Interleukin-27 Gene Delivery for Modifying Malignant Interactions Between Prostate Tumor and Bone

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Abstract

We have examined the role of a novel cytokine, interleukin-27 (IL-27), in mediating interactions between prostate cancer and bone. IL-27 is the most recently characterized member of the family of heterodimeric IL-12-related cytokines and has shown promise in halting tumor growth and mediating tumor regression in several cancer models, including prostate cancer. Prostate cancer is frequently associated with metastases to the bone, where the tumor induces a vicious cycle of communication with osteoblasts and osteoclasts to induce bone lesions, which are a significant cause of pain and skeletal-related events for patients, including a high fracture risk. We describe our findings in the effects of IL-27 gene delivery on prostate cancer cells, osteoblasts, and osteoclasts at different stages of differentiation. We applied the IL-27 gene delivery protocol *in vivo* utilizing sonoporation (sonodelivery) with the goal of treating and reducing the growth of prostate cancer at a bone metastatic site *in vivo*. We used a new model of immune-competent prostate adenocarcinoma and characterized the tumor growth reduction, gene expression, and effector cellular profiles. Our results suggest that IL-27 can be effective in reducing tumor growth, can help normalize bone structure, and can promote enhanced accumulation of effector cells in prostate tumors. These results are promising, because they are relevant to developing a novel IL-27-based strategy that can treat both the tumor and the bone, by using this simple and effective sonodelivery method for treating prostate tumor bone metastases.

Introduction

METASTASES TO BONE TISSUE are frequent in breast and prostate cancer patients (Mundy, 2002), resulting in incurable bone lesion development and significant morbidity (Clark and Torti, 2003; Roodman, 2004). Bone metastases also can lead to significant pain, fractures, nerve compression syndromes, and hypercalcemia. There are many factors that worsen the outcome of skeletal complications in patients with prostate cancer, including decreased bone integrity and density resulting from androgen deprivation (Smith, 2007), which is associated with an increased risk of fractures within 2 years posttherapy (Daniell, 1997; Hatano *et al.*, 2000). Bone metastases have been reported in ~65–90% of patients with advanced prostate cancer (Bubendorf *et al.*, 2000; Carlin and Andriole, 2000; Coleman, 2001). The types of bone lesions associated with prostate cancer include increased immature bone formation (osteoblastic lesions) (Berruti *et al.*, 2000), and also enhanced osteolysis by osteoclasts (OC; osteoclastic lesions) (Goltzman, 1997; Mundy and Guise, 1997; Boyce *et al.*,

1999), which promote a high level of bone resorption (Berruti *et al.*, 2001). A key objective in managing skeletal morbidity in patients with prostate cancer is to reestablish the homeostasis of bone metabolism by inhibiting excess osteolysis. Unfortunately, current treatments for malignant bone disease are limited and include palliative radiotherapy (Coleman, 1997), whereby spinal cord compression requires immediate intervention to avoid neurologic sequelae (Osborn *et al.*, 1995). New therapeutics have included bisphosphonates and denosumab, agents that can slow down the rate of bone resorption by acting on OC (Saad *et al.*, 2004). Denosumab, a fully human monoclonal anti-RANK antibody, is able to significantly delay the time to first skeletal-related event in patients with breast or prostate cancer metastases (Iranikhah *et al.*, 2012); however, evidence is still insufficient to prove its efficacy over zoledronic acid (ZA), as well as cost considerations. Therefore, ZA remains the agent most commonly used in the clinic for skeletal complications of solid tumors. Despite promising clinical results, disadvantages to using ZA or denosumab include the increased risk of osteonecrosis of the jaw (Wong

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et al., 2012), especially when used in combination with docetaxel (Miyazaki *et al.*, 2012), and a potential for ZA to enhance tumor cell invasion since it elevates $\beta 3$ integrin in PC3 cells (Sameni *et al.*, 2008; Mani *et al.*, 2012). Also, ZA alone, the standard of care for preventing skeletal-related effects in men with bone metastatic prostate cancer, has shown no significant improvement in survival in clinical trials (Saad, 2002).

Therefore, since no available therapies have shown to either improve survival or significantly delay tumor progression in advanced prostate cancer patients, an ideal agent likely would have to display the dual function of restoring bone health while impairing interactions between the tumor and the associated bone lesions. An emerging area of research, osteoimmunology, aims to investigate new immune stimulatory strategies with the potential to combat cancer while also stimulating regeneration of bone. We propose the use of a novel osteoimmune cytokine, interleukin-27 (IL-27), for achieving this therapeutic purpose. IL-27 halts tumor growth and mediates tumor regression preclinically in studies that include prostate cancer (Zolochovska *et al.*, 2011), neuroblastoma (Salcedo *et al.*, 2004), colorectal cancer (Engel and Neurath, 2010; Zhu *et al.*, 2010), and breast cancer (Zhu *et al.*, 2010). IL-27 is a heterodimeric cytokine composed of subunit IL-27p28, related to IL-12 p35, and subunit EB13 (Epstein-Barr virus-induced gene 3), related to IL-12 p40. IL-27 is immunomodulatory, is predominantly produced by antigen-presenting cells in response to microbial or host immune stimuli, and is involved in the regulation of immune responses against tumor development. IL-27 synergizes with IL-12 in interferon gamma ($\text{IFN}\gamma$) production from naïve T and NK cells. The receptor for IL-27 is composed of WSX1 and gp130 subunits, and is highly expressed in lymphoid organs, particularly in naïve CD4⁺ T and NK cells. IL-27 signaling induces T-bet, $\text{IFN}\gamma$, and IL12-R $\beta 2$ expression, promoting initiation of Th1 differentiation (Lucas *et al.*, 2003; Takeda *et al.*, 2003). Moreover, expression of IL-27 in tumor cells inhibits tumor development and induces T cell-dependent antitumor immune memory (Hisada *et al.*, 2004; Salcedo *et al.*, 2004), with either systemic (Zhu *et al.*, 2010) or intratumoral (I.Tu.) (Zolochovska *et al.*, 2011) IL-27 treatments eliminating tumors without any observed toxicity. IL-27 also shows antitumor activity through indirect mechanisms such as induction of natural killer and CTL response or inhibition of angiogenesis through induction of CXCL10 and CXCL9 (Feng *et al.*, 2007; Zhu *et al.*, 2010).

We have reported recently a direct role for IL-27 on reducing the communication between prostate tumor cells and bone cells (Zolochovska *et al.*, 2013). The IL-27 actions involved direct effects on STAT1 and $\text{IFN}\gamma$ signaling in tumor, T cells, osteoblasts (OB), and OC (OC). That report was the first evidence that IL-27 could directly mediate bone cell signaling that enhanced proliferation and differentiation of mature OB and prevented OC formation in coculture with tumor cells. Other direct effects of IL-27 reported on bone cells have included blocking human bone marrow OC formation, through induction of c-Fos and NFATc1, indispensable transcription factors for osteoclastogenesis (Furukawa *et al.*, 2009), as well as IL-27 downregulation of RANK and TREM-2 and blockage of RANKL-mediated ERK, p38, and NF- κ B activation in OC precursors (Kallioli *et al.*, 2010). The role of IL-27 in mediating bone cell phenotypic changes also has been described as indirect, via inhibition of OC differentiation (Kamiya *et al.*, 2007),

partially through activated T cell effects on survival and OC cell fusion. In summary, IL-27 inhibits osteoclastogenesis by suppressing multiple pathways that impact the response of OC precursors to RANKL, suggesting that this cytokine plays a strong homeostatic role in restraining bone erosion.

Other supporting studies implicated a dual role for IL-27 as an antitumor agent in multiple myeloma, where it reduced malignant plasma cell proliferation, with beneficial effects on bone cells, including inhibition of osteoclastogenesis and amplification of mature functional OB (Cocco *et al.*, 2010). Moreover, their results indicated that IL-27 could simultaneously treat myeloma and the surrounding bone, without any evidence of inflammation. For prostate cancer treatment, the present work is the first to report the effect of IL-27 on tumor and bone *in vivo*. We propose that IL-27 represents a novel, promising therapeutic agent for prostate tumors that have metastasized to the bone, based on its multifunctional activity. We show here a role for the cytokine IL-27 in preventing OC formation and in promoting OB differentiation and also that *in vivo* gene delivery of IL-27 can significantly reduce the rate of tumor growth as well as normalize bone density. In combination, we propose that IL-27 can be used to simultaneously treat prostate tumor metastases and the surrounding bone lesions.

Materials and Methods

Cell culture and transfections

Mouse TRAMP-C2 cells were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium-nutrient mixture F-12 (DMEM:F12; Mediatech) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). TRAMP-C2 cells were transduced with a lentivirus expressing activated H-ras^{G12V} (Lv-Hras) (Zolochovska *et al.*, 2011) at a multiplicity of infection of 1 and a lentivirus expressing mouse androgen receptor at a multiplicity of infection of 1, resulting in TC2R. The RM1 murine prostate cancer cell line (McCabe *et al.*, 2008) has the ability to aggressively grow in the bone microenvironment upon intratibial implantation. Prostate cancer cells were cultured in DMEM:F12 (Cellgro) with 10% FBS (GeneMate) and 1% PS (Invitrogen), and regularly passaged by trypsinization (0.05% [v/v] trypsin, 0.53 mM EDTA; Cellgro). The conditioned culture medium from prostate cancer cells was obtained as follows: RM1 or TC2R cells were grown to 70% confluence and washed 3 $\times$ in phosphate-buffered saline, phosphate-buffered saline was replaced with 2% FBS DMEM:F12 medium (Cellgro), and then the conditioned culture medium was collected 48 hr later. MC-3T3-E1 clone 14 mouse pre-OB were obtained from ATCC and cultured in 10% heat inactivated FBS plus alpha-minimum essential medium (a-MEM; Invitrogen) with 1% PS. Heat inactivation of FBS (ATCC) was carried out at 55°C for 30 min followed by storage at 4°C before addition to the medium. Raw264.1 mouse cells were cultured in DMEM (Cellgro)/10% FBS (GeneMate) with 1% PS (Invitrogen) and gently scraped for passaging.

Vectors

The mouse androgen lentivirus was polymerase chain reaction (PCR)-cloned with specific primers using *NheI* and

BglII ends and cloned into a pccl-CMV-ires-EGFP vector. Lentiviruses were prepared as described (Nelson *et al.*, 2011). For therapeutic studies, plasmid DNA vectors were prepared using a pCpG_{free} backbone (Invivogen) and consisted of either control empty vector (multiple cloning site or MCS) or vector containing humanized renilla luciferase (Rluc). For therapeutic studies, IL-27 vector contained mouse EBI3/p28 gene (hyperIL-27 excised from pORF9-EBI3/p28; Invivogen) in the pCpG_{free} backbone. Vectors were prepared for all experiments using Endofree kits (Qiagen).

Cell growth and coculture assays

For bone cell proliferation in coculture with prostate cancer cells, assays were performed in 24-well plates using 10^4 – 10^5 prostate cancer cells plated in the top chamber in 0.5 ml DMEM/10% FBS (for coculture with OC) or α -MEM/10% FBS heat inactivated (for OB coculture). The bottom chamber received 0.5 ml of the same medium and 10^4 – 10^5 OC or OB cells, with cells fixed and stained using a crystal violet assay for cell proliferation with absorbance changes read at 550 nm (A_{550}) as described (Zolochovska and Figueiredo, 2011). For each cell line, the fold change over control sample was determined. For bone cell differentiation assays, 1.5×10^5 cells were seeded in 12-well format in triplicate and cultured with differentiation supplements as per manufacturer's instructions using supplements for differentiation. For inducing pre-OB (MC-3T3) differentiation, 50 μ g/ml ascorbic acid and 10 mM beta-glycerol phosphate were used when cells reached confluency in a plate using 0.2% heat-inactivated FBS (ATCC). Cells were assayed at either day 14 or day 21 postsupplement treatment. After culturing in differentiation supplements, cells were fixed in ice-cold 70% ethanol for 1 hr at room temperature and stained for Alizarin S Red stain (Millipore) using manufacturers' protocols. For inducing Raw264.1 cell fusion and differentiation into OC, we cultured cells in 100 ng/ml RANKL for 7 days and stained for detection of tartrate-resistant alkaline phosphatase (TRAP; Sigma-Aldrich). Images of differentiated cells were acquired for 7–10 independent microscopic fields under a 10 $\times$ lens using an epi-fluorescence microscope (Bioexpress), and the total area fraction covered by cells was determined using NIH ImageJ software.

Flow cytometry and ELISA procedures

Cells for flow cytometry detection were assayed at the University of Texas Medical Branch (UTMB) Flow Cytometry Core facility. Tumor-infiltrating lymphocytes were isolated as described by Zolochovska *et al.* (2011) following a single sonoporation treatment *in vivo* delivered either intratumorally or intramuscularly. TC2R tumors were collected 7 days after treatment, and 2 – 4×10^6 cells were stained, with 2.5 – 5×10^5 cells collected during FACS analysis. Cells were stained in cell staining buffer (BioLegend), and anti-mouse antibodies (0.25 μ g) were used to detect surface marker expression, including antimouse CD8a-APC, CD3-PE, CD19-APC, NK1.1-APC, TCRV β 7-PE, and antirat TCR γ / δ -PE (BioLegend). For T regulatory (Treg) cell detection, a kit was used with FoxP3-A467, CD25-PE, and CD4-FITC anti-mouse antibodies (BioLegend). The corresponding isotype antibodies were used as controls. Flow cytometry was performed on a Becton Dickinson FACSCanto using Cell Quest

Software (BD Biosciences), and data analysis was performed using FlowJo 9.1 (Tree Star Inc.).

In vivo studies

Animal care and procedures were performed in accordance with the UTMB institutional review board guidelines. Mouse prostate cancer cells (10^6 TC2R) were delivered intratibially in 10 μ l sterile 0.9% saline to 6–8-week-old C57/BL6 male mice (Taconic). The tumor growth was monitored over time using vernier calipers. For micro-computed tomography (μ CT), a Siemens Inveon multimodality scanner (Siemens Medical Solutions) was used with a time course of CT imaging of tibia at high resolution (FOV 37 $\times$ 56 mm) with routine acquisition parameters (80 kVp, 500 μ A, and voxel size of 56 $\times$ 56 $\times$ 56 μ m), exposure time 800 ms per projection, and 520 equidistant steps covering 360 degrees of rotation. Step-and-shoot mode of acquisition was used to provide better stability of images compare with continuous imaging. The scan time is about 20–30 min. All scans were carried out using isoflurane anesthesia and data analyzed using the Siemens Inveon Research Workplace software version 3.0 with 3D Visualization option. Isotropic resolution of the reconstructed images was 0.056 mm. Dark-field, light-field, and flat-field corrections were applied during calibration. Center offset was applied during positioning calibration. Intensity was in linear mode according to histogram calibration. Data were analyzed using the Siemens Inveon Research Workplace software with 3D Visualization option.

For gene delivery studies, we utilized NLS2, a polycyclooctene polymer with pendant tetralysine and NLS (nuclear localization signal) oligopeptide, synthesized as described in (Parelkar *et al.*, 2011). We prepared polymers in low retention Eppendorf tubes, dissolved in nuclease-free water, and sterilized by filtration. The stock solution of NLS2 was diluted to enable complexation with pLuc plasmid DNA at an N/P ratio = 6 (i.e., the ratio of protonatable nitrogens in the polymer, N , to DNA phosphates, P). DNA (12.5 μ g) was added to nuclease-free water 1:1 to the polymer solution and allowed to equilibrate for a minimum of 35 min under sterile conditions. After polyplex formation, 2.2 μ l sterile Micro-marker microbubbles (VisualSonics) were added per tube and injected intramuscularly to the hind legs of male mice. After applying ultrasound (US) gel, we irradiated the muscle to mediate sonoporation and thus gene delivery of pRluc plasmid using a Sonigene sonoprotector (VisualSonics) using settings of 1 MHz, 20% duty cycle, 3 W/cm², and 60 sec. *In vivo* imaging for renilla luciferase expression in muscle was performed on day 6 after sonoporation using previously published procedures by intravenous coelenterazine substrate administration and collection of images within 10 min (Zolochovska and Figueiredo, 2009; Zolochovska *et al.*, 2011).

Real-time quantitative reverse transcription-PCR (qPCR) analyses

Total RNA from 1 mg tissue was extracted using the Trizol method using polytron homogenization (Figueiredo *et al.*, 2006) followed by a SurePrep kit (Fisher Scientific); 2 μ g RNA was reverse transcribed using TaqMan (Applied Biosystems); 1 μ l template cDNA was used in a real-time qPCR reaction with 2 $\times$ Sybr green master mix (Applied Biosystems),

and 10 μ M each forward and reverse primers for experimental or β -actin control housekeeping gene. Reactions were run on an Eppendorf Realplex 2S, using 40 $\times$ 95°C/15 sec, 56°C/30 sec, and 72°C/30 sec, and analyzed using Realplex software. Primer sequences are available upon request. All samples were normalized to the internal β -actin control and expressed as change in C_t relative to β -actin.

Histology

Injected tibiae were fixed and decalcified overnight in decalcification (CalEX) solution (Fishersci), cut longitudinally, and incubated overnight again in Decalcifier I. Tissue was then embedded in paraffin and sectioned (5 μ m). Sections were stained with toluidine blue, and visualization and imaging were done using a photomicroscope at the Research Histopathology Core.

Statistical analysis

Assays were performed in triplicate, and values provided as mean $\pm$ SEM or 95% confidence interval. Comparisons were performed using an unpaired *t*-test, and $p < 0.05$ was considered to indicate a significant difference.

Results

IL-27 enhances proliferation of OB and reduces proliferation of OC

We examined the effect of autocrine or paracrine IL-27 expression on bone cells *in vitro* by using coculture models. Pre-OB (MC3T3E1 clone 14) or RM1 cells were transfected with control (empty vector) or IL-27-expressing plasmid. Then, cells were cocultured in the absence or presence of osteoblastic differentiation stimuli (ascorbic acid plus beta-glycerol phosphate supplements). The coculture of pre-OB or OB with RM1 resulted in no change in proliferation compared with control on day 14 (Fig. 1A and Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/hum). Expression of IL-27 in differentiating OB (OB-27) enhanced their proliferation, although this difference was not significant compared with pre-OB (pOB-27) ($p = 0.06$) (Fig. 1A). RM1 cells expressing IL-27 (RM1-27) significantly enhanced the proliferation of maturing OB but not pre-OB ($p < 0.02$). Interestingly, the expression of IL-27 in both prostate tumor (paracrine) and OB (autocrine; RM1-27+OB-27), appeared to promote proliferation of OB even further ($p < 0.04$) compared with control (pOB-27). Therefore, although providing paracrine IL-27 might be sufficient to promote mature OB proliferation, combined expression of IL-27 in both OB and RM1 target cells appears to induce an enhanced proliferation of mature OB, which would likely be helpful to restoring bone tissue.

In OC, the same coculture model was used but with a different differentiation supplement, RANKL, to induce OC formation from RAW264.1 monocytic lineage cells. In all groups, IL-27 exposure inhibited OC proliferation by day 7 relative to day 2 (Fig. 1B). Although there was a trend toward reduced proliferation, expression of IL-27 in OC (autocrine) did not significantly reduce OC proliferation compared with pre-OC control ($p = 0.2$). However, expression of IL-27 in a paracrine manner (in prostate cancer cells

FIG. 1. The effect of IL-27 on osteoblast (OB) and osteoclast (OC) proliferation. **(A)** OB proliferation was examined in coculture with prostate cancer cell line RM1. IL-27 was either absent or expressed in the cell groups indicated. The effect of IL-27 on OB proliferation was examined on day 14 post-treatment relative to day 2 control using a crystal violet assay. MC-3T3E1 clone 14 pre-OBs were used and treated with either presence (+) or absence (-) of differentiation supplements (50 μ g/ml ascorbic acid and 10 mM beta-glycerol phosphate). Significant difference in OB proliferation (* $p < 0.02$, # $p < 0.04$) compared with control OB (OB/RM1 transfected with empty plasmid) on day 14. **(B)** Effect of IL-27 was examined on OC proliferation in coculture with prostate cancer cell line RM1. IL-27 was either absent or expressed in the cell groups indicated. The effect of IL-27 in OC proliferation was examined on day 7 posttreatment relative to day 3 control OC using a crystal violet assay. The Raw264.1 mouse monocyte precursor cell line was used and treated with 100 ng/ml RANKL differentiation supplement. Significant difference in OC proliferation (* $p < 0.01$) compared with control OC (OC/RM1 transfected with empty plasmid) on day 7.

cocultured with OC) resulted in a significant reduction of OC proliferation ($p < 0.03$) (Fig. 1B). Expression of IL-27 in both OC and RM1 cells (OC-27+RM1-27) further reduced the OC proliferation compared with control ($p < 0.02$) and this difference was also significantly reduced compared with IL-27

expression in RM1 alone (RM1-27) ($p < 0.04$). Therefore, although providing paracrine IL-27 (via tumor cell transfection) might be sufficient to prevent OC proliferation, interestingly, combined expression in both OC and RM1 target cells appears to help achieve higher cytokine efficacy against OC proliferation. This effect would likely support efforts to halt bone erosion by using IL-27.

Impact of IL-27 on OB and OC differentiation

The normal differentiation from pre-OB to OB can be observed by utilizing stains such as alizarin S red for detecting OB differentiation and mineralization by day 21 of culture. In Fig. 2A, we show that coculture of RM1 with pre-OB does

not seem to stimulate pre-OB differentiation (panel 1). When differentiation supplements are added (+AAB, ascorbic acid, and β -glycerol phosphate), some mineralization can be detected (panel 2). When pre-OB are transfected with IL-27 (autocrine) (Fig. 2A, panel 3), some differentiation can be seen compared with control transfected with empty vector (RM1/pOB). When differentiation supplements are added, the effect of IL-27 is further enhanced, as evidenced by darker extent of mineralization staining (Fig. 2A, panel 4). When IL-27 is provided in a paracrine manner (RM1-27/OB), differentiation of pOB is enhanced, but only in the presence of supplements (Fig. 2A, panel 6) compared with without (panel 5). Finally, when IL-27 is provided in both RM1 and OB cells (Fig. 2A, panels 7–8), the differentiation effect is

FIG. 2. Effect of the conditioned medium from prostate cancer cells on OB differentiation. **(A)** OB differentiation assessed by alizarin S red staining. Cells were seeded in a 24-well plate format and either kept undifferentiated (pre-OB) or differentiated with supplements (OB, ascorbic acid + β -glycerol phosphate as per Fig. 1) in coculture with RM1 prostate cancer cells. The effect of IL-27 was assessed by either direct expression in pre-OB or OB (autocrine) or from prostate cancer cell transfection (paracrine). **(B)** Plot showing the semiquantification of alizarin red stain from **(A)** using NIH ImageJ analyses. * $p < 0.03$ compared with pre-OB control images. **(C)** Effect of the conditioned medium from prostate cancer cells on OC differentiation. RANKL (100 ng/ml) stimulus promotes osteoclastogenesis of Raw264.1 (pre-OC) cells into OC, which present as cells bearing more than three nuclei (>3n), that is, multinucleated cells (MNC) staining positive for the enzyme tartrate-resistant alkaline phosphatase (TRAP). Prostate cancer cell (RM1) culture conditioned medium (CCM) also enhances differentiation of pre-OC into OC. Quantification of TRAP+ MNC forming in pre-OC cultured in the presence of 100 ng/ml RANKL for 7 days showing that RM1-CCM significantly enhances the MNC+ % ($p < 0.04$), while IL-27 expression in either prostate cancer cells or OCs via plasmid transfection reduces the MNC+ % (* $p < 0.02$). Color images available online at www.liebertpub.com/hum

magnified further compared with expression in either cell type alone. Analyses of these coculture image data yields a semiquantitative comparison plot that is shown in Fig. 2B, with significant differences at $p < 0.04$.

The extent of osteoclastogenesis also can be assessed by staining OC cultures for the expression of TRAP and scoring the extent of multinucleated cells (MNC) or mature OC per 20 $\times$ field. Raw264.1 cells pre-OC can differentiate and fuse into MNC in the presence of 100 ng/ml RANKL differentiation supplements (Hsu *et al.*, 1999; Zolochovska *et al.*, 2013). Prostate cancer cells also can induce osteoclastogenesis in coculture with pre-OC in the absence of RANKL (Fig. 2C). Interestingly, RANKL addition did not enhance the effect of coculturing with RM1 prostate cancer cells in inducing TRAP+ MNC cells ($p < 0.04$). We examined the effect of expressing IL-27 on MNC formation as a measure of the impact of the cytokine therapy on osteoclastogenesis. IL-27 or empty plasmid control was expressed in either prostate cancer cells or bone cells and induced to differentiate by RANKL addition. First, OC displayed an ~ 2.5 -fold increase in MNC formation during differentiation with either RANKL treatment or during coculture with tumor cells, indicating osteoclastogenesis compared with untreated control ($p < 0.03$). When IL-27 was expressed in OC (autocrine), this effect was suppressed and RANKL could not mediate osteoclastogenesis (Fig. 2C). When IL-27 was expressed in a paracrine manner (RM1-27), RANKL could not exert its osteoclastogenic effects either, suggesting that IL-27 expression can inhibit osteoclastogenesis in the presence of both strong prostate tumor cell and/or RANKL signals. There were no additive effects observed in the coculture when IL-27 was expressed in a combined paracrine and autocrine manner (RM1-27+OC-27), suggesting no added benefit to administering IL-27 to both cell compartments for halting osteoclastogenesis.

Development of an IL-27 gene delivery system in vivo

With the objective of developing a gene delivery system that would enable us to treat distant bone metastases of prostate cancer, we developed an intramuscular (I.M.) US-mediated gene transfer method utilizing plasmid DNA in complex with a novel polymer, oligolysine, and sonoporation in the presence of microbubbles (Fig. 3A). We first developed a protocol for Renilla Luc reporter plasmid DNA delivery to the hind leg muscle using sonoporation gene delivery (sonodelivery). For efficient sonodelivery, we used polyplexes of the novel comb-type oligolysine polymer, termed NLS2 complexed with plasmid DNA (Parelkar *et al.*, 2011). The polymer consists of a polyolefin backbone with multiple copies of pendent tetralysine and NLS (nuclear localization signal) groups strung along the backbone. The NLS group consists of the PKKKRKV amino acid sequence, and the molar ratio of the two pendent groups (tetralysine and NLS) was approximately 1:1. This enhanced sonodelivery method resulted in ~ 4 -fold higher levels of reporter gene expression in muscle as compared with controls (Fig. 3B). The most effective gene delivery formulations with transfection exceeding that of naked DNA control contained the NLS2 polymer complexed either with sonovue microbubbles (SV+NLS2) or with micro-marker microbubbles (MM+NLS2) (Fig. 3B, $p < 0.03$). Re-

presentative images of naked DNA or MM+NLS2 are shown in the presence (+US) or absence (–US) of US stimulus (Fig. 3C).

Next, we compared the efficiency of I.Tu. and I.M. sonodelivery and observed that the I.M. route was $\sim 2\times$ as efficient *in vivo* in reducing tumor volume using a subcutaneous prostate tumor model as proof of concept (Fig. 3D). Because of the higher efficacy of the gene delivery observed, we selected the I.M. route to further examine efficacy of IL-27 gene delivery in treating distant bone metastases of prostate cancer. For the following studies, we developed a new cell line that could better mimic the interaction of bone cells with prostate tumors. The cell line we have developed (TC2R) can form bone lesions representative of the human disease, including osteoblastic and osteolytic reactions *in vivo* after intratibial implantation into syngeneic C57/BL6 mice. TC2R allows us to explore the full effects of immune therapies for skeletal metastasis of prostate cancer and might be better suited for testing immunotherapy efficacy compared with other available immunocompetent models (RM1, RM1.BM), which are predominantly osteolytic (McCabe *et al.*, 2008; Hung *et al.*, 2011). Also, TC2R may be useful for testing prostate cancer therapies targeting the androgen receptor via drugs or transcriptional-targeted gene therapy (Figueiredo *et al.*, 2007).

Using I.M. sonodelivery, we proceeded to examine whether IL-27 could affect the interaction of tumor and bone compared with delivery of control Rluc vector to mice bearing intratibial 1.5-week TC2R tumors. IL-27 treatment reduced tumor weight by $\sim 40\%$ and reduced tumor volume by $\sim 75\%$ (Fig. 4A). We monitored the effect of IL-27 treatment on bone structure and density using two-dimensional μ CT (Fig. 4B). IL-27-treated mice had significantly less bone degradation over time compared with control mice treated with Rluc plasmid. Also, quantification of the bone density over time showed that IL-27 prevented bone loss ($p < 0.02$, Fig. 4C). The bone density loss in controls was sharp, going from ~ 1400 to ~ 100 HU within 4 weeks. In contrast, the IL-27 treatment prevented this density decline, with an ~ 4 -fold slower rate of density loss. Three-dimensional μ CT analyses showed that the extent of bone degradation as well as uneven ossification patterns (rounded protrusions) was very high in control animals bearing TC2R tumors by week 3 compared with control baseline (Fig. 4B). In contrast, IL-27-treated animals displayed a significant improvement in bone surface appearance (Fig. 4B), which was more uniform compared with controls, and bone density (Fig. 4C). μ CT thus allowed us to qualitatively and quantitatively examine bone changes in response to the IL-27 sonodelivery strategy *in vivo*. Histological examination showed that IL-27 therapy improved osteoblastic immature bone lesions compared with Rluc control (Fig. 4D), with a significant normalization of bone toward control levels (Fig. 4E).

IL-27 sonodelivery enhances the accumulation of effector cells in prostate tumors

We had compared two routes of sonodelivery (Fig. 3D) and had shown that the I.M. IL-27 delivery was more effective than the I.Tu. route in inhibiting tumor growth. To continue to characterize and determine potential

FIG. 3. Route of administration of IL-27 gene delivery impacts efficacy *in vivo*. **(A)** Schematic depicting the mechanism of ultrasound-mediated sonoporation as a method of gene delivery (adapted from and reprinted with permission from Frontiers in Bioscience). **(B)** Intramuscular gene delivery of a reporter plasmid DNA encoding renilla luciferase (RLuc) is shown. The protocol of sonoporation or ultrasound-mediated cavitation for gene delivery was applied to muscles of the hind legs. Shown are naked DNA control, sonovue (SV) microbubbles, micromarker (MM) microbubbles, polyethyleneimine (PEI), or NLS2 polymer. Combinations of PEI+SV, SV+NLS2, or MM+NLS2 were also examined, with the latter two being most effective ($p < 0.03$) compared with naked DNA control. Light (yellow) bars represent mice that did not receive any ultrasound (no US), and dark (red) bars represent mice that received ultrasound stimulus to enhance transfection of muscle (+US). **(C)** Representative bioluminescence images of FVB mice transduced with reporter genes (naked compared with MM+NLS2 groups) obtained from 5 min acquisition using a Xenogen IVIS100 CCD apparatus. Color bar, light units represented in photons/sec/cm²/steradian (p/sec/cm²/sr). **(D)** Effect of different routes of administration for sonodelivery of plasmid expressing IL-27 *in vivo*. Tumor volume 20 days postdelivery of pCpG-free IL-27 or control empty vector (pCpG-free-MCS). Controls are saline or polymer:MM MB (ok.NLS) alone. Intramuscular (I.M.) sonodelivery is efficient in treating aggressive subcutaneous TC2R tumors (starting size 250 mm³) compared with controls ($*p < 0.04$) and surpasses efficacy of intratumoral (I.Tu.) sonodelivery ($*p < 0.02$). Color images available online at www.liebertpub.com/hum

FIG. 4. *In vivo* intramuscular IL-27 gene delivery reduces tumor size and bone resorption. Shown are three-dimensional micro-computed tomography (μ CT) images for the tibial bones of C57/BL6 mice implanted with TC2^{Ras} cells (10^6). (A) IL-27 sonodelivery reduces TC2^{Ras} tumor growth and reduces bone resorption. (B) INVEON-Siemens μ CT 3D reconstruction. (C) Bone density quantification (HU, huntington units) is shown compared with control vectors (pRLuc). (D) IL-27 sonodelivery reduces immature osteoblastic bone lesions compared with pRLuc control. Staining shows toluidine blue, 20 $\times$ magnification. (E) Quantification of osteoblastic lesions (OBL) using NIH ImageJ (* $p < 0.02$). Color images available online at www.liebertpub.com/hum

mechanisms for this observation, we examined levels of IL-27 and its target genes in the target tissue (tumors) or the site of sonodelivery (muscle). In tumors, I.M. IL-27 sonodelivery induced 2–4-fold higher levels of IL-27 subunits (p28 and EBI3), and the cytokine's target genes T-bet and IFN γ , as compared with I.Tu. as assessed by qPCR (Fig. 5A). At the site of sonodelivery (muscle), I.M. IL-27 sonodelivery also induced IL-27p28, EBI3, and T-bet (although at lower levels than in tumor), but interestingly, IFN γ levels were unchanged. We hypothesized that these differences might indicate a mechanism by which I.M. IL-27 promotes infiltration of immune effector cells into tumors. These effectors would act to further augment IL-27 signaling through a feedforward loop of transcriptional activation of p28 and EBI3 via IFN γ upregulation of IRF1/4, as we have reported (Zolochovska *et al.*, 2011). When we compared the levels of IL-27 in serum between groups treated with IL-27 I.M. and I.Tu.,

we observed that IL-27 levels were higher following the I.M. sonodelivery route (Fig. 5C). FACS data utilizing immune cells isolated from TC2R tumors suggest that efficacy of IL-27 sonodelivery might be related to an enhanced accumulation of NKT and/or $\gamma\delta$ T cells, and this is observed with either sonodelivery route used (Fig. 5D). Interestingly, $\gamma\delta$ T cells were significantly upregulated (~ 3 -fold) in tumors treated with I.M. IL-27 sonodelivery (Fig. 5D) as compared with the I.Tu. route. These results suggest a role for $\gamma\delta$ T effectors in mediating the effects of I.M. IL-27 sonodelivery *in vivo*.

Discussion

The application of a single "osteimmune" cytokine that could simultaneously treat metastatic lesions while restoring bone homeostasis makes IL-27 a very attractive therapeutic

FIG. 5. Modulation of immune effector cell accumulation in TC2Ras subcutaneous prostate tumors by IL-27 intramuscular sonoporation gene delivery *in vivo*. **(A)** Tumor IL-27 levels (IL-27p28 and EBI3), IFN γ , T-bet after sonodelivery by real-time quantitative polymerase chain reaction (qPCR) (* $p < 0.03$ from I.M. as compared with I.Tu.). **(B)** Muscle levels after I.M. sonodelivery (qPCR). IFN γ levels unchanged ($p = 0.2$) (* $p < 0.05$ compared with pCpGfreeMCS control). Sonodelivery used 12.5 μ g pDNA and either I.Tu. (1 MHz, 60 sec, 50% duty cycle) or I.M. (2 MHz, 30 sec, 20% duty cycle). **(C)** Levels of IL-27 in serum as detected by ELISA 8 days post-pCpGfree-IL-27 gene delivery by sonoporation (I.Tu. or I.M. route) relative to levels of control vector (pCpGfreeMCS) (* $p < 0.05$). **(D)** Increased accumulation of NKT and $\gamma\delta$ T cells was observed infiltrating TC2R tumors treated using both routes of pCpGfree-IL-27 gene delivery (I.Tu. and I.M.) (* $p < 0.01$ compared with MCS control). Presented is the percent change observed for each treatment group as compared with the respective control (pCpGfreeMCS) group. Intramuscular pIL-27 delivery modulated a higher percentage of $\gamma\delta$ T cells than the I.Tu. route (# $p < 0.03$). Color images available online at www.liebertpub.com/hum

candidate for prostate cancer. We have previously shown that IL-27 has antitumor effects on prostate cancer *in vivo* (Zolochovska *et al.*, 2011), an effect supported by other tumor models (Yoshimoto *et al.*, 2008; Cocco *et al.*, 2010; Zhu *et al.*, 2010). In this context, the present work extends the use of IL-27 in reducing osteoclastogenesis and in enhancing OB differentiation when present in coculture with prostate tumor cells. This type of experimental design was chosen as a suitable model for mimicking *in vivo* applications, where the tumor interacts actively with the bone microenvironment. For preclinical testing, the tumor is typically the primary target of gene delivery/therapy, and the cytokine expressed is provided to the bone microenvironment in a paracrine manner. We thus examined the effect of paracrine IL-27 on bone cells of different differentiation stages. This design is relevant to assessing the different effects of IL-27 on immature (Roudier *et al.*, 2004, 2008) or mature bone cells. We also wanted to assess whether autocrine IL-27 (expressed directly in bone cells) could modify proliferation or differentiation of OB. Paracrine IL-27 enhanced the proliferation of maturing OB, augmented OB differentiation, and inhibited OC proliferation. Autocrine IL-27 expression further promoted the proliferation of mature OB. For OC, paracrine IL-27 reduced OC proliferation. Autocrine IL-27 further reduced the OC proliferation, suggesting that combined IL-27 delivery to both OC and prostate cancer cells appears to help achieve higher cytokine efficacy against OC proliferation. Regarding effects on bone cell differentiation, IL-27 also was able to

promote pre-OB differentiation even in the absence of differentiation supplements, and augmented differentiating OB mineralization, suggesting that IL-27 can directly induce and augment osteogenesis. Both autocrine and paracrine IL-27 were able to inhibit osteoclastogenesis by the potent inducing agent RANKL, suggesting promising translational applications of this molecule.

Regarding IL-27 therapy delivery *in vivo*, we selected a method that utilizes clinically safe US frequencies to induce cellular cavitation and deliver plasmid DNA via sonoporation for gene delivery (sonodelivery) (Zolochovska and Figueiredo, 2012). We have observed that this is an efficient method for gene delivery that approximates the efficiency of adenovirus (Zolochovska *et al.*, 2011). In the present report, we devised a strategy for I.M. sonodelivery since we needed to treat the bone tumor lesions and these are difficult to reach via I.Tu. injections. The muscle is an excellent site of gene administration because of long-term gene expression and was shown to successfully deliver IL-27 plasmid *in vivo* by electroporation in colon and breast tumors (Zhu *et al.*, 2010). In the current report, we optimized several I.M. sonodelivery conditions using reporter gene plasmids, finding that the best approach consisted of complexing pDNA with a novel cationic polymer, NLS2, in the presence of sonoporation assisted by microbubbles (Zolochovska *et al.*, 2011). This novel polymer leads to a transfection efficiency higher or comparable to JetPEI and in some cases, Lipofectamine 2000 (Parlekar *et al.*, 2011). Ours is the first use of the novel DNA:NLS2

polyplex *in vivo*. We observed efficient gene delivery in muscle and a reduced rate of bone loss in mice bearing TC2R intratibial tumors. These results are promising, since IL-27 was able to slow the rate of bone destruction and inhibit tumor growth within bone. However, one limitation lies in the partial efficacy, whereby some tumors still grew, although at a ~75% slower rate. The IL-27 I.M. delivery appears to reduce tumor-induced immature osteoblastic lesions, suggesting that IL-27 normalizes bone tissue and restores its homeostasis. Our data indicate that both I.Tu. and I.M. IL-27 sonodelivery mediate significant recruitment of infiltrating $\gamma\delta$ T and NKT cells to subcutaneous TC2R tumors. Interestingly, I.M. IL-27 sonodelivery recruited ~3-fold as many $\gamma\delta$ T cells to the subcutaneous tumor, suggesting that this could be one mechanism for the different efficacy observed. This difference may be related to the higher expression levels of IL-27 found in tumors of animals treated with I.M. sonoporation, although the mechanism for this elevated expression pattern remains uncharacterized at this time. Regarding effector cells recruited to tumors, we have previously reported the antitumor effects of IL-27 on prostate cancer through enhancement of CD8+ T cells, reduction in Treg, and myeloid-derived suppressor cells in tumors *in vivo* after gene delivery (Zolochovska *et al.*, 2011). rIL-27 has been recently shown to enhance $\gamma\delta$ T cytotoxicity (Morandi *et al.*, 2012). $\gamma\delta$ T cells recognize and kill tumor cells in a tumor antigen-independent manner, therefore, these are an interesting and relatively novel effector cell type that may provide protective immunosurveillance against metastatic tumors (Dieli *et al.*, 2007, 2008). Future studies will include examination of the potential infiltration of other organs by $\gamma\delta$ T effector cells, although we have not observed any significant lymphocytic infiltration in organs other than tumors, even when delivering IL-27 at a higher DNA dose (45 μ g) (Zolochovska *et al.*, 2011). However, such studies will be critical to determining toxicity potential for this therapeutic modality.

We also characterized the potential of IL-27 therapy and its impact on tumor growth as well as bone density. We chose μ CT for examining structural bone changes since this technique has several advantages, including high spatial resolution and contrast for imaging mineralized tissues in small animals (Freeman *et al.*, 2009; Morgan *et al.*, 2009; Campbell *et al.*, 2011; Johnson *et al.*, 2011). μ CT allows longitudinal monitoring of tumor-associated bone destruction in mouse models and also detection of newly ossified bone in prostate cancer models. However, although promising, the IL-27 therapeutic response was partial; therefore, we propose that strategies still must be devised to enhance IL-27 expression and secretion after I.M. sonodelivery, as well as strategies to enhance retention of IL-27 at bone metastases in order to achieve an efficient and complete therapeutic effect. We have observed that a variety of normal cell types appear to tolerate treatment with IL-27 at relatively high doses *in vitro* without significantly losing viability (Supplementary Fig. S1), suggesting that the dose delivered can be increased further, and future experiments *in vivo* will assess the exposure limit of persistent IL-27 after sonodelivery to help characterize any potential toxicity. Other strategies by which the therapy efficacy can be augmented are by utilizing cytokine combinations proven successful for other preclinical studies, such as IL-27 + IL-12 (Zhu *et al.*, 2010) or IL-27 + IL-2

(Salcedo *et al.*, 2009), and ligand-mediated targeting strategies in order to enhance cytokine efficacy via increased retention within tumors [reviewed by Corti *et al.* (2011)].

Conclusions

We describe a novel gene delivery strategy that is simple to perform, is cost-effective, and is clinically applicable, since US-mediated gene delivery or sonodelivery can be accomplished using any conventional clinical US apparatus. Our findings are promising for I.M. delivery of a novel osteoimmune cytokine, IL-27, which shows great promise for simultaneously treating tumor and bone. Our results are promising since there are no current therapies that can successfully achieve these outcomes for treating patients with bone metastatic prostate cancer.

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Author Disclosure Statement

No competing interests exist for the authors.

References

- Berruti, A., Dogliotti, L., Bitossi, R., *et al.* (2000). Incidence of skeletal complications in patients with bone metastatic prostate cancer and hormone refractory disease: predictive role of bone resorption and formation markers evaluated at baseline. *J. Urol.* 164, 1248–1253.
- Berruti, A., Dogliotti, L., Tucci, M., *et al.* (2001). Metabolic bone disease induced by prostate cancer: rationale for the use of bisphosphonates. *J. Urol.* 166, 2023–2031.
- Boyce, B.F., Yoneda, T., and Guise, T.A. (1999). Factors regulating the growth of metastatic cancer in bone. *Endocr. Relat. Cancer* 6, 333–347.
- Bubendorf, L., Schopfer, A., Wagner, U., *et al.* (2000). Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. *Hum. Pathol.* 31, 578–583.
- Campbell, G.M., Ominsky, M.S., and Boyd, S.K. (2011). Bone quality is partially recovered after the discontinuation of RANKL administration in rats by increased bone mass on existing trabeculae: an *in vivo* micro-CT study. *Osteoporos. Int.* 22, 931–942.
- Carlin, B.I., and Andriole, G.L. (2000). The natural history, skeletal complications, and management of bone metastases in patients with prostate carcinoma. *Cancer* 88, 2989–2994.
- Clark, P.E., and Torti, F.M. (2003). Prostate cancer and bone metastases: medical treatment. *Clin. Orthop. Relat. Res.* S148–S157.

- Cocco, C., Giuliani, N., Di Carlo, E., *et al.* (2010). Interleukin-27 acts as multifunctional antitumor agent in multiple myeloma. *Clin. Cancer Res.* 16, 4188–4197.
- Coleman, R.E. (1997). Skeletal complications of malignancy. *Cancer* 80, 1588–1594.
- Coleman, R.E. (2001). Metastatic bone disease: clinical features, pathophysiology and treatment strategies. *Cancer Treat. Rev.* 27, 165–176.
- Corti, A., Pastorino, F., Curnis, F., *et al.* (2011). Targeted drug delivery and penetration into solid tumors. *Med. Res. Rev.* 32, 1078–1091.
- Daniell, H.W. (1997). Osteoporosis after orchiectomy for prostate cancer. *J. Urol.* 157, 439–444.
- Dieli, F., Vermijlen, D., Fulfaro, F., *et al.* (2007). Targeting human $\{\gamma\}\delta$ T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Res.* 67, 7450–7457.
- Dieli, F., Caccamo, N., and Meraviglia, S. (2008). Advances in immunotherapy of castration-resistant prostate cancer: bisphosphonates, phosphoantigens and more. *Curr. Opin. Investig. Drugs* 9, 1089–1094.
- Engel, M.A., and Neurath, M.F. (2010). Anticancer properties of the IL-12 family—focus on colorectal cancer. *Curr. Med. Chem.* 17, 3303–3308.
- Feng, X.M., Chen, X.L., Liu, N., *et al.* (2007). Interleukin-27 up-regulates major histocompatibility complex class II expression in primary human endothelial cells through induction of major histocompatibility complex class II transactivator. *Hum. Immunol.* 68, 965–972.
- Figueiredo, M.L., Dayan, S., Kim, Y., *et al.* (2006). Expression of cell-cycle regulator CDK2-associating protein 1 (p12CDK2AP1) in transgenic mice induces testicular and ovarian atrophy *in vivo*. *Mol. Reprod. Dev.* 73, 987–997.
- Figueiredo, M.L., Kao, C., and WU, L. (2007). Advances in pre-clinical investigation of prostate cancer gene therapy. *Mol. Ther.* 15, 1053–1064.
- Freeman, T.A., Patel, P., Parvizi, J., *et al.* (2009). Micro-CT analysis with multiple thresholds allows detection of bone formation and resorption during ultrasound-treated fracture healing. *J. Orthop. Res.* 27, 673–679.
- Furukawa, M., Takaishi, H., Takito, J., *et al.* (2009). IL-27 abrogates receptor activator of NF- κ B ligand-mediated osteoclastogenesis of human granulocyte-macrophage colony-forming unit cells through STAT1-dependent inhibition of c-Fos. *J. Immunol.* 183, 2397–2406.
- Goltzman, D. (1997). Mechanisms of the development of osteoblastic metastases. *Cancer* 80, 1581–1587.
- Hatano, T., Oishi, Y., Furuta, A., *et al.* (2000). Incidence of bone fracture in patients receiving luteinizing hormone-releasing hormone agonists for prostate cancer. *BJU Int.* 86, 449–452.
- Hisada, M., Kamiya, S., Fujita, K., *et al.* (2004). Potent antitumor activity of interleukin-27. *Cancer Res.* 64, 1152–1156.
- Hsu, H., Lacey, D.L., Dunstan, C.R., *et al.* (1999). Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc. Natl. Acad. Sci. USA* 96, 3540–3545.
- Hung, T.T., Chan, J., Russell, P.J., and Power, C.A. (2011). Zoledronic acid preserves bone structure and increases survival but does not limit tumour incidence in a prostate cancer bone metastasis model. *PLoS One* 6, e19389.
- Iranikhan, M., Wilborn, T.W., Wensel, T.M., and Ferrell, J.B. (2012). Denosumab for the prevention of skeletal-related events in patients with bone metastasis from solid tumor. *Pharmacotherapy* 32, 274–284.
- Johnson, L.C., Johnson, R.W., Munoz, S.A., *et al.* (2011). Longitudinal live animal micro-CT allows for quantitative analysis of tumor-induced bone destruction. *Bone* 48, 141–151.
- Kalliolias, G.D., Zhao, B., Triantafylloulou, A., *et al.* (2010). Interleukin-27 inhibits human osteoclastogenesis by abrogating RANKL-mediated induction of nuclear factor of activated T cells c1 and suppressing proximal RANK signaling. *Arthritis Rheum.* 62, 402–413.
- Kamiya, S., Nakamura, C., Fukawa, T., *et al.* (2007). Effects of IL-23 and IL-27 on osteoblasts and osteoclasts: inhibitory effects on osteoclast differentiation. *J. Bone Miner. Metab.* 25, 277–285.
- Lucas, S., Ghilardi, N., Li, J., and De Sauvage, F.J. (2003). IL-27 regulates IL-12 responsiveness of naive CD4⁺ T cells through Stat1-dependent and -independent mechanisms. *Proc. Natl. Acad. Sci. USA* 100, 15047–15052.
- Mani, R.S., Iyer, M.K., Cao, Q., *et al.* (2012). TMPRSS2-ERG-mediated feed-forward regulation of wild-type ERG in human prostate cancers. *Cancer Res.* 71, 5387–5392.
- McCabe, N.P., Madajka, M., Vasanji, A., and Byzova, T.V. (2008). Intraosseous injection of RM1 murine prostate cancer cells promotes rapid osteolysis and periosteal bone deposition. *Clin. Exp. Metastasis* 25, 581–590.
- Miyazaki, H., Nishimatsu, H., Kume, H., *et al.* (2012). Leukopenia as a risk factor for osteonecrosis of the jaw in metastatic prostate cancer treated using zoledronic acid and docetaxel. *BJU Int.* 110, E520–E525.
- Morandi, F., Prigione, I., and Airolidi, I. (2012). Human TCR γ delta + T cells represent a novel target for IL-27 activity. *Eur. J. Immunol.* 42, 1547–1552.
- Morgan, E.F., Mason, Z.D., Chien, K.B., *et al.* (2009). Micro-computed tomography assessment of fracture healing: relationships among callus structure, composition, and mechanical function. *Bone* 44, 335–344.
- Mundy, G.R. (2002). Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat. Rev. Cancer* 2, 584–593.
- Mundy, G.R., and Guise, T.A. (1997). Hypercalcemia of malignancy. *Am. J. Med.* 103, 134–145.
- Nelson, P.L., Zolocheska, O., Figueiredo, M.L., *et al.* (2011). Regulation of Ca(2⁺)-entry in pancreatic alpha-cell line by transient receptor potential melastatin 4 plays a vital role in glucagon release. *Mol. Cell Endocrinol.* 335, 126–134.
- Osborn, J.L., Getzenberg, R.H., and Trump, D.L. (1995). Spinal cord compression in prostate cancer. *J. Neurooncol.* 23, 135–147.
- Parekar, S.S., Chan-Seng, D., and Enrick, T. (2011). Reconfiguring polylysine architectures for controlling polyplex binding and non-viral transfection. *Biomaterials* 32, 2432–2444.
- Roodman, G.D. (2004). Mechanisms of bone metastasis. *N. Engl. J. Med.* 350, 1655–1664.
- Roudier, M.P., Corey, E., True, L.D., *et al.* (2004). Histological, immunophenotypic and histomorphometric characterization of prostate cancer bone metastases. *Cancer Treat. Res.* 118, 311–339.
- Roudier, M.P., Morrissey, C., True, L.D., *et al.* (2008). Histopathological assessment of prostate cancer bone osteoblastic metastases. *J. Urol.* 180, 1154–1160.
- Saad, F. (2002). Zoledronic acid significantly reduces pathologic fractures in patients with advanced-stage prostate cancer metastatic to bone. *Clin. Prostate Cancer* 1, 145–152.
- Saad, F., Gleason, D.M., Murray, R., *et al.* (2004). Long-term efficacy of zoledronic acid for the prevention of skeletal complications in patients with metastatic hormone-refractory prostate cancer. *J. Natl. Cancer Inst.* 96, 879–882.
- Salcedo, R., Stauffer, J.K., Lincoln, E., *et al.* (2004). IL-27 mediates complete regression of orthotopic primary and metastatic

- murine neuroblastoma tumors: role for CD8+ T cells. *J. Immunol.* 173, 7170–7182.
- Salcedo, R., Hixon, J.A., Stauffer, J.K., *et al.* (2009). Immunologic and therapeutic synergy of IL-27 and IL-2: enhancement of T cell sensitization, tumor-specific CTL reactivity and complete regression of disseminated neuroblastoma metastases in the liver and bone marrow. *J. Immunol.* 182, 4328–4338.
- Sameni, M., Dosescu, J., Yamada, K.M., *et al.* (2008). Functional live-cell imaging demonstrates that beta1-integrin promotes type IV collagen degradation by breast and prostate cancer cells. *Mol. Imaging* 7, 199–213.
- Smith, M.R. (2007). Androgen deprivation therapy for prostate cancer: new concepts and concerns. *Curr. Opin. Endocrinol. Diabetes Obes.* 14, 247–254.
- Takeda, A., Hamano, S., Yamanaka, A., *et al.* (2003). Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. *J. Immunol.* 170, 4886–4890.
- Wong, M.H., Stockler, M.R., and Pavlakis, N. (2012). Bisphosphonates and other bone agents for breast cancer. *Cochrane Database Syst. Rev.* 2, CD003474.
- Yoshimoto, T., Morishima, N., Mizoguchi, I., *et al.* (2008). Antiproliferative activity of IL-27 on melanoma. *J. Immunol.* 180, 6527–6535.
- Zhu, S., Lee, D.A., and Li, S. (2010). IL-12 and IL-27 sequential gene therapy via intramuscular electroporation delivery for eliminating distal aggressive tumors. *J. Immunol.* 184, 2348–2354.
- Zolochevska, O., and Figueiredo, M.L. (2009). Expression of cell cycle regulator cdk2ap1 suppresses tumor cell phenotype by non-cell-autonomous mechanisms. *Oral Oncol.* 45, e106–e112.
- Zolochevska, O., and Figueiredo, M.L. (2011). Cell-cycle regulators cdk2ap1 and bicalutamide suppress malignant biological interactions between prostate cancer and bone cells. *Prostate* 71, 353–367.
- Zolochevska, O., and Figueiredo, M.L. (2012). Sonoporation-based strategies for cancer therapy. *Front. Biosci. (Schol. Ed.)* 4, 988–1006.
- Zolochevska, O., Xia, X., Williams, B.J., *et al.* (2011). Sonoporation delivery of interleukin-27 gene therapy efficiently reduces prostate tumor cell growth in vivo. *Hum. Gene Ther.* 22, 1537–1550.
- Zolochevska, O., Ellis, J., and Figueiredo, M.L. (2013). Interleukin-27 expression modifies prostate cancer cell crosstalk with bone and immune cells. *J. Cell Physiol.* 228, 1127–1136.

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