Published OnlineFirst September 16, 2019; DOI: 10.1158/2159-8290.CD-19-0154

RESEARCH BRIEF

# Infiltrating Myeloid Cells Drive Osteosarcoma Progression via GRM4 Regulation of IL23

Maya Kansara[1,2], Kristian Thomson[1], Puiyi Pang[1], Aurelie Dutour[3], Lisa Mirabello[4], Francine Acher[5], Jean-Philippe Pin[6], Elizabeth G. Demicco[7], Juming Yan[8], Michele W.L. Teng[8], Mark J. Smyth[9], and David M. Thomas[1,2]

**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.

*See related commentary by Jones, p. 1484.*

## INTRODUCTION

Osteosarcoma is an aggressive primary malignant tumor of the bone and a significant cause of cancer-related death in the young. Patients are commonly treated with multimodal approaches, including surgery and adjuvant chemotherapy. Few effective systemic therapies have emerged in the last four decades for relapsed or metastatic osteosarcoma (1, 2). Osteosarcomas represent a promising indication for strategies that target the immune system (2); however, a recent clinical trial

[1]The Kinghorn Cancer Centre, Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia. [2]St. Vincent's Clinical School, Faculty of Medicine, UNSW Sydney, New South Wales, Australia. [3]Cancer Research Center of Lyon, INSERM UMR 1052, CNRS UMR 5286, Centre Leon Berard, Lyon, France. [4]Clinical Genetics Branch, Division of Cancer Epidemiology and Genetics, NCI, NIH, Bethesda, Maryland. [5]IGF, Universite de Montpellier, CNRS, INSERM, Montpellier, France. [6]Laboratoire de Chimie et de Biochimie Pharmacologiques et Toxicologiques, CNRS UMR8601, Université Paris Descartes, Sorbonne Paris Cité, Paris, France. [7]Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada. [8]Cancer Immunoregulation and Immunotherapy Laboratory, QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia. [9]Immunology in Cancer and Infection Laboratory, QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia.

**Note:** Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

**Corresponding Authors:** Maya Kansara, Garvan Institute of Medical Research, 370 Victoria Street, Darlinghurst, NSW, Sydney, Australia. Phone: 61-0-2-9355-5779; E-mail: m.kansara@garvan.org.au; and David M. Thomas, Phone: 61-0-2-9355-5770; E-mail: d.thomas@garvan.org.au

Cancer Discov 2019;9:1511–9

**doi:** 10.1158/2159-8290.CD-19-0154

Published OnlineFirst September 16, 2019; DOI: 10.1158/2159-8290.CD-19-0154



using immune-checkpoint blockade has failed so far in the treatment of advanced osteosarcoma (3).

Hereditary factors play an important role, and osteosarcoma is a feature of families with rare mutations in *TP53, RB1, RECQL4,* and *BLM* (4). A genome-wide association study (GWAS) investigating the role of common genetic variation 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 with susceptibility to osteosarcoma (5), which was 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 negatively regulates the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway. GRM4 plays a role in the central nervous system and is highly expressed in the cerebellum by cerebellar granule cells, but also by immune cells, and has been implicated in both neurodegenerative and autoimmune diseases (8, 9). Studies of the biological role of GRM4 in cancer are limited. Its expression has been associated with poor prognosis in several cancers, including malignant gliomas, colorectal cancer, and rhabdomyosarcoma (10), whereas GRM4 agonists have shown *in vitro* or xenograft activity in medulloblastoma (11) and glioblastoma cell lines (12), and recently in bladder cancer (13). In osteosarcoma, a small study has shown GRM4 expression correlates with better survival (14). Here, we sought to investigate the biological and therapeutic roles of GRM4 *in vivo* using genetic models of osteosarcoma.

## RESULTS

### *Grm4* Gene–Targeted Mice Have Accelerated Osteosarcoma Development and Identify Role for Inflammatory Cytokine IL23

We first asked whether GRM4 had tumor-suppressive or oncogenic effects on tumor development. Wild-type (WT; $n = 26$) or *Grm4*$^{-/-}$ mice ($n = 25$) were injected with $^{45}$Ca, a low-energy β-emitter that localizes to bone (ref. 15; Fig. 1A; Supplementary Fig. S1A–S1C). Ionizing radiation is the strongest environmental risk factor for osteosarcoma in humans (4). Tumor latency in this model is consistently 10 to 12 months (16, 17). *Grm4*$^{-/-}$ mice had accelerated tumor development [Fig. 1B; hazard ratio (HR) 0.41; 95% CI, 0.22–0.76, $P = 0.0006$; median survival in WT mice 89 weeks vs. 65 weeks in *Grm4*$^{-/-}$ mice). Outside the central nervous system, GRM4 is highly expressed by dendritic cells (DC), as well as CD4$^+$ T cells (8). In the mouse osteosarcomas, we observed GRM4 is predominantly expressed by CD45$^+$CD11c$^+$MHC$^+$ myeloid cells, but not by tumor cells (Fig. 1C). Too few CD4$^+$ T cells were detectable to characterize GRM4 expression (not shown).

GRM4 regulates DC expression of the cytokines IL1, IL6, IL23, and IL27 in experimental autoimmune encephalomyelitis (8). Using a standard strategy enriching for bone marrow DCs (18), *Grm4*$^{-/-}$ DCs showed selectively increased expression of the related proinflammatory cytokines IL12 and IL23 relative to WT DCs (Fig. 1D). Sharing a common p40 subunit, both IL12 and IL23 are secreted by human and mouse DCs and tissue-resident macrophages in response to exogenous or endogenous signals (19, 20). Increased expression of IL23 is observed in many human cancers (21–23), whereas IL12 has potent antitumor activity (24). Primary $^{45}$Ca osteosarcomas and allografted cell lines had high IL23 expression relative to normal bone, but *ex vivo*–cultured osteosarcoma cell lines did not express IL23 (Supplementary Fig. S2A and S2B). In addition, IL23 is not expressed within primary tumor cells (Fig. 1E). To show that these findings were not a function of a radiocarcinogen model, the expression of IL23 was also examined in osteosarcomas from genetically defined mouse models (*Osx/Cre Trp53/Rb* and *Osx/Cre Trp53.1224 pRb* mice, ref. 25; Supplementary Fig. S3A). Again, the majority of tumors had increased IL23 compared with normal bone. $^{45}$Ca radiation-induced osteosarcomas from *Grm4*$^{-/-}$ mice ($n = 5$) have higher expression of IL23 compared with WT tumors ($n = 6$; $P = 0.0358$; Supplementary Fig. S3B). Flow-cytometry analysis of $^{45}$Ca spontaneous tumors identified GRM4$^+$ MHC II$^+$ CD11c$^+$ cells as the predominant source of IL23 in the tumor microenvironment. To identify more precisely the tumor myeloid subpopulations and expression of GRM4, IL12, and IL23, we phenotyped osteosarcomas for infiltration of conventional DCs (cDC1: MHCII$^+$CD11c$^+$CD64$^-$Ly6c$^{lo}$CD11b$^{lo}$ or cDC2: MHCII$^+$CD11c$^+$CD64$^-$Ly6c$^{lo}$CD11b$^+$) and monocyte-derived DCs (MoDC), defined as MHCII$^+$CD11c$^+$Ly6c$^{hi}$CD64$^+$CD24$^{int}$ CD11b$^+$ (Supplementary Fig. S4 gating strategy; ref. 20). In the OS18 allograft, the predominant source of IL23 was found to be from GRM4$^+$ MoDCs (Supplementary Fig. S5). Similar results were observed in primary $^{45}$Ca tumors (Fig. 1E; Supplementary Fig. S6A), and the non-radiocarcinogen K7M2 allograft tumors (Supplementary Fig. S6B). By contrast with IL23, IL12 was virtually undetectable in tumor-derived MoDCs, and neither IL12 nor IL23 was observed in conventional DCs. Taken together, these data suggest that both GRM4 and IL23 are expressed by MoDCs within tumors.

To determine whether osteosarcoma cells influence the expression of IL12 and IL23 *in vitro*, bone marrow–derived DCs (BMDC) were exposed to conditioned media from cultured mouse osteosarcoma cells (OS-CM). Lipopolysaccharide (LPS) was used as a positive control (26, 27). In WT BMDCs, LPS increased cAMP (Supplementary Fig. S7A) and IL12 and IL23. OS-CM significantly induced IL23 expression in BMDCs, while suppressing IL12 (Fig. 1F and G). The cAMP/PKA pathway mediates, and LPS increases cAMP whereas GRM4 decreases cAMP production (8). Consistent with an intermediate role for the cAMP/PKA pathway, forskolin, a cAMP agonist, recapitulated the effect of OS-CM by inducing *Il23* expression by BMDCs (Supplementary Fig. S7B). GRM4 agonists are being developed for neurologic disorders, including depression and Parkinson disease (28). PHCCC [(–)-*N*-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide)] is a positive allosteric modulator of GRM4, whereas cinnabarinic acid (CIN) is an orthosteric regulator. Pretreatment with GRM4 agonists attenuated cAMP expression (Supplementary Fig. S7A) and suppressed LPS-induced (Supplementary Fig. S8A) or OS-CM–induced IL23 expression (Fig. 1H–K); PHCCC induced IL12 expression higher than that observed for LPS alone, but not OS-CM. PHCCC also downregulated IL23 in human peripheral blood DCs stimulated with LPS (Supplementary Fig. S8B). Collectively, these data support the interpretation that osteosarcoma cells repress IL23 production by MoDCs, an effect that is modulated by GRM4 signaling.

Published OnlineFirst September 16, 2019; DOI: 10.1158/2159-8290.CD-19-0154



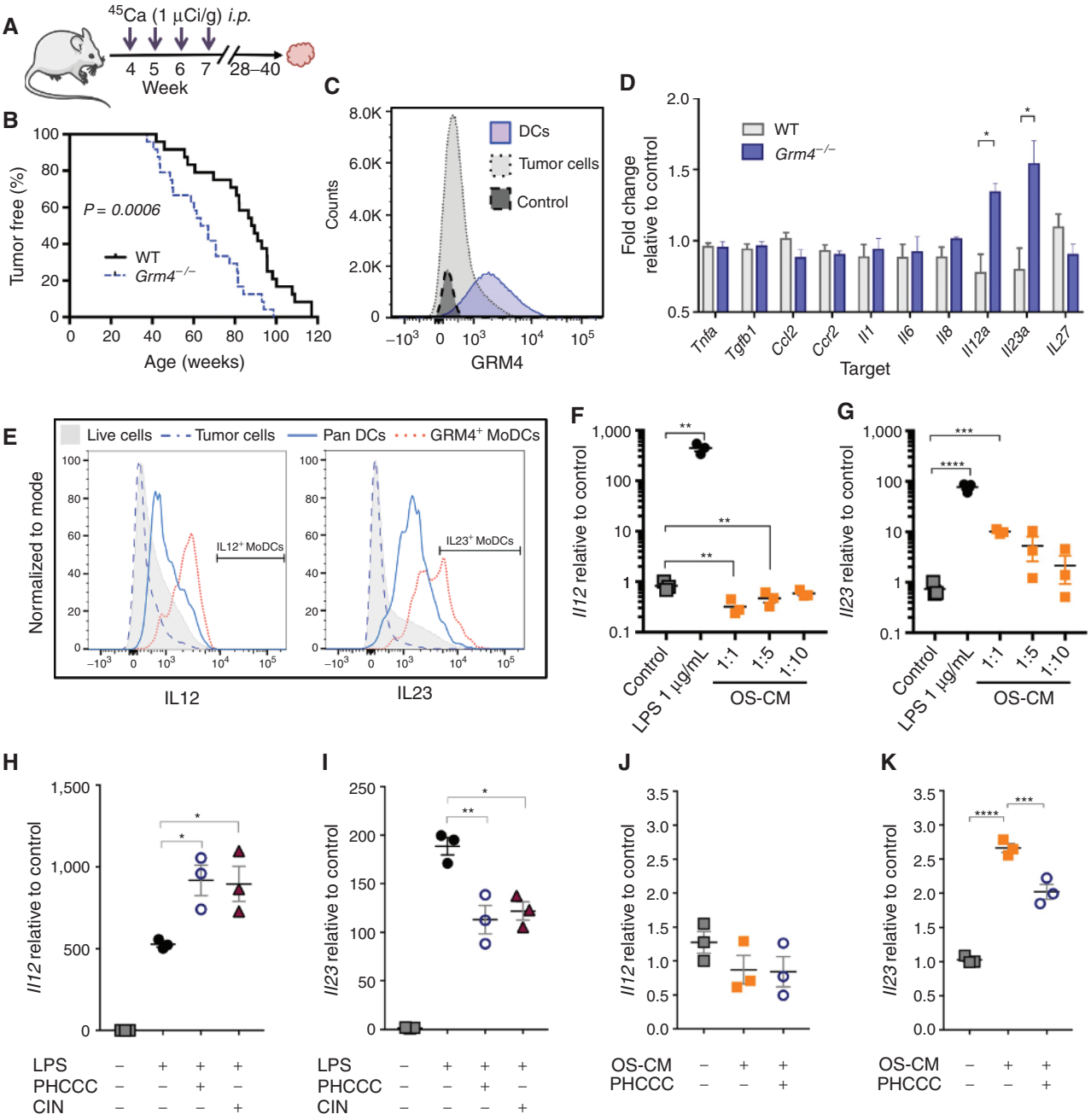


**Figure 1.** *Grm4*$^{-/-}$ mice have accelerated osteosarcoma development. **A,** Schematic of the radiation-induced mouse model of osteosarcoma; mice at 28 days of age were injected with 1 μCi/g $^{45}$Ca intraperitoneally once weekly for 4 consecutive weeks and monitored for the growth of tumors. **B,** Kaplan–Meier plots showing that *Grm4*$^{-/-}$ mice ($n = 25$) are predisposed to the development of $^{45}$Ca-induced osteosarcomas compared with WT mice ($n = 26$; $P = 0.0006$). **C,** Flow cytometry analysis of WT $^{45}$Ca tumors reveals that GRM4 is predominantly expressed by tumor-infiltrating myeloid cells defined as CD45$^+$, MHC class II$^+$, CD11c$^+$ (blue line) and with limited expression in tumor cells (CD45-negative cells), dotted line; control fluorescence minus one (FMO), dashed line. A representative tumor is shown with ~19.4 % of MHC class II$^+$, CD11c$^+$ cells positive for extracellular GRM4 expression. **D,** *Grm4*$^{-/-}$ BMDCs have higher transcript levels of *Il12a* and *Il23a* at baseline. Bone marrow was isolated from WT or *Grm4*$^{-/-}$ mice, cultured in the presence of IL4 and GMCSF for 7 days to enrich for BMDCs, and cytokine transcriptional profiling was conducted. *Grm4*$^{-/-}$, blue bars; WT, gray bars; data are normalized to house-keeping genes. Values are means and SEM of triplicate determinations; duplicate experiments were performed. **E,** Flow-cytometry analysis of a $^{45}$Ca WT osteosarcoma; tumor cells are defined as CD45-negative, Pan DCs CD11c$^+$MHCII$^+$, MoDCs (Supplementary Fig. S4 gating strategy). A representative tumor is shown. The crossbar shows gating for IL12 or IL23 FMO defining positive cytokine staining cells. **F** and **G,** BMDCs derived from WT mice have attenuated *Il12* expression and increased *Il23* expression when treated with OS-CM. WT BMDCs were treated with LPS 1 μg/mL or OS-CM for 24 hours, and transcript levels of cytokines were assessed using qRT-PCR as outlined in the Methods. GRM4 agonists suppress the expression of *Il23* by BMDCs. **H** and **I,** BMDCs were treated with GRM4 agonists PHCCC or CIN (40 μmol/L) for 1 hour and stimulated with LPS at 1 μg/mL for 24 hours, and transcript levels of cytokines measured. **J** and **K,** BMDCs were treated with PHCCC (40 μmol/L) for 1 hour and stimulated with OS-CM at a 1:1 ratio for 24 hours, and transcript levels were measured. Data are means ± SEM, representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Published OnlineFirst September 16, 2019; DOI: 10.1158/2159-8290.CD-19-0154



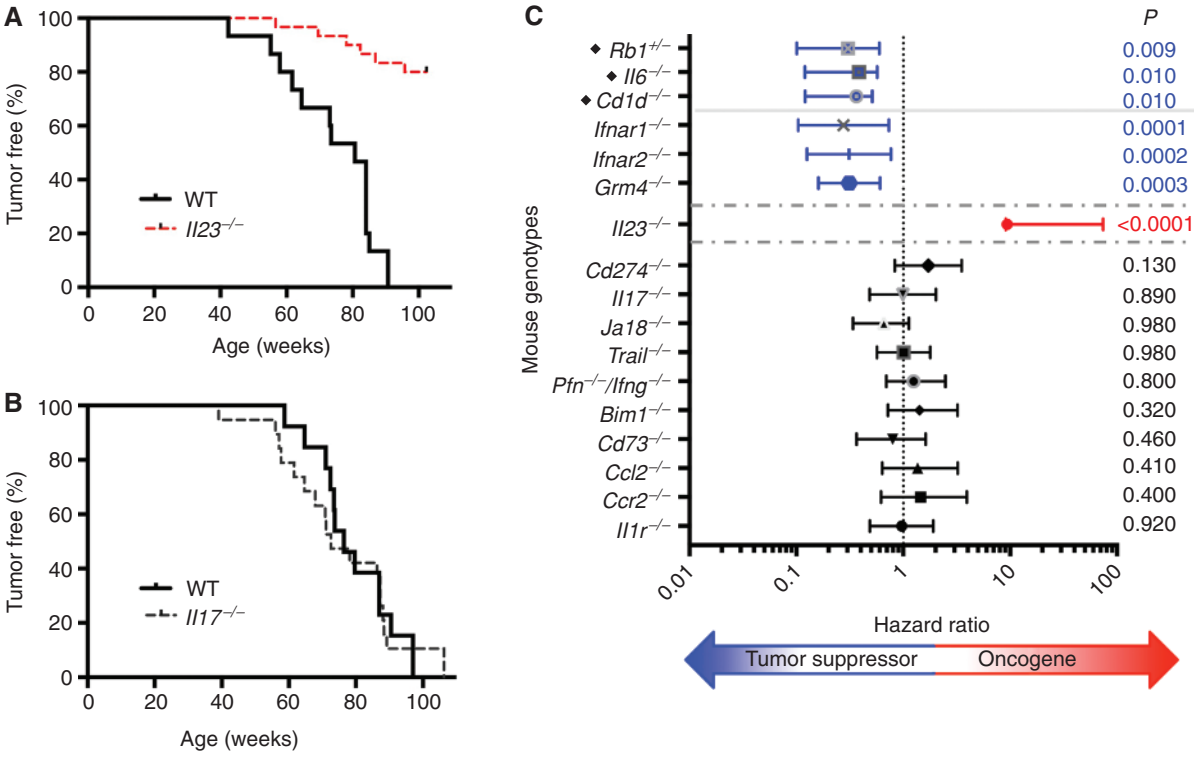


**Figure 2.** *Il23*$^{-/-}$ mice are protected from the development of tumors. **A,** Kaplan–Meier plot showing that IL23$^{-/-}$ mice are significantly protected from the development of $^{45}$Ca-induced osteosarcomas compared with WT mice ($n$ = 25–26/cohort, respectively; $P < 0.0001$). **B,** *Il17*$^{-/-}$ mice show no predisposition to or protection from the development of $^{45}$Ca-induced tumors. **C,** Forest plot of groups of 14–25 WT and litter-matched gene-targeted mice that were injected with $^{45}$Ca and then aged. Mice were monitored for tumor development for up to 2 years. Hazard ratio from log-rank test, 95% confidence interval, and Mantel–Cox test for significance compared with WT. ♦, published (see ref. 11).

## *Il23* Gene–Targeted Mice Are Protected from the Development of Tumors

Given that GRM4 suppresses tumor development (Fig. 1B), and IL23 is negatively regulated by GRM4, we tested whether IL23 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 with 0 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 $^{18}$F 2-deoxyglucose PET scanning, without any subclinical evidence of tumors (data not shown). IL6 drives the generation of IL17-expressing T-helper cells ($T_H17$), and IL23 differentiates $T_H17$ cells so that they acquire effector function (29, 30). However, unlike *Il6*$^{-/-}$ mice (16), *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 sarcoma (31). To put our findings of a protumorigenic role for IL23 in context, we tested 15 mouse genotypes of pathways implicated in immune control of cancer development [*Il1r*$^{-/-}$, *Il6*$^{-/-}$, *Il17*$^{-/-}$, *Pdl1*$^{-/-}$*(Cd274*$^{-/-}$*)*, *Ifnar1*$^{-/-}$, *Ifnar2*$^{-/-}$, *IFNγ*$^{-/-}$*/perforin*$^{-/-}$, *Trail*$^{-/-}$, *Ccl2*$^{-/-}$, *Ccr2*$^{-/-}$, *Jα18*$^{-/-}$, and *CD1d*$^{-/-}$)], apoptosis (*Bim1*$^{-/-}$), and adenosine metabolism (*Cd73/NTE5*$^{-/-}$; Supplementary Fig. S9A–S9J). Among these, only *Il23*$^{-/-}$ mice were protected against osteosarcoma development (Fig. 2C). Notably, the effect sizes observed in this model in both *Il23*$^{-/-}$ and *Grm4*$^{-/-}$ mice were comparable with or greater than those observed in *Rb*$^{+/-}$ mice (16).

## High *IL23* in the Tumor Correlates with Worse Overall Survival in Humans

We next examined the expression of *IL23* in a series of human osteosarcomas using *in situ* hybridization. More than 60% of samples demonstrated focal staining for *IL23A* (p19) expression (12% high, 24% medium), whereas little staining was observed in normal human bone (NHB; Fig. 3A; Supplementary Fig. S10). In an independent cohort quantitated by qRT-PCR, increased *IL23A* expression was noted in tumors compared with NHB, accompanied by reduced *IL12A* (p35) expression. More than 70% of samples exhibited significantly increased expression *of IL23A* over NHB, with 53% having >2-fold increase and 34% having >5-fold greater expression. By contrast, *IL12A* (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; $P = 0.0427$).

## IL23 and GRM4 Are Therapeutically Targetable in Osteosarcoma

Both IL23 and GRM4 are potential therapeutic targets, and IL23 blockade has been successful in treating psoriasis (32, 33). The antitumor activity of a neutralizing antibody (16E5)

Published OnlineFirst September 16, 2019; DOI: 10.1158/2159-8290.CD-19-0154



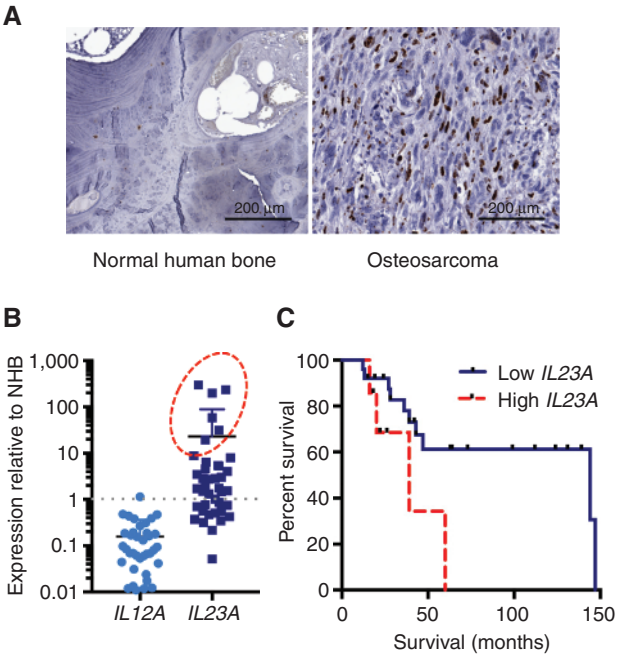


**Figure 3.** High *IL23A* in the tumor correlates with worse overall survival in humans. **A,** *In situ* hybridization was used to evaluate *IL23A* expression in a human osteosarcoma tissue microarray (40 samples in duplicate) and normal human bone (10 samples in duplicate). A representative image of human osteosarcoma and normal human bone expressing *IL23A*. **B,** *Il23A* and *IL12A* transcript levels were measured relative to normal human bone using qRT-PCR in an independent cohort with corresponding survival data. **C,** High *IL23A* expression trends with worse overall survival in human osteosarcoma. Kaplan–Meier curve of high *IL23A* expressors shown in **B** ($P = 0.0427$).

targeting the p19 subunit of IL23 (αIL23) was compared with a control antibody (αAGP3). Following implantation of osteosarcoma cells into the flank of the leg, mice were treated with αIL23 or αAGP3. IL23 inhibition moderately slowed tumor growth ($P = 0.0425$) and prolonged survival ($P = 0.0322$; Fig. 4A and Fig. B). Neutralizing IL23 significantly decreased intratumor *Il23* transcript as well as downstream targets (transcripts encoding IL22, MMP9, and TGFβ, but not IL17; Supplementary Fig. S11; refs. 21, 34). Markers of cytotoxic T-cell activity, *Gzma* and *Cxcl9*, were significantly increased in tumors following treatment with αIL23 (Supplementary Fig. S12). To enhance the single-agent activity of αIL23, it was combined with doxorubicin, one of the most active drugs in the treatment of osteosarcoma. These studies used PEGylated liposomal doxorubicin (DOX), which has a more favorable toxicity profile for treatment of relapsed patients (35). Targeting IL23 suppressed tumor growth compared with both controls and DOX-treated mice (Fig. 4C; $P = 0.009$). To test whether GRM4 also represented a potential therapeutic target, mice transplanted with osteosarcomas were treated with PHCCC or DOX. PHCCC significantly suppressed tumor growth (Fig. 4D; $P = 0.0001$) with a potency comparable to DOX, but without the weight loss associated with DOX, suggesting PHCCC was better tolerated (Supplementary Fig. S13). Treatment with PHCCC was associated with increased IL12 transcript levels (Supplementary Fig. S14A and S1B). Similar effects were observed with another specific GRM4 agonist, LSP2-9166 (Supplementary Fig. S15). Taken together, these data support the therapeutic potential of targeting the GRM4–IL23 axis (Fig. 4E). Neither an IL23 blocking antibody nor GRM4 agonists affected the growth of primary osteosarcoma cells cultured *ex vivo*, consistent with a key role for the host immune system in mediating their antitumor effects (Supplementary Fig. S16).

## DISCUSSION

Cancer immunoediting studies over two decades suggest the immune system has secondary modifier effects on tumor development relative to the contribution of cell-autonomous, classic tumor suppressors and oncogenes (36). Here, we present direct human and mouse genetic evidence for a non–cell-autonomous role for GRM4 and IL23 within MoDCs in spontaneous tumor development. Strikingly, the magnitude of the effect of loss of GRM4 is comparable with the loss of the canonical tumor suppressor RB1. The modest disease association of polymorphisms in GWAS, presumably due to weak effects on gene expression, does not predict the biological or therapeutic effect due to complete loss-of-function mutations in mice. Of the 17 gene-targeted genotypes tested, the size of the oncogenic effect of IL23 in tumor development was marked. Although the oncogenic effects of IL23 have been reported in multiple cancer types (2, 21, 22, 37), it is interesting that subjects with psoriasis, an autoimmune disease driven by IL23, are specifically at risk of osteosarcoma and chondrosarcomas (HR 4.97; 95% CI, 2.32–10.62; $P < 0.0001$; ref. 38).

We propose that tumor-infiltrating MoDCs responding to inflammatory signals in the tumor microenvironment secrete IL23, contributing to an immune-suppressed environment. GRM4 and IL23 are primarily coexpressed within monocytic dendritic cells, and do not appear to be expressed by tumor cells. This is consistent with our previous observations (39), although we cannot exclude the role of other stromal cells in tumor development. GRM4 activation downregulates IL23 and suppresses tumor growth. In human osteosarcomas, tumor-infiltrating myeloid cells, including dendritic cells and macrophages, connote a worse survival outcome (40). DCs comprise diverse progeny of the myeloid lineage, including antigen-presenting cells (APC) required for efficient activation of T cells and maintenance of immune tolerance (41, 42). APCs are best known in cancer through their pivotal role in therapeutic vaccination strategies (43). Distinct DC populations have shown opposing effects on tumor immunity, driving antitumor immunity or contributing to immune nonresponsiveness in cancer (20). The understanding of myeloid subpopulations within tumors and their functional role is a rapidly evolving field (20, 42). Our data linking GRM4 to IL23 build on emerging evidence that glutamate signaling regulates IL23 expression (44), probably via GRM4 (8). It is important to note that IL23 is likely regulated by other mechanisms than GRM4, and also that GRM4 has actions independent of IL23.

There are significant therapeutic implications of these findings. To date, immune-checkpoint inhibitors targeting PD-1/PD-L1 have been disappointing in osteosarcoma (3).

Published OnlineFirst September 16, 2019; DOI: 10.1158/2159-8290.CD-19-0154



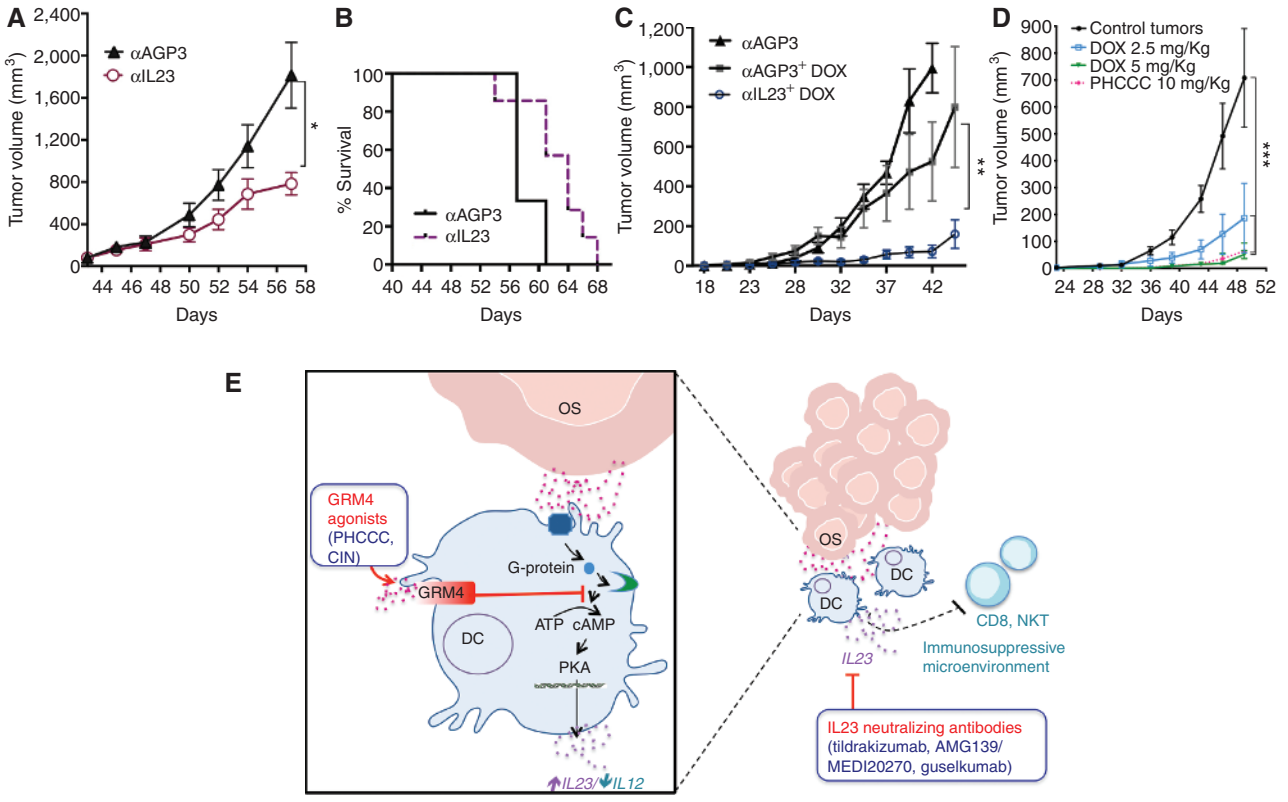


**Figure 4.** IL23 and GRM4 are therapeutically targetable in osteosarcoma. **A,** C57BL/6 mice were injected subcutaneously in the flank of the leg with osteosarcoma cell line OS18 until tumors were palpable. Mice were divided into cohorts of 6 and treated with αAGP3 or αIL23 (500 μg/mouse; 1, 3, 5, 7, 9, 12, 16, and 21). Mean tumor volume is shown ± SEM; *, $P = 0.0425$. Survival of the same, a representative experiment is shown **(B). C,** Synergistic effect of anti-IL23 and doxorubicin (DOX). Mice were treated with αIL23 or αAGP3 (500 μg/mouse), as above and liposomal DOX (5 mg/kg/once a week). Treatment started day 23 after tumor-cell implantation. Mean tumor volume shown ± SEM ($n = 6$/cohort); a representative experiment is shown. **D,** PHCCC suppresses the growth of osteosarcoma. Six mice per cohort were treated with PHCCC 10 mg/kg in 20% DMSO s.c. or vehicle alone (days 8, 10, 12, 14, 16, 18, 20, 30, 32, 34 i.p. and DOX 2.5 mg/kg or 5 mg/kg day 10, 17, 24, 30 i.v.; 5 doses in total). The Wilcoxon matched test was used to test the statistical significance of difference between control and each treatment group, PHCCC and DOX (all $P = 0.0001$). LPS29166, another GRM4 agonist, also suppressed tumor growth (Supplementary Fig. S13). **E,** Schematic of the proposed role for GRM4 in modifying tumor growth. Malignant cells produce inflammatory signals 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 increases the expression of inflammatory cytokines, including IL23. Activation of GRM4 by glutamate binding or GRM4 agonists inhibits adenynyl cyclase and the production of cAMP, inhibiting the expression of IL23. Modified from neuroinflammation model in ref. 8. Therapeutic intervention by GRM4 agonists that downregulate IL23 or direct inhibition of IL23 activity with neutralizing antibodies can suppress tumor growth. NKT, natural killer T cell.

The therapeutic effect of both IL23 antagonists and GRM4 agonists appeared comparable in our system to doxorubicin, one of the most active drugs currently used for the treatment of osteosarcoma. The fact that both *Grm4*$^{-/-}$ and *Il23*$^{-/-}$ mice apparently develop normally suggests drugs targeting these genes may be well tolerated. IL23 antagonists are already approved for the treatment of plaque psoriasis, with favorable toxicity profiles (33). Our data suggest the balance of IL23:IL12 is important, because IL12 alone has potent antitumor activity (24). The first-generation agent IL23 antagonists (e.g., ustekinumab) targeted both IL23 and IL12 through a shared p40 subunit and may be less effective cancer therapeutics than second-generation agents selectively targeting the p19 subunit of IL23 (e.g., tildrakizumab and guselkumab; ref. 45). Of note, GRM4 agonists not only downregulate IL23, but also increase IL12. Foliglurax (46), a novel positive allosteric activator of GRM4, is currently in phase II trials for Parkinson disease (NCT03162874A). In summary, our findings identify a non–cell-autonomous pathway linking GRM4 to the proinflammatory IL23–IL12 axis, with the potential to be therapeutically targeted in osteosarcoma.

## METHODS

### Mice

Inbred wild-type C57B6/J (C57BL/6 WT) and C57BL6 GRM4-deficient (*Grm4*$^{-/-}$) mice were purchased from The Jackson Laboratory; C57BL/6 *Il23p19*-deficient (*Il23*$^{-/-}$), C57BL/6 *Cd1d*-deficient (*Cd1d*$^{-/-}$), C57BL/6 *IfnαR1*-deficient (*IfnαR1*$^{-/-}$), C57BL/6 *IfnαR2*-deficient (*IfnαR2*$^{-/-}$), C57BL/6 *Il17*-deficient (*Il17*$^{-/-}$), C57BL/6 perforin and *Ifnγ*-deficient (*pfpifnγ*$^{-/-}$), 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*$^{-/-}$), and C57BL/6 *Il1r*-deficient (*Il1r*$^{-/-}$) mice were either generated using C57BL/6 embryonic stem cells or back-crossed to at least 10 generations to C57BL/6. All mice were genotyped using published protocols. Mice were bred at Australian BioResources (Moss Vale, NSW, Australia) and

Published OnlineFirst September 16, 2019; DOI: 10.1158/2159-8290.CD-19-0154



maintained at the Garvan Institute Biological Testing Facility, 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, 18/31, 18/38). 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 experiments using the radiocarcinogen model were conducted as previously described (15, 16). Briefly, mice were injected with 1 μCi/g $^{45}$Ca (GE Healthcare) or 0.9% saline intraperitonially 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 for up to two years. Mice developed tumors in the spine (70%) and limbs (18%), and then pelvis, cranium, scapula, and clavicle (12%). In some instances, X-ray imaging was conducted using a Faxitron system. Mice were sedated with isoflurane inhalation and scanned.

### Tumor Implantation Model and Treatment Studies

Osteosarcoma cell lines were derived from tumors from $^{45}$Ca experiments (OS18, OS25); these cell lines generate osteosarcoma when implanted into C57BL/6 mice. The OS cell lines were derived by culturing tumor pieces in Minimum Essential Medium with alpha modification, 10% heat-inactivated fetal calf serum, 1× penicillin/streptomycin, 2 mmol/L Glutamax, and 1 mmol/L sodium pyruvate until a monolayer grew out. Cells were passaged 20 to 25 times and were checked for *Mycoplasma* contamination by qRT-PCR, and aliquots were frozen. New aliquots were thawed for each experiment and passaged up to 8 times. OS lines cultured *in vitro* were mixed with 1:1 Matrigel:media (GIBCO), and a total volume of 100 μL ($10^6$ cells) was injected subcutaneously in the flank. Mice were monitored for tumor growth relative to adjacent non–tumor cell injected leg using digital calipers (United Precision Machine, Inc.). Mice were treated as described in the legends; mice were treated intraperitoneally with anti-IL23p19 (αIL23) or control antibody (αAGP3) at 500 μg/mouse (Amgen) weekly, liposomal doxorubicin (Calyx) 2.5–5 mg/kg or PHCCC in vehicle DMSO 20% 10 mg/kg or LSP2-9166 10 mg/kg in saline as described in the legends.

### 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 RNeasy Mini Kit per the manufacturer's instructions. RNA was 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 Supplementary Table S1. Statistical analysis was performed using GraphPad Prism software. Human osteosarcoma samples with correlative survival data were collected and approved under ethics application project CMT 2018-010 approved by HREC CRB Centre Léon Bérard, Lyon, France.

### Cytokine Assay

Cell culture media from control and treated cells were frozen at −80°C. The concentration of cytokines was quantitatively determined using Cytometric Bead Array (CBA) Cytokine Kits, mouse or human (BD Biosciences), as per kit instructions. A standard calibration curve was established for each kit. The maximum and minimum detection limits for cytokines were 1 to 5,000 pg/mL.

### Flow-Cytometry Immune Cell–Infiltration Analysis

Tumors were washed in PBS and cut into 1-mm$^3$ pieces, and tissue was digested in DMEM supplemented with 2% FCS and 5 mg/mL collagenase A for 50 minutes at 37°C. Cells were passed through a 70-μm then a 40-μm cellular sieve and labeled with surface antibodies and intracellular antibodies. Mouse splenocytes were used as positive controls for immune cells. Cells were analyzed using a Fortessa system (BD Biosciences). Data were analyzed using FlowJo software. Antibodies are listed in Supplementary Table S2.

### Histology

Tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned and stained with hematoxylin and eosin, via routine method. Human bone and osteosarcoma tissue microarrays were purchased from Biomax (BO244b, OS804). *In situ* hybridization was carried out using probes for mouse and human IL23-encoding RNA (ACD Bio-Techne). Slides were scanned on ScanScope XT (Aperio).

### DC Enrichment and Stimulation

BMDCs were generated as described (Abcam protocol modified from ref. 18). 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 (PeproTech); 80% of the medium was removed, and new medium 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). Values are reported as means ± SEM. When comparing two groups, $P$ values were calculated using two-tailed Student $t$ 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 the Tukey *post hoc* multiple comparisons test, where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

### Data and Materials Availability

All data are available in the main text or the supplementary materials.

## Disclosure of Potential Conflicts of Interest

J.-P. Pin reports receiving a commercial research grant from CisBio Bioassays and has ownership interest in patents on mGlu4 assays and pharmacological compounds. E.G. Demicco is a consultant/advisory board member for Bayer. M.W.L. Teng has received honoraria from the speakers bureaus of Bristol-Myers Squibb, Roche, and Merck. M.J. Smyth reports receiving commercial research grants from Bristol-Myers Squibb and Tizona Therapeutics, has ownership interest (including patents) in Tizona Therapeutics, and is a consultant/advisory board member for Tizona Therapeutics and Compass Therapeutics. D.M. Thomas reports receiving commercial research support from Amgen. No potential conflicts of interest were disclosed by the other authors.

## Authors' Contributions

**Conception and design:** M. Kansara, M.J. Smyth, D.M. Thomas
**Development of methodology:** M. Kansara, J.-P. Pin, D.M. Thomas
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** K. Thomson, P. Pang, A. Dutour, F. Acher, E.G. Demicco, J. Yan, M.J. Smyth

Published OnlineFirst September 16, 2019; DOI: 10.1158/2159-8290.CD-19-0154



**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** M. Kansara, K. Thomson, L. Mirabello, M.W.L. Teng, M.J. Smyth, D.M. Thomas
**Writing, review, and/or revision of the manuscript:** M. Kansara, A. Dutour, F. Acher, J.-P. Pin, M.W.L. Teng, M.J. Smyth, D.M. Thomas
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** M. Kansara, K. Thomson, P. Pang, D.M. Thomas
**Study supervision:** M. Kansara, D.M. Thomas

## Acknowledgments

This work was supported by grants from the National Health and Medical Research Council (NHMRC), NHMRC Program Grant APP1113482. M. Kansara and D.M. Thomas were supported by a Cancer Council NSW grant 1107784, Tour de Cure foundation project grant, and Shriver Immunosarc International Collaborative Grant. D.M. Thomas was supported by an NHMRC Principal Research Fellowship and M.J. Smyth was supported by an NHMRC Australia Fellowship and Senior Principal Research Fellowship. M.J. Smyth and M.W. Teng were supported by a donation from the Summit to Sarcoma and a grant from the Hare Foundation. We thank Torsten Nielsen and Amanda Dancsok, University of British Columbia, Vancouver, Canada, for helpful discussion, and Carl Walkley, St. Vincent's Institute, Melbourne, Australia, for providing mouse tumor tissue.

Received February 3, 2019; revised June 3, 2019; accepted July 18, 2019; published first September 16, 2019.

## REFERENCES

1. Harris MB, Gieser P, Goorin AM, Ayala A, Shochat SJ, Ferguson WS, et al. Treatment of metastatic osteosarcoma at diagnosis: a Pediatric Oncology Group study. J Clin Oncol 1998;16:3641–8.
2. Kansara M, Teng MW, Smyth MJ, Thomas DM. Translational biology of osteosarcoma. Nat Rev Cancer 2014;14:722–35.
3. Tawbi HA, Burgess M, Bolejack V, Van Tine BA, Schuetze SM, Hu J, et al. Pembrolizumab in advanced soft-tissue sarcoma and bone sarcoma (SARC028): a multicentre, two-cohort, single-arm, open-label, phase 2 trial. Lancet Oncol 2017;18:1493–501.
4. Thomas DM, Ballinger ML. Diagnosis and management of hereditary sarcoma. Recent Results Cancer Res 2016;205:169–89.
5. Savage SA, Mirabello L, Wang Z, Gastier-Foster JM, Gorlick R, Khanna C, et al. Genome-wide association study identifies two susceptibility loci for osteosarcoma. Nat Genet 2013;45:799–803.
6. Jiang C, Chen H, Shao L, Dong Y. GRM4 gene polymorphism is associated with susceptibility and prognosis of osteosarcoma in a Chinese Han population. Med Oncol 2014;31:50.
7. Wang K, Zhao J, He M, Fowdur M, Jiang T, Luo S. Association of GRM4 gene polymorphisms with susceptibility and clinicopathological characteristics of osteosarcoma in Guangxi Chinese population. Tumour Biol 2015;37:1105–12.
8. Fallarino F, Volpi C, Fazio F, Notartomaso S, Vacca C, Busceti C, et al. Metabotropic glutamate receptor-4 modulates adaptive immunity and restrains neuroinflammation. Nat Med 2010;16:897–902.
9. Marino MJ, Hess JF, Liverton N. Targeting the metabotropic glutamate receptor mGluR4 for the treatment of diseases of the central nervous system. Curr Top Med Chem 2005;5:885–95.
10. Chang HJ, Yoo BC, Lim SB, Jeong SY, Kim WH, Park JG. Metabotropic glutamate receptor 4 expression in colorectal carcinoma and its prognostic significance. Clin Cancer Res 2005;11:3288–95.
11. Iacovelli L, Arcella A, Battaglia G, Pazzaglia S, Aronica E, Spinsanti P, et al. Pharmacological activation of mGluR4 metabotropic glutamate receptors inhibits the growth of medulloblastomas. J Neurosci 2006;26:8388–97.
12. Zhang Z, Zheng X, Luan Y, Liu Y, Li X, Liu C, et al. Activity of metabotropic glutamate receptor 4 suppresses proliferation and promotes apoptosis with inhibition of gli-1 in human glioblastoma cells. Front Neurosci 2018;12:320.
13. Zhang Z, Liu Y1, Wang K, Zhu K, Zheng X, Wang L, et al. Activation of type 4 metabotropic glutamate receptor promotes cell apoptosis and inhibits proliferation in bladder cancer. J Cell Physiol 2019;234:2741–55.
14. Wang S, Wei X, Chen B, Zhao M, Song G, Zhang Z, et al. Expression of metabotropic glutamate receptor 4 in osteosarcoma. Mol Clin Oncol 2016;4:65–9.
15. Finkel MP, Jinkins PB, Biskis BO. Parameters of radiation dosage that influence production of osteogenic sarcomas in mice. Nat Cancer Inst Monogr 1964;14:243–69.
16. Kansara M, Leong HS, Lin DM, Popkiss S, Pang P, Garsed DW, et al. Immune response to RB1-regulated senescence limits radiation-induced osteosarcoma formation. J Clin Invest 2013;123:5351–60.
17. Kansara M, Tsang M, Kodjabachian L, Sims NA, Trivett MK, Ehrich M, et al. Wnt inhibitory factor 1 is epigenetically silenced in human osteosarcoma, and targeted disruption accelerates osteosarcomagenesis in mice. J Clin Invest 2009;119:837–51.
18. Labeur MS, Roters B, Pers B, Mehling A, Luger TA, Schwarz T, et al. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. J Immunol 1999;162:168–75.
19. Teng MW, Bowman EP, McElwee JJ, Smyth MJ, Casanova JL, Cooper AM, et al. IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. Nat Med 2015;21:719–29.
20. Laoui D, Keirsse J, Morias Y, Overmeire EV, Geeraerts X, Elkrim Y, et al. The tumour microenvironment harbours ontogenically distinct dendritic cell populations with opposing effects on tumour immunity. Nat Commun 2016;7:13720.
21. Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, et al. IL-23 promotes tumour incidence and growth. Nature 2006;442:461–5.
22. Langowski JL, Kastelein RA, Oft M. Swords into plowshares: IL-23 repurposes tumor immune surveillance. Trends Immunol 2007;28:207–12.
23. Yan J, Smyth MJ, Teng MW L. Interleukin (IL)-12 and IL-23 and their conflicting roles in cancer. Cold Spring Harb Perspect Biol 2018;10:pii:a028530.
24. Lasek W, Zagozdzon R, Jakobisiak M. Interleukin 12: still a promising candidate for tumor immunotherapy? Cancer Immunol Immunother 2014;63:419–35.
25. Walkley CR, Qudsi R, Sankaran VG, Perry JA, Gostissa M, Roth SI, et al. Conditional mouse osteosarcoma, dependent on p53 loss and potentiated by loss of Rb, mimics the human disease. Genes Develop 2008;22:1662–76.
26. Li K, Anderson KJ, Peng Q, Noble A, Lu B, Kelly AP, et al. Cyclic AMP plays a critical role in C3a-receptor-mediated regulation of dendritic cells in antigen uptake and T-cell stimulation. Blood 2008;112:5084–94.
27. Schnurr M, Toy T, Shin A, Wagner M, Cebon J, Maraskovsky E. Extracellular nucleotide signaling by P2 receptors inhibits IL-12 and enhances IL-23 expression in human dendritic cells: a novel role for the cAMP pathway. Blood 2005;105:1582–9.
28. Marino MJ, Williams DL Jr, O'Brien JA, Valenti O, McDonald TP, Clements MK, et al. Allosteric modulation of group III metabotropic glutamate receptor 4: a potential approach to Parkinson's disease treatment. Proc Natl Acad Sci U S A 2003;100:13668–73.
29. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 2003;278:1910–4.
30. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005;6:1123–32.
31. Teng MW, Swann JB, von Scheidt B, Sharkey J, Zerafa N, McLaughlin N, et al. Multiple antitumor mechanisms downstream of prophylactic regulatory T-cell depletion. Cancer Res 2010;70:2665–74.
32. Kopp T, Riedl E, Bangert C, Bowman EP, Greisenegger E, Horowitz A, et al. Clinical improvement in psoriasis with specific targeting of interleukin-23. Nature 2015;521:222–6.
33. Papp K, Thaçi D, Reich K, Riedl E, Langley RG, Krueger JG, et al. Tildrakizumab (MK-3222), an Anti-IL-23p19 monoclonal antibody,

Published OnlineFirst September 16, 2019; DOI: 10.1158/2159-8290.CD-19-0154



improves psoriasis in a phase 2b randomized placebo-controlled trial. Br J Dermatol 2015;173:930.
34. Sherlock JP, Joyce-Shaikh B, Turner SP, Chao CC, Sathe M, Grein J, et al. IL-23 induces spondyloarthropathy by acting on ROR-gammat+ CD3+CD4-CD8- entheseal resident T cells. Nat Med 2012;18: 1069–76.
35. Rafiyath SM, Rasul M, Lee B, Wei G, Lamba G, Liu D. Comparison of safety and toxicity of liposomal doxorubicin vs. conventional anthracyclines: a meta-analysis. Exp Hematol Oncol 2012;1:10.
36. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011;331:1565–70.
37. Teng MW, von Scheidt B, Duret H, Towne JE, Smyth MJ. Anti-IL-23 monoclonal antibody synergizes in combination with targeted therapies or IL-2 to suppress tumor growth and metastases. Cancer Res 2011;71: 2077–86.
38. Jensen P, Egeberg A, Gislason G, Thyssen JP, Skov L. Risk of uncommon cancers in patients with psoriasis: a Danish nationwide cohort study. J Eur Acad Dermatol Venereol 2017;32:601–5.
39. von Scheidt B, Leung PS, Yong MC, Zhang Y, Towne JE, Smyth MJ, et al. Combined anti-CD40 and anti-IL-23 monoclonal antibody therapy effectively suppresses tumor growth and metastases. Cancer Res 2014;74:2412–21.
40. Koirala P, Roth ME, Gill J, Piperdi S, Chinai JM, Geller DS, et al. Immune infiltration and PD-L1 expression in the tumor microenvironment are prognostic in osteosarcoma. Scientific Rep 2016;6:30093.
41. Audiger C, Rahman MJ, Yun TJ, Tarbell KV, Lesage S. The importance of dendritic cells in maintaining immune tolerance. J Immunol 2017;198:2223–31.
42. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Ann Rev Immunol 2000;18: 767–811.
43. Santos PM, Butterfield LH. Dendritic cell-based cancer vaccines. J Immunol 2018;200:443–9.
44. Mogilenko DA, Haas JT, L'homme L, Fleury S, Quemener S, Levavasseur M, et al. Metabolic and innate immune cues merge into a specific inflammatory response via the UPR. Cell 2019;177:1201–16.
45. Bilal J, Berlinberg A, Bhattacharjee S, Trost J, Riaz IB, Kurtzman DJB. A systematic review and meta-analysis of the efficacy and safety of the interleukin (IL)-12/23 and IL-17 inhibitors ustekinumab, secukinumab, ixekizumab, brodalumab, guselkumab and tildrakizumab for the treatment of moderate to severe plaque psoriasis. J Dermatolog Treat 2018;29:569–78.
46. Charvin D, Di Paolo T, Bezard E, Gregoire L, Takano A, Duvey G, et al. An mGlu4-positive allosteric modulator alleviates parkinsonism in primates. Mov Disord 2018;33:1619–31.

Published OnlineFirst September 16, 2019; DOI: 10.1158/2159-8290.CD-19-0154

AACR American Association for Cancer Research

CANCER DISCOVERY

# Infiltrating Myeloid Cells Drive Osteosarcoma Progression via GRM4 Regulation of IL23

Maya Kansara, Kristian Thomson, Puiyi Pang, et al.

*Cancer Discov* 2019;9:1511-1519. Published OnlineFirst September 16, 2019.