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Infiltrating Myeloid Cells Drive Osteosarcoma Progression via GRM4 Regulation of IL23

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Highlights

- Dendritic cell GRM4 has tumor suppressor properties in human and mouse osteosarcoma
- GRM4 inhibits IL23 expression, while increasing the related cytokine, IL12
- IL23 has oncogenic properties in tumor development
- Both GRM4 and IL23 are novel therapeutic targets in osteosarcoma.

Abstract

The glutamate metabotropic receptor 4 (*GRM4*) locus is linked to susceptibility to human osteosarcoma, through unknown mechanisms. We show that *Grm4*^{-/-} gene-targeted mice demonstrate accelerated radiation-induced tumor development to an extent comparable with *Rb1*^{+/-} mice. GRM4 is expressed in myeloid cells, selectively regulating expression of IL23 and the related cytokine IL12. Osteosarcoma-conditioned media induce myeloid cell *Il23* expression in a GRM4-dependent fashion, while suppressing the related cytokine *Il12*. Both human and mouse osteosarcomas express an increased IL23:IL12 ratio, whereas higher IL23 expression is associated with worse survival in humans. Consistent with an oncogenic role, *Il23*^{-/-} mice are strikingly resistant to osteosarcoma development. Agonists of GRM4 or a neutralizing antibody to IL23 suppressed osteosarcoma growth in mice. These findings identify a novel, druggable myeloid suppressor pathway linking GRM4 to the proinflammatory IL23/IL12 axis.

SIGNIFICANCE:

Few novel systemic therapies targeting osteosarcoma have emerged in the last four decades. Using insights gained from a genome-wide association study and mouse modeling, we show that GRM4 plays a role in driving osteosarcoma via a non-cell-autonomous mechanism regulating IL23, opening new avenues for therapeutic intervention.

Teaser

GRM4 regulates the myeloid IL-12:IL-23 inflammatory axis to control tumor development

Osteosarcoma is an aggressive bone cancer that is incurable in relapsed or metastatic disease (1). After chemotherapy (2), no new drug targets have emerged over the past four decades(3), including immune checkpoint inhibition (4). A genome-wide association study identified a locus at 6p21.3 (rs1906953; odds ratio 1.57, 95% confidence intervals [95% CI] 1.35-1.83; $P=8.1 \times 10^{-9}$) in the *GRM4* gene(5), validated in two subsequent studies (6, 7). GRM4 (metabotropic glutamate receptor-4 or mGluR4) is a member of the group III family of G-protein coupled receptors that play a role in the cyclic adenosine monophosphate (cAMP)/Protein kinase A (PKA) pathway. GRM4 is implicated in neurodegenerative and autoimmune diseases(8, 9), but its biological role in tumorigenesis is unknown.

We first asked whether GRM4 had tumor suppressor or oncogenic effects on tumor development. Wild-type (WT) or *Grm4*^{-/-} mice ($n=25/\text{cohort}$) were injected with ⁴⁵Ca, a low energy β -emitter that localizes to bone (10, 11) (Fig 1A and Fig S1). Tumor latency in this model is consistently 10-12 months (11, 12). Loss of *Grm4* accelerated tumor development (Fig. 1B; Hazard ratio [HR] 0.41, 95% 95%CI 0.22-0.76, $P=0.0006$; median survival in WT mice 89 vs 65 weeks in *Grm4*^{-/-} mice). Outside the central nervous system, GRM4 is expressed by dendritic cells (DCs) and CD4⁺ T cells (8). Using flow cytometry, GRM4 was predominantly expressed by intratumoral DCs (defined as CD45⁺CD11c⁺MHC class II⁺ cells (13), and not by CD45⁺ tumor cells (Fig 1C). Few CD4⁺ T cells were found in tumors (not shown).

Grm4 was shown to regulate DC expression of *Il1*, *Il6*, *Il23* and *Il27* in experimental autoimmune encephalomyelitis (8). Bone marrow DCs cultured with IL4 and GM-CSF for 7 days, showed that *Grm4*^{-/-} DCs had selectively increased expression of the related pro-inflammatory cytokines *Il12* and *Il23* relative to WT DCs (Fig 1D). A common p40 subunit is covalently linked to p35 to form *Il12*, or to p19 to form *Il23*, both of which are primarily expressed by activated DCs (14). Increased expression of *Il23* is observed in many human cancers (15, 16), while *Il12* has potent anti-tumor activity (17). *Grm4*, *Il12*, and *Il23* expression were next studied in conventional DCs, cDC1 and cDC2 (defined as MHCII⁺CD11c⁺CD64⁺Ly6c^{low}CD11b^{low} and MHCII⁺CD11c⁺CD64⁺Ly6c^{low}CD11b⁺ respectively) and monocyte-derived DCs (MoDCs; defined as MHCII⁺CD11c⁺Ly6c^{hi}CD64⁺CD24^{int}CD11b⁺; gating strategy Fig S2 (13)). Tumor-derived MoDCs expressed both *Grm4* and high levels of *Il23*, but little *Il12* relative to splenic MoDCs (Fig 1E; Figs S3A-C). To show these findings were not a function of the model system, the expression of *Il23A* was also examined in osteosarcomas from genetically defined mouse models (*osx/cre p53/Rb* and *osx/cre p53.1224 pRb* mice (18); Fig S4). Again, the majority of tumors had increased *Il23A* compared to normal bone.

To determine whether osteosarcoma cells influence expression by DCs of *Il12* and *Il23* in vitro, bone marrow-derived DCs (BMDCs) were exposed to conditioned media (OSCM) from cultured mouse osteosarcoma cells. Lipopolysaccharide (LPS) was used as a positive control (19, 20). In WT BMDCs, LPS increased cAMP (Fig S5A) and *Il12* and *Il23*. OSCM significantly induced *Il23* expression in BMDCs, while suppressing *Il12* (Fig. 1F and 1G). Both LPS and GRM4 act via the cAMP/ PKA

pathway (8, 11). Consistent with an intermediate role for the cAMP/ PKA pathway, forskolin, a cAMP agonist, recapitulated the effect of OSCM by inducing *Il23* expression by BMDCs (Fig S5B).

Since *Grm4* also regulates *Il23* and *Il12*, we treated BMDCs with GRM4 agonists, with or without LPS or OSCM. GRM4 agonists are being developed for neurological disorders, including depression and Parkinson's disease (21). PHCCC ((-)-N-phenyl-7-(hydroxyimino) cyclopropa[b]chromen-1a-carboxamide) is a positive allosteric modulator of GRM4, while cinnabarinic acid (CIN) is an orthosteric regulator. Pretreatment with GRM4 agonists attenuated cAMP expression, and suppressed both basal (Fig S3), and LPS- or OSCM-induced *Il23* protein and transcript levels (Fig 1H-K). PHCCC also down-regulated *Il23* in human peripheral blood DCs stimulated with LPS (Fig S6). Collectively, these data suggest that osteosarcoma cells repress *Il23* production by DCs via the GRM4-mediated pathway.

Given that GRM4 suppresses tumor development (Fig 1B), and IL-23 is negatively regulated by GRM4, we tested whether IL-23 itself had oncogenic properties in the osteosarcoma model. *Il23p19^{-/-}* (*Il23^{-/-}*) mice were strikingly protected from tumor development, with 24/30 (80%) of *Il23^{-/-}* mice tumor-free at 104 weeks compared to no control mice at 90.7 weeks (Fig 2A: HR 9.4, 95%CI 3.3-27; $P < 0.0001$). To enhance sensitivity, a subset of *Il23^{-/-}* mice >2 years of age were screened by ¹⁸F 2-deoxyglucose PET scanning, without any subclinical evidence of tumors. Similar to IL6, IL23 contributes to the generation of IL17 expressing T-helper cells (TH17) (22, 23). However, unlike *Il6^{-/-}* mice (12), *Il17a^{-/-}* mice did not display accelerated tumor development (Fig 2B: HR 0.95, 95%CI 0.47-1.9, $P = 0.89$). These findings recapitulate our observations in a mouse model of soft-tissue sarcomas (3). To put our findings of a pro-tumorigenic role for IL23 in context, we tested 15 mouse genotypes of pathways implicated in immune control of cancer development (*Il1r^{-/-}*, *Il6^{-/-}*, *Il17^{-/-}*, *Pd1^{-/-}* (*cd276^{-/-}*), *Ifnar1^{-/-}*, *Ifnar2^{-/-}*, *IFN γ ^{-/-}/perforin^{-/-}*, *Trail^{-/-}*, *Ccl2^{-/-}*, *Ccr2^{-/-}*, *J α 18^{-/-}*, and *CD1d^{-/-}*), apoptosis (*Bim1^{-/-}*), and adenosine metabolism (*Cd73/NTE5^{-/-}*). Among these, only *Il23^{-/-}* mice were protected against osteosarcoma development, notably including *Pd1^{-/-}* (Fig 2C). Notably, the effect sizes observed in this model in both *Il23^{-/-}* and *Grm4^{-/-}* mice were comparable to or greater than those observed in *Rb^{+/-}* mice.

In mouse osteosarcomas, increased expression of *Il23* was observed relative to normal bone. To confirm these results, *in situ* hybridization for *Il23* transcript was undertaken in a series of human osteosarcomas. More than 60% of samples demonstrated focal staining for *Il23* (12% high, 24% medium), while little staining was observed in normal human bone (NHB) (Fig 3A and Fig S7). In an independent cohort quantitated by qRT-PCR, increased *Il23A* (p19) expression was noted in tumors compared to NHB, accompanied by reduced *Il12A* (p35) expression. More than 70% of samples exhibited significantly increased expression of *Il23* over NHB, with 53% having >2-fold increase and with 34% have >5-fold greater expression. By contrast, *Il12* p35 transcript expression was significantly lower in tumor samples relative to NHB (Fig 3B). Finally, high *Il23* expression (>8-fold) was associated with worse survival (Fig 3C: HR 0.33, 95%CI 0.06-1.69, $P = 0.0427$).

Both IL23 and GRM4 are potential therapeutic targets. Notably, IL23 blockade has been successful in treating psoriasis (24, 25). The anti-tumor activity of an antibody (16E5) targeting the p19 subunit of IL23 (α IL23) was compared to a control antibody (α AGP3, Amgen). Following autografting of osteosarcomas into the flank of the leg, mice were treated with α IL23 or α AGP3³. IL23 inhibition moderately slowed tumor growth ($P=0.0425$), and lengthened survival ($P=0.0322$; Fig 3D and Fig 3E). Neutralizing IL23 significantly decreased intratumor IL23 transcript as well downstream-targets (*Il22*, *Mmp9*, and *Tgf β* , but not *Il17*; Fig S8) (15, 26). Markers of cytotoxic T-cell activity, *Gzma* and *Cxcl9*, were significantly increased in tumors following treatment with α IL23 (Fig S9). To enhance the single agent activity of α IL23, it was combined with doxorubicin, an active agent in the treatment of osteosarcoma. These studies used pegylated liposomal doxorubicin (DOX), which has a more favourable toxicity profile for treatment of relapsed patients (27). In two independent cell lines, α IL23 and DOX significantly suppressed tumor growth compared to controls (Fig 3F, $P=0.009$). To test whether GRM4 also represented a potential therapeutic target, mice transplanted with osteosarcomas were treated with PHCCC, significantly suppressing tumor growth (Fig 3G, $P=0.0273$). This effect was associated with increased IL12 transcript levels (Figure S10). Similar effects were observed with another specific GRM4 agonist, LSP29166 (Fig S11). Taken together, these data support the therapeutic potential of targeting GRM4/IL23 axis.

Our studies show that GRM4 and IL-23 are rate-limiting for osteosarcoma development through non-cell autonomous mechanisms involving DCs. Although their role is unclear, intratumoral DCs connote a worse outcome in osteosarcoma(28). DCs are best known as antigen-presenting cells required for efficient activation of T-cells and maintenance of immune tolerance (29, 30)). To date, there is little genetic evidence for a rate-limiting role for DCs in cancer development. Polymorphisms in the DC-SIGN gene, expressed in DCs, are associated with nasopharyngeal carcinoma(31), while variants in the FGR1 gene affect intratumoral dendritic cell migration and response to chemotherapy (32). Here we present direct human and mouse genetic evidence for a rate-limiting pathway within dendritic cells for spontaneous tumor development. Surprisingly, the magnitude of the effect of *Grm4* was comparable to the canonical cell autonomous tumor suppressor, *Rb1*, a reminder that polymorphic effects in GWAS do not reflect the biological or therapeutic significance of associated genes.

GRM4 acts via the cAMP/PKA pathway, reported recently to possess tumor suppressor activity in osteosarcoma (33). GRM4 regulates the pro-inflammatory cytokines, IL23 and IL12 to create an immune-suppressive tumor environment. The oncogenic effects of IL-23 are seen in multiple cancer types (3, 15, 16, 34). In the context of our data on osteosarcoma, subjects with psoriasis, an autoimmune disease driven by IL23 are specifically at risk of cancers of bone and cartilage (HR 4.97, 95%CI 2.32-10.62, $P<0.0001$) (35). Critically, IL23 antagonists have favourable safety and toxicity profiles and are approved for the treatment of plaque psoriasis (25). Since immune checkpoint blockade has been disappointing in osteosarcoma (4), GRM4 agonists and/or IL23 antagonists, may be of therapeutic interest, perhaps combined with chemotherapy. The balance of IL23:IL12 is important, since IL-12 alone has potent anti-tumor activity (17). Since the first-generation agents

targeting IL23 inhibited both IL23 and IL12, second-generation agents with greater specificity for IL23 should probably be the agents of choice in future cancer trials. In this context, we note that targeting GRM4 agonists not only down-regulate IL23, but also increase IL12. In summary, our findings identify a non-cell autonomous pathway linking Grm4 to the pro-inflammatory IL23/IL12 axis, with the potential to be therapeutically targeted in osteosarcoma.

Fig. 1. *Grm4*^{-/-} mice have accelerated osteosarcoma development (A) Schematic of radiation-induced mouse model of osteosarcoma, mice at 28 days of age were injected with 1 μ Ci/g ⁴⁵Ca intraperitoneally once weekly for 4 consecutive weeks and monitored for the growth of tumors. (B) Kaplan Meier plot showing *Grm4*^{-/-} mice are predisposed to the development of ⁴⁵Ca induced osteosarcomas compared to WT mice (n=25-26/cohort) ($p=0.0003^{***}$). (C) Flow cytometry analysis of disaggregated tumors reveal that *Grm4* is predominantly expressed by tumor infiltrating Dcs (CD45⁺, MHCII⁺, CD11c⁺)(blue line) and with limited expression in tumor cells (CD45 negative cells)(dotted line) control fmo (dashed line). WT ⁴⁵Ca tumors were disaggregated into single cells and flow cytometry conducted to identify GRM4 expressing cells, representative tumor shown 19.4 % of MHC class II⁺, CD11c are positive for extracellular GRM4 expression. (D) Quantitative mRNA expression of DCs revealed that *Grm4*^{-/-} DCs (blue bars) had increased expression of inflammatory cytokines *Il12A* and *Il23A* compared to WT DCs (gray bars). Bone marrow DCs were isolated from WT or *Grm4*^{-/-} mice and cultured in the presence of IL4 and GM-CSF for 7 days and profiling conducted. (E) moDCs (markers as outlined in the text) coexpressed GRM4 and IL23 in the tumor microenvironment. Two ⁴⁵Ca tumors are shown (dashed and solid line) compared to control fmo. *Grm4*⁺ moDCs had limited IL12 expression. (F) BMDCs derived from wild type mice have attenuated IL12 expression and increased IL23 expression when treated with osteosarcoma cell lines condition media (CM) quantitative mRNA expression. (G & H) *Grm4* agonists inhibit IL23 protein expression in BMDC. BMDC were treated with GRM4 agonists PHCCC or Cinabarrinic acid for 1 hr followed by treatment with LPS at 1 μ g/ml or osteosarcoma cell line condition media (I & J).

Figure 2. *IL23*^{-/-} mice are protected from the development of tumors. (A) Kaplan Meier plot *IL23*^{-/-} mice are significantly protected from the development of ⁴⁵Ca induced osteosarcoma compared to wild type mice (n=25-26/cohort) ($P<0.0001$). (B) *Il17*^{-/-} mice (C) Forest plot of groups of 15-25 WT and knockout mice were injected with ⁴⁵Ca and aged, mice were monitored for tumor development for up to 2 years. Hazard ratio (log rank) 95% confidence interval, Mantel-Cox test for significance compared to WT.

Figure 3. *IL23* is expressed in human osteosarcoma and correlates with worse overall survival in humans (A) Representative image human osteosarcoma expressing *IL23A* relative to normal human bone. (B) Independent French cohort of osteosarcomas examined *IL23A* and *IL12* transcript levels relative to normal human bone (RT-PCR). We found that 53% (17/32) of the samples 2-fold or greater expression compared to control normal human bone (NHB) and that expression was 8-fold higher in 25% (7/32) samples, note that *IL12p35* transcript expression was significantly lower in tumor samples relative to NHB. (C) **High *IL23* expression trends with worse overall survival in human osteosarcoma.** Kaplan Meier curve of high expressors shown in B $P=0.0427$. ***IL23* and *Grm4* are targetable in osteosarcoma** (D) C57BL/6 mice were injected subcutaneously in the flank of the leg with osteosarcoma cell line OS18, mice were divided onto cohorts of 6 and treated with aAGP3 or α IL-23 or 500 μ g/mouse days 1, 3, 5, 7, 9, 12, 16, 21, mean tumor volume shown \pm SEM $*P=0.0425$ and prolongs survival Fig (E). Representative figure shown. (F) Synergistic effect of α IL-23 and

doxorubicin. Treatment starting day 23 after tumor cell implantation. Mice were treated with α IL-23 or α AGP3 500 μ g/mouse and liposomal DOX 10 mg/Kg/week, mean tumor volume shown \pm SEM ($n=6$ /cohort). **G**) PHCCC can suppress the growth of osteosarcoma. Seven mice per cohort were treated with PHCCC 10 mg/Kg in 20% DMSO or vehicle alone, once every second day (seven doses in total) (starting day 28) The Wilcoxon matched test was used to test statistical significance of difference between treatment groups ($p=0.0273$). LPS29166 another Grm4 agonist showed similar results to PHCCC Fig S12.

Fig 4 Schematic of proposed role for GRM4 in modifying tumor growth. Malignant cells produce inflammatory signals (e.g DAMPs (damage associated molecular patterns) in the microenvironment in turn recruiting myeloid cells. Inflammatory signals in the tumor microenvironment acting on DCs activate adenylyl cyclase and increase the production of cAMP, which in turn activates Protein Kinase A (PKA), and increase the expression of inflammatory cytokines, including IL23. Activation of GRM4 by glutamate binding or GRM4 agonists inhibits adenylyl cyclase and the production of cAMP, preventing the expression of IL23. Modified from neuroinflammation model (8). Therapeutic intervention by GRM4 agonists that down-regulate IL23 direct suppression of IL23 with neutralizing antibodies can suppress tumor growth. Down-regulation of IL23 in combination with chemotherapeutics may synergistically suppress tumor growth.

Materials and Methods

Mice. Inbred wild-type C57B6/J (C57BL/6 WT), C57BL6 GRM4 deficient (GRM4^{-/-}) were purchased from The Jackson Laboratory, C57BL/6 heterozygous (Rb^{+/-}), C57BL/6 Il23p19-deficient (Il23^{-/-}), C57BL/6 Wif1-deficient (Wif1^{-/-}), C57BL/6 Cd1d-deficient (Cd1d^{-/-}), C57BL/6 Ja18 (Ja18^{-/-}), C57BL/6 Ifn α R1 -deficient (Ifn α R1^{-/-}), C57BL/6 Ifn α R2-deficient (Ifn α R2^{-/-}), C57BL/6 Il17-deficient (IL17^{-/-}), C57BL/6 perforin and Ifn γ -deficient (pfpifn γ ^{-/-}), C57BL/6 CD274 /pdl1^{-/-}, C57BL/6 Trail-deficient (Trail^{-/-}), C57BL/6 Bim-deficient (Bim^{-/-}), C57BL/6 CD73-deficient (CD73^{-/-}), C57BL/6 Ccl2-deficient (Ccl2^{-/-}), C57BL/6 Ccr2-deficient (Ccr2^{-/-}), C57BL/6 Il1R-deficient (Il1r^{-/-}) mice were either generated using C57BL/6 ES cells or backcrossed to at least 10 generations to C57BL/6. All mice were genotyped using published protocols. Mice were bred at ABR and maintained at the Garvan Institute BTF, with all animal experiments carried out according to guidelines contained within the NSW (Australia) Animal Research Act 1985, the NSW (Australia) Animal Research Regulation 2010 and the Australian code of practice for the care and use of animals for scientific purposes, (8th Edition 2013, National Health and Medical Research Council (Australia)) and approved by Garvan/St Vincent's Animal Ethics and Experimentation Committees (approval number 15/21, 15/30). Some experiments were performed at the Peter MacCallum Cancer, Melbourne Australia, all procedures using mice were reviewed and approved by the Peter MacCallum animal ethics experimentation committee.

Mouse models of osteosarcoma. The radiocarcinogen model was conducted as previously described (10, 12). Briefly mice were injected with 1 μ Ci/g ⁴⁵Ca (GE Healthcare) or 0.9% saline intraperitoneally at 28 35, 42 and 49 days postpartum. Mice were aged and monitored for signs of tumorigenesis (limping, paralysis, loss of condition, poor feeding or grooming, or weight loss) twice weekly up to two years. Mice develop tumors in the spine (70%) and limbs (18%), and then pelvis, cranium, scapula, and clavicle (12%). ¹⁸F 2-deoxyglucose positron emission tomography (PET) scanning was undertaken as previously described (12). In some instances X-ray imaging was conducted using Faxitron. Mice were sedated with isoflurane inhalation and scanned. Short-term high dose ⁴⁵Ca studies used a single dose of 4 μ Ci/g ⁴⁵Ca at day 28 postpartum. Mice were sacrificed 14 days after exposure to ⁴⁵Ca, and blood, calvaria, spines, and sera were collected for analysis. **Tumor implantation model and treatment studies.** Osteosarcoma cell lines derived from the ⁴⁵Ca experiments (OS18, OS5) and cultured ex vivo were mixed with 1:1 Matrigel:Media (GIBCO) and a total volume of 100 μ l (10⁶ cells) was injected subcutaneously in the flank. Mice were monitored for tumor growth using digital calliper measurement (United Precision Machine, Inc). Mice were treated intraperitoneally with anti-Il23p19 (α IL23) or control antibody (AGP3) 500 mg/mouse (Amgen) weekly, liposomal doxorubicin (Calyx) 5-10 mg/kg or PHCCC in vehicle DMSO 20% 10 mg/kg or LSP29166 10 mg/kg in saline every second day for 14 days.

Gene expression analysis and statistical methods. Transcript levels of cytokines were determined using quantitative RT-PCR. Total RNA was extracted from cells using TRIzol™ and Qiagen RNAeasy mini kit as per manufacturer's instructions. RNA converted to cDNA using standard techniques. Real-time RT-PCR was carried out using SyBr Green (Applied Biosystems) according to the manufacturer's instructions using an ABI-Prism 7000 Sequence Detection System. All primer sequences are listed in Supplemental Table 1. Statistical analysis was performed using GraphPad Prism software.

Cytokine assay. Cell culture media from control and treated cells were frozen at -80°C. The concentration of cytokines was quantitatively determined using CBA IL23 kit mouse or human (BD Biosciences, San Jose, C, USA) as per kit instructions. A standard calibration kit was established for each kit. The maximum and minimum detection limits for cytokines was 1-5000 pg/mL.

Flow cytometry immune cell infiltration analysis. Tumors were washed in PBS, cut into 1 mm³ pieces, and tissue digested in DMEM supplemented with 2% FCS and 5 mg/ml collagenase A for 50 minutes at 37°C. Cells were passed through a 40 µm cellular sieve and labelled with surface antibodies and intracellular antibodies. Mouse splenocytes were used as positive controls for immune cells. Cells were analyzed using the Fortessa (BD Biosciences). Data was analyzed using Flowjo software®. Antibodies are listed in Supplemental Table 2.

Histology. Tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin, routine method. Human bone and osteosarcoma tissue microarrays were purchased from Biomax.USA (BO244b, OS804). In situ hybridization was carried out using probes for mouse and human IL23 RNA scope (ACD Bio-technique brand, USA, Newark, CA). Slides were scanned on Scan Score XT (Aperio).

DC enrichment and stimulation. BMDCs were generated as described (Abcam protocol). Briefly bone marrow was flushed out of mouse tibia and femur and single cell suspension plated in the presence of 50 ng/mL GM-CSF and 50 ng /mL IL4, 80% of the media was removed and media and new media added at day 3, assays were conducted on day 7. Flow cytometry analysis of enriched dendritic cells was conducted. Cells were plated in 6 well plates and treated.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software. (V7.0a, GraphPad, La Jolla, California, USA). Values are reported as means ± SEM. When comparing two groups, *P*-values were calculated using 2-tailed Student's *t* tests. For time to event and survival analysis *P*-values for the Kaplan-Meier survival curves were calculated with a log-rank (Mantel-Cox) test. Significance was conventionally accepted at *P*-values equal to or less than 0.05. For multiple treatment group comparisons, significance was determined by one-way analysis of variance, followed by Tukey post hoc multiple comparisons test where **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001.

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