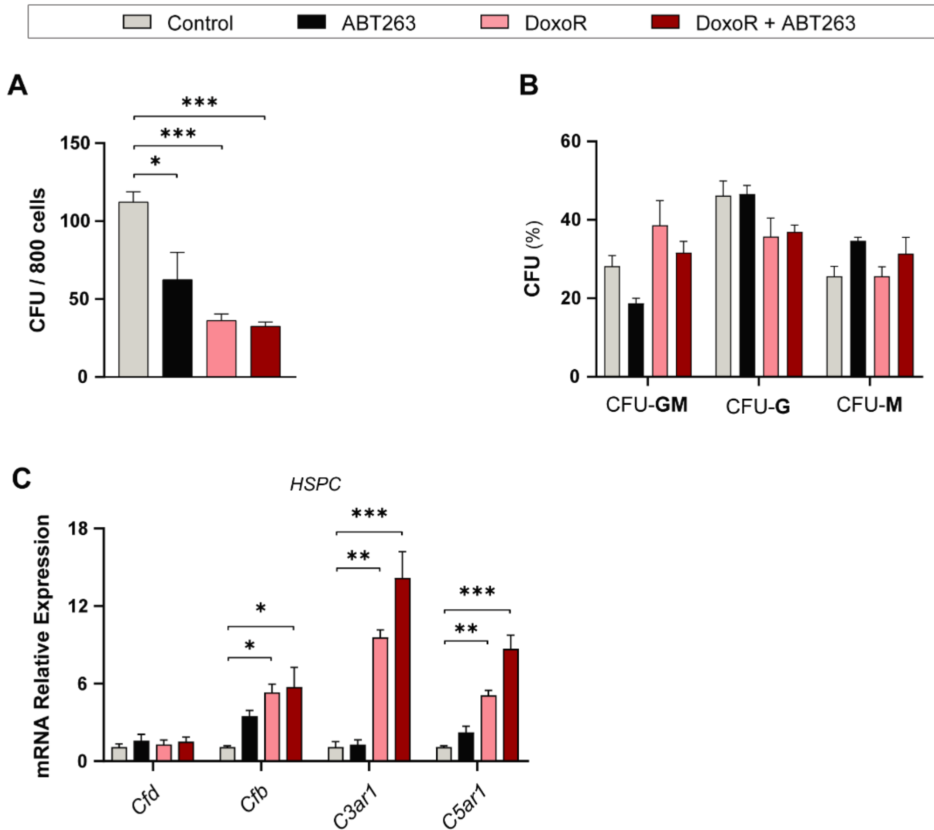


Figure S4: Senescence and senolytic impact on HSPCs (A-C) HSPCs untreated (Control) or treated with: 1 µM ABT263, 100 nM DoxoR or 100nM DoxoR + 1 µM ABT263 **(A)** Colony forming units (CFUs) per 800 HSPCs seeded after 7 days. n=4. **(B)** % of CFU lineages -GM (Granulocytes and Monocytes), -G (Granulocytes) and -M (Monocytes) derived from purified HSPCs after 7-10 days. n=4. **(C)** Complement genes (*Cfd, Cfb, C3ar1and C5ar1*) relative expression. n = 4. Data shown as mean ± SEM *p < 0.05, **p < 0.01 and *** p < 0.001. D, one-way ANOVA.

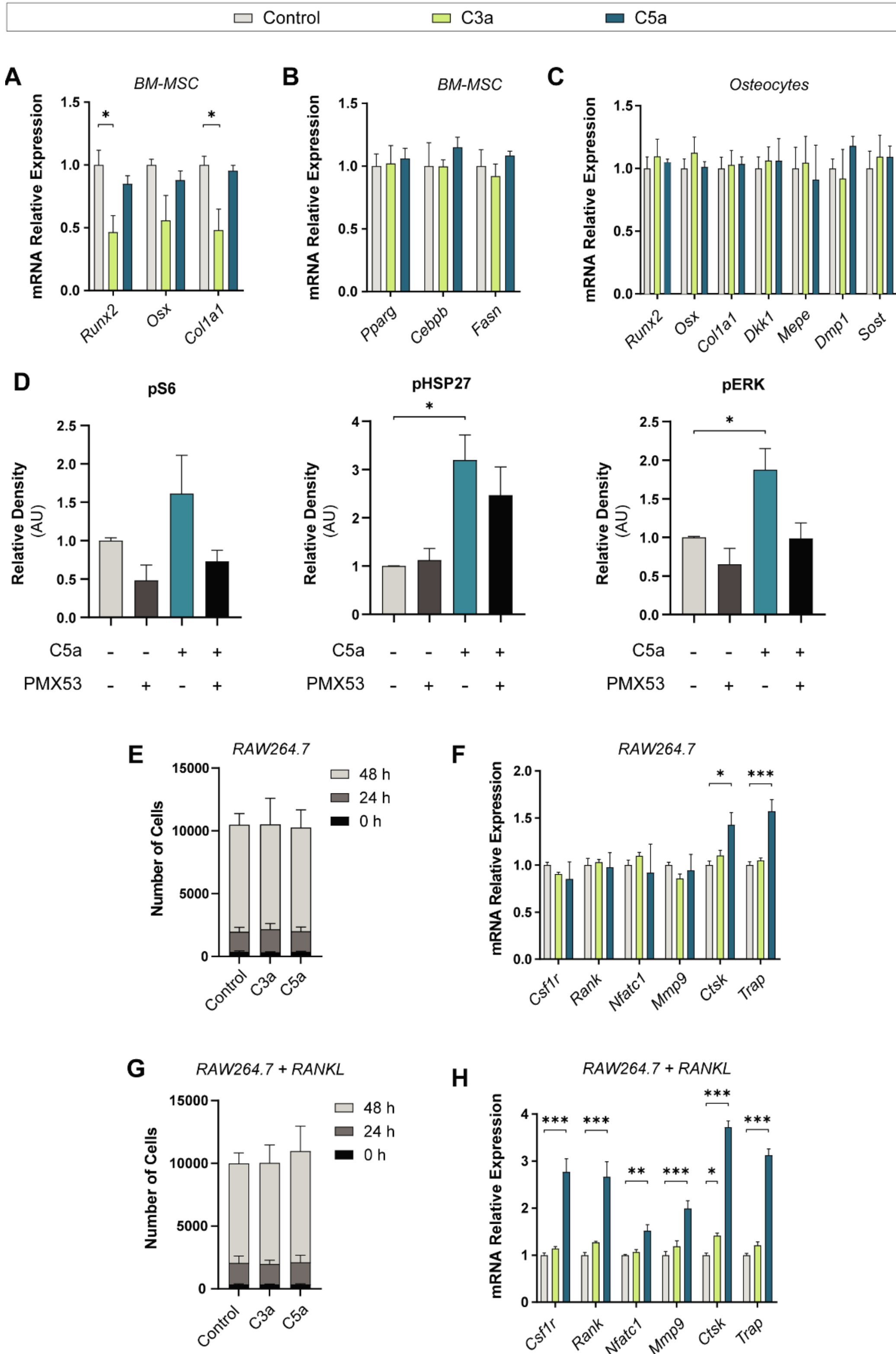


Figure S5: **Complement anaphylatoxin's impact *in vitro*. (A,B)** Relative gene expression of osteoblastic (*Runx2, Osx* and *Col1a1*) and adipogenic (*Pparg, Cebpa, Fasn* and *Pnpla2*) in BM-MSCs exposed daily to 1 µg/ml C3a or 0.1 µg/ml C5a for 5 days. n = 3-4. **(C)** RT-qPCR of osteoblastic and osteocytic markers (*Runx2, Osx, Col1a1, Dkk1,*

Mepe, Dmp1 and *Sost*). RNA isolated from osteocytes exposed daily to 1 μg/ml C3a or 0.1 μg/ml C5a for 5 days. n = 3-4. **(D)** Quantification of western blots from Figure 4D. Protein levels are represented as relative density in arbitrary units quantified from bands of each marker (phosphoS6, phosphoHSP27 and phosphoERK) versus β-actin. n = 3. **(E, F)** RAW264.7 were untreated (control) or treated daily with 1 μg/ml of C3a or 0.1 μg/ml C5a for 3 to 5 days. **(E)** Number of RAW264.7 cells at 0, 24 and 48 hours. n = 4. **(F)** Osteoclastic gene expression (*Csf1r, Rank, Nfatc1, Mmp9, Ctsk and Acp5/Trap*) was assessed by RT-qPCR. n = 4. **(G)** Number of RAW264.7 cells treated with 15 ng/ml RANKL and the indicated anaphylatoxins, at 0, 24 and 48 hours. n = 4. **(H)** Fold change in osteoclastic genes (*Csf1r, Rank, Nfatc1, Mmp9, Ctsk* and Acp5/*Trap*). RNA isolated from RAW264.7 differentiated into osteoclasts for 5 days with 15 ng/ml RANKL. Cells were exposed daily to 1 μg/ml C3a or 0.1 μg/ml C5a. n = 3-4. Data are shown as mean ± SEM *$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$; one-way ANOVA.

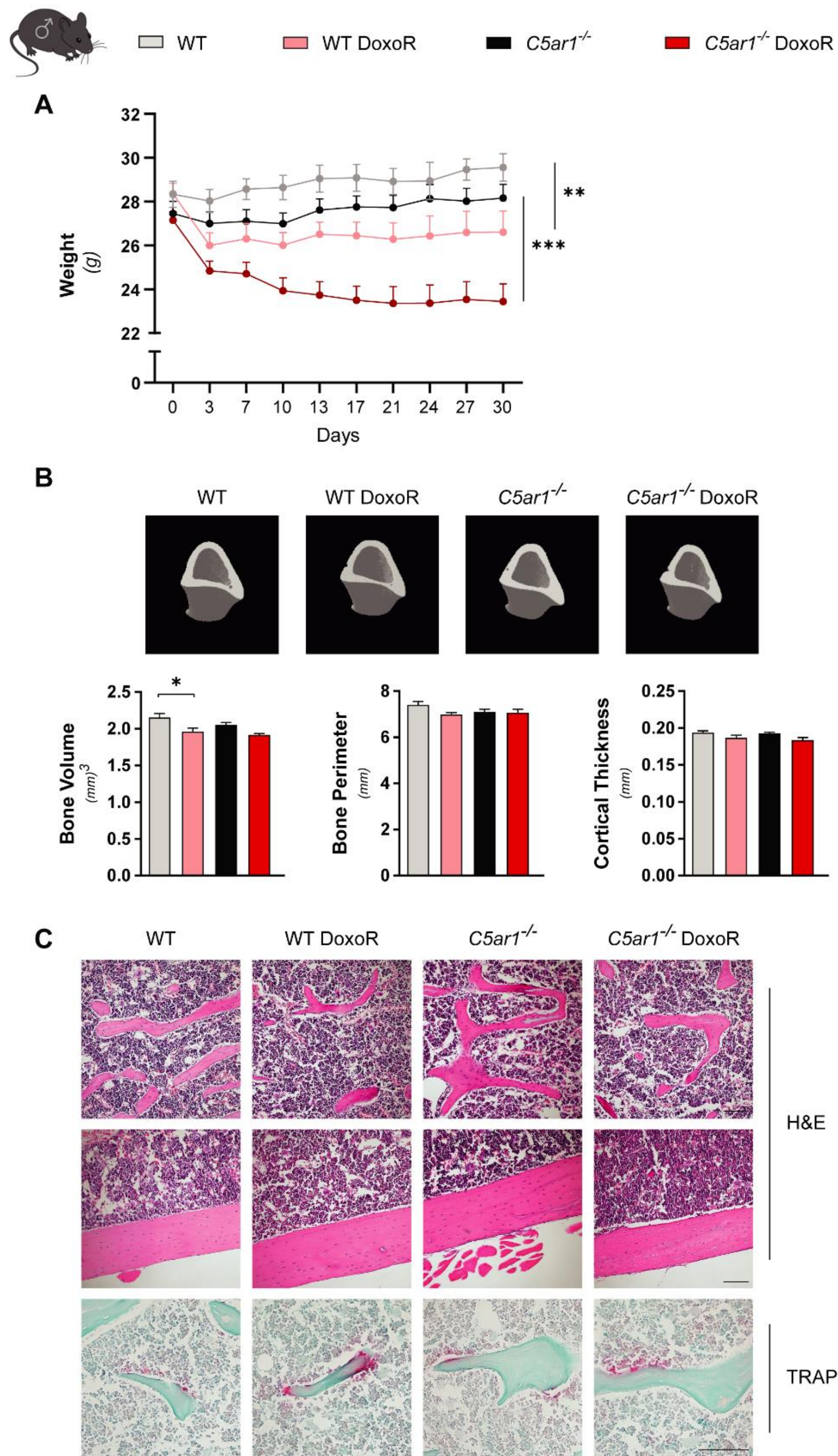
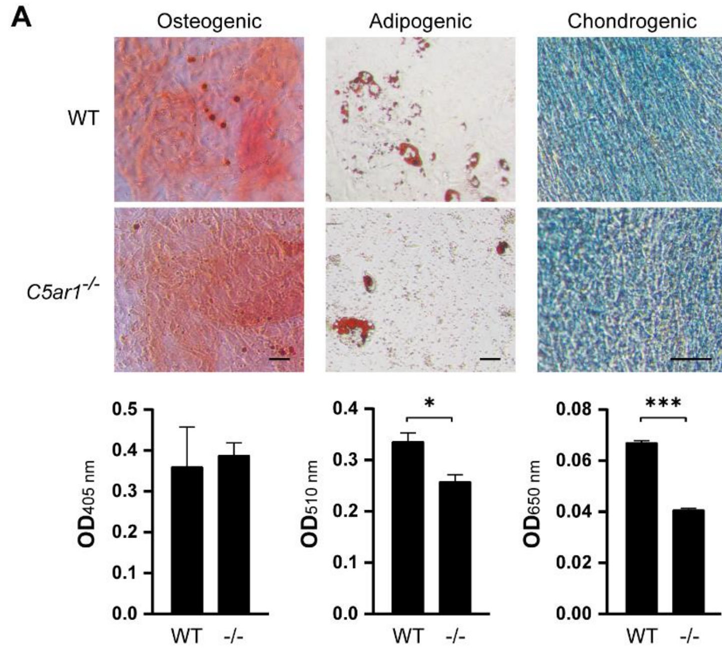


Figure S6: **Therapy-induced senescence in *C5aR1$^{-/-}$* mice.** Body weight curves of **(A)** WT, WT DoxoR, C5aR1$^{-/-}$ and C5aR1$^{-/-}$ DoxoR-treated mice. n = 9-13. **(B)** Quantitative parameters and 3D representation of cortical bone of tibiae measured by µ-CT. n = 9-13. **(C)** Histological images of WT, WT doxorubicin, C5aR1$^{-/-}$ and C5aR1$^{-/-}$ DoxoR-treated mice bone samples stained with hematoxylin and eosin (H&E), and tartrate-resistant acid phosphatase (TRAP) staining. Scale bars = 100 µm. Data are shown as mean ± SEM **p < 0.01 and *** p < 0.001. Body weight statistics were calculated considering the overall area under the curve.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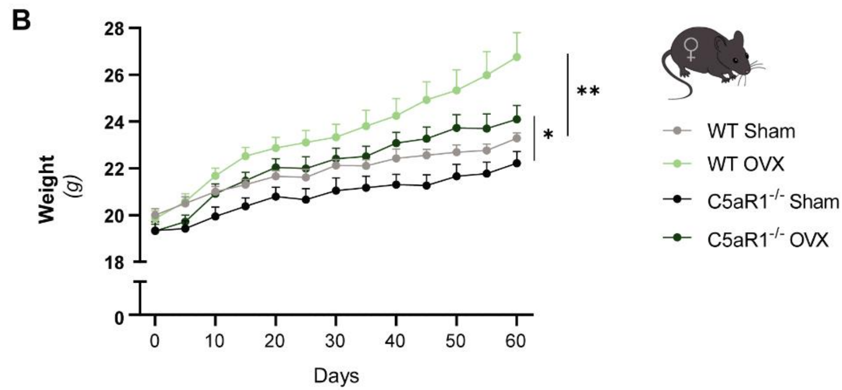
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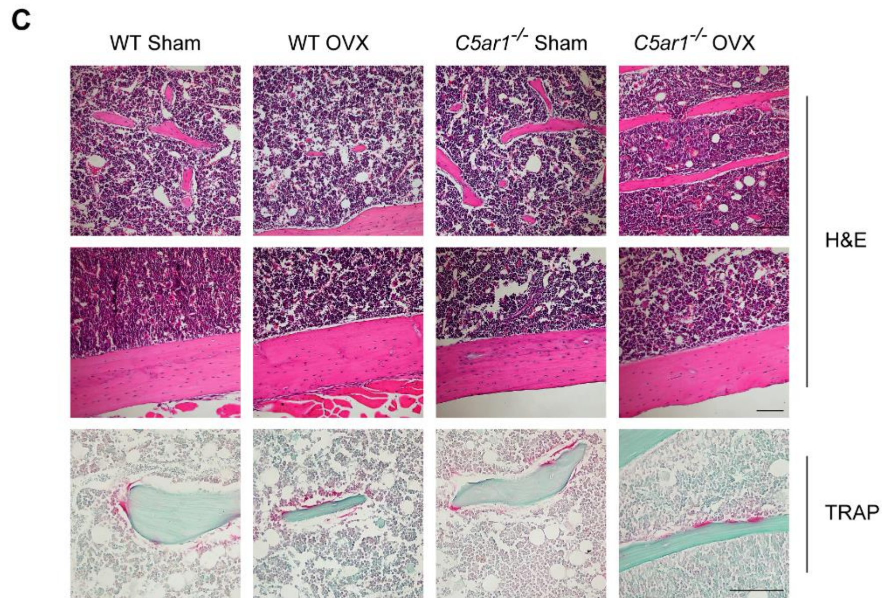
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WT Sham
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C5ar1-/- OVX
H&E
TRAP

Figure S7: ***C5aR1$^{-/-}$* BM-MSCs differentiation potential and OVX-induced bone loss in *C5aR1$^{-/-}$* mice. (A)** Trilineage differentiation of WT and C5aR1$^{-/-}$ BM-MSCs. Staining and quantification of osteogenic (Alizarin Red S at 450 nm), adipogenic (Oil Red O at 510 nm) and chondrogenic (Alcian Blue at 650 nm) differentiation. n = 4. **(B)** Body weight curves of WT Sham, WT OVX, C5aR1$^{-/-}$ Sham and C5aR1$^{-/-}$ OVX mice. n = 10-13. **(C)** Histological images of bone samples stained with hematoxylin and eosin (H&E), and tartrate-resistant acid phosphatase (TRAP) staining. Scale bars = 100 µm. Data are shown as mean ± SEM *$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$. Student's t-test. Body weight statistics were calculated considering the overall area under the curve.

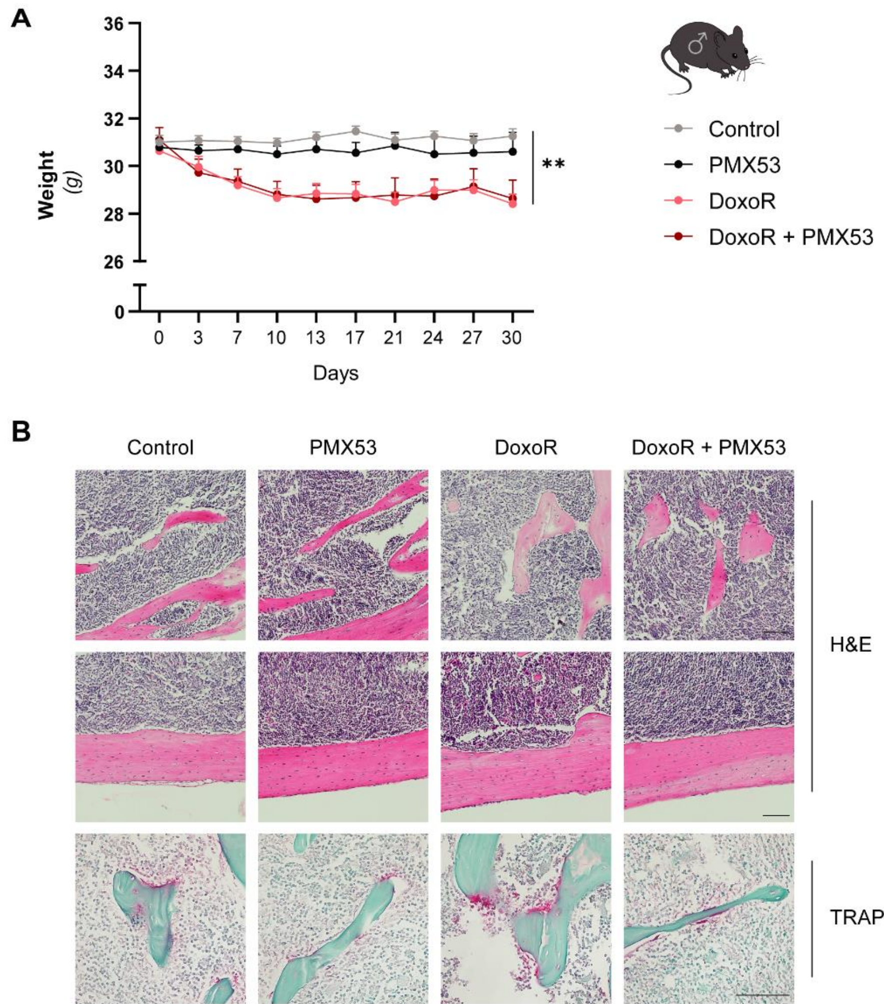

Figure S8: **Pharmacological C5AR1 inhibition in doxorubicin-induced bone loss.** Body weight curves of **(A)** Control, PMX53-, doxorubicin- and doxorubicin + PMX53-treated mice. n = 8-18. **(B)** Histological images of bone samples stained with hematoxylin and eosin (H&E), and tartrate-resistant acid phosphatase (TRAP) staining. Scale bars = 100 µm. Data are shown as mean ± SEM $^{**}p < 0.01$. Body weight statistics were calculated considering the overall area under the curve.

Supplemental Tables

Supplemental Table S1: List of genes differentially expressed between control and doxorubicin-treated mice.

Supplemental Methods

FACS analysis

Cell morphology was visualized in live cells using an inverted light microscope. Differences in morphology were quantified using the forward scatter parameter of flow cytometry. Briefly, cells were rinsed twice with PBS. Cells were incubated with 0.25% Trypsin and 0.02% EDTA at 37 °C for 5 min until cells became rounded and started to detach. Cell suspensions were collected and centrifuged at 250 g for 5 min. Cell pellets were resuspended in PBS. Before flow cytometry analysis, cell suspensions were filtered through a 0.70-µm nylon mesh to remove aggregates.

Senescence-associated beta-galactosidase staining

SA-β-gal staining was performed using the SA-β-gal staining kit (Cell Signaling) following the manufacturer's instructions. Senescent cells appeared as blue-stained cells under a light microscope. The percentage of SA-β-gal^{+} cells was calculated by defining an intensity threshold for blue cells in ImageJ.

Trilineage differentiation of BM-MSCs

For osteogenic differentiation, BM-MSCs were cultured in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, P/S, 0.16 mM ascorbic acid, and 6mM β-glycerophosphate. Cells were cultured for 14 days and the medium was changed every 2 days. Undifferentiated cells were treated with α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, P/S. At the end of the differentiation period cells were fixed with 4% paraformaldehyde for 30 minutes and stained with 2% Alizarin Red S, pH 4.2 (Sigma-Aldrich) for 1 hour. After removal of the unincorporated dye, samples were washed at least 3 times with PBS. Images of stained monolayers were captured with inverted-phase microscopy. For quantification of staining, each well was incubated for 30 min with 10% acetic acid. The cell lysates were collected, centrifuged at 20000g for 15 min and supernatant was transferred into a new tube and neutralized with 10% ammonium hydroxide. The absorbance was measured at 405 nm.

For adipogenic stimulation, BM-MSCs were cultured in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, P/S, 1 µM dexamethasone, 500 µM IBMX (3-isobutyl-1-methylxanthine), and 10 µM insulin (all from Sigma-Aldrich). Undifferentiated cells were treated with α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, P/S. Cells were differentiated for 14 days and medium was changed every 2 days. After 14 days, BM-MSC were stained with 0.35% Oil Red O. Briefly, after fixation, cells were exposed to 60% isopropanol for 5 minutes and stained with 0.35% Oil Red O (diluted 3:2 in water and filtered with a 0.2 0.22 µm nitrocellulose membrane) for 15 minutes. After 3 washes with PBS, lipid droplets were observed with an inverted microscope. The staining was quantified by extracting the Oil Red O stain with 100% isopropyl alcohol, after which the absorbance was measured using a spectrophotometer at 510 nm.

Chondrogenic differentiation of BM-MSCs was performed for 21 days with D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, P/S, 10 µM dexamethasone, 0.35 mM L-Proline, ITSX 1X, and 0.4 nM TGF-β1. Undifferentiated cells were treated with D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, P/S. The medium was changed every two days and after 21 days cells were fixed and stained with 1% Alcian Blue 8GX (diluted in HCl 0.1M and filtered) overnight at room temperature with gentle shaking. For quantification, BM-MSCs were incubated with guanidine HCl 6M for 2 hours and the extracted staining was quantified using a spectrophotometer at 650 nm.

All the quantifications were performed using the Tecan Sunrise Microplate Reader and measures are expressed as optical density (ODx), x = at a given wavelength.

Haematopoietic Stem Progenitor Cells assays

Purified HSPCs were untreated (Control) or treated for 24 h with 100 nM DoxoR to induce cellular senescence. At day 4 and 6 after senescence HSPCs were exposed to 1 µM ABT263. The experiment was stopped at day 7. For the Colony Forming Units (CFU) assay, control or DoxoR-treated HSPCs were seeded in MethoCult GF M3434 (Stem Cell Technologies) following the protocol described by Rodríguez, et al. (Rodríguez et al., 2021).

Supplemental References

Rodríguez, A., Filiatrault, J., Flores-Guzmán, P., Mayani, H., Parmar, K., & D'Andrea, A. D. (2021). Isolation of human and murine hematopoietic stem cells for DNA damage and DNA repair assays. STAR Protocols, 2(4). https://doi.org/10.1016/j.xpro.2021.100846