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Research Paper

β2ARs stimulation in osteoblasts promotes breast cancer cell adhesion to bone marrow endothelial cells in an IL-1β and selectin-dependent manner

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ABSTRACT

Progression and recurrence of breast cancer, as well as reduced survival of patients with breast cancer, are associated with chronic stress, a condition known to impact the hypothalamic-pituitary axis and the autonomic nervous system. Preclinical and clinical evidence support the involvement of the sympathetic nervous system in the control of bone remodeling and in pathologies of the skeleton, including bone metastasis. In experimental mouse models of skeletal metastasis, administration of the βAR agonist isoproterenol (ISO), used as a surrogate of norepinephrine, the main neurotransmitter of sympathetic neurons, was shown to favor bone colonization of metastatic breast cancer cells via an increase bone marrow vascularity. However, successful extravasation of cancer cells into a distant organ is known to be favored by an activated endothelium, itself stimulated by inflammatory signals. Based on the known association between high sympathetic outflow, the expression of inflammatory cytokines and bone metastasis, we thus asked whether βAR stimulation in osteoblasts may alter the vascular endothelium to favor cancer cell engraftment within the skeleton. To address this question, we used conditioned medium (CM) from PBS or ISO-treated bone marrow stromal cells (BMSCs) in adhesion assays with bone marrow endothelial cells (BMECs) or the endothelial cell line C166. We found that ISO treatment in differentiated BMSCs led to a robust induction of the pro-inflammatory cytokines interleukin-1 beta (IL-1β) and interleukin-6 (IL-6). The CM from ISO-treated BMSCs increased the expression of E- and P-selectin in BMECs and the adhesion of human MDA-MB-231 breast cancer cells to these cells in short-term static and dynamic adhesion assays, and a blocking antibody against IL-1β, but not IL-6, reduced this effect. Direct IL-1β treatment of BMECs had a similar effect, whereas the impact of IL-6 treatment on the expression of adhesion molecules by BMECs and on the adhesion of cancer cells to BMECs was negligible. Collectively, these *in vitro* results suggest that in the context of the multicellular and dynamic bone marrow environment, sympathetic activation and subsequent βAR stimulation in osteoblasts may profoundly remodel the density but also the activation status of bone marrow vessels to favor the skeletal engraftment of circulating breast cancer cells.

1. Introduction

Breast cancer, the most common cancer diagnosed among women, frequently metastasizes to bone, causing osteolytic lesions, pain, fractures and poor life quality [1–3]. Current treatment options for breast cancer-associated skeletal-related events mainly target the action of osteoclasts with bisphosphonates [4,5], but the 5-year survival rate associated with the use of this class of drugs is improved in postmenopausal women only [6]. In addition, the use of adjuvant denosumab in an intense dosing schedule did not improve bone metastasis-free survival in patients with early-stage breast cancer who were receiving optimal locoregional and standard-of-care systemic adjuvant therapy [7]. Hence, despite the overall success of treating primary breast cancer, new treatments options are needed to reduce recurrence and increase the survival of women with advanced breast cancer.

Metastatic cancer cells circulate throughout the body at very early

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stages of the disease and can lodge into distant organs in a dormant stage for years until unknown mechanisms trigger their re-entry into the cell cycle and growth. These early disease stages represent an opportunity for treatment [8], as disrupting the cross-talk between disseminated cancer cells and their microenvironment might prevent progression to macrometastases and recurrence. Cancer patients, including those with triple-negative breast cancer (TNBC), have increased survival metrics when their treatment regimen includes a β-blocker [9–12]. β-blockers are a widely used drug for the treatment of high blood pressure, arrhythmia and anxiety, and work by blocking the communication between sympathetic nerves and target cells. Activation of the hypothalamic-pituitary axis (HPA) and of the sympathetic nervous system (SNS) are hallmarks of prolonged stress, and recent studies have shown that chronic stress exacerbates cancer progression in animal models of prostate [13], ovarian [14,15] and breast cancers [16–18], as well as in some cancer patient and survivor cohort studies [19–24]. Patients diagnosed with cancer experience significant psychological stress at the time of diagnosis and all along treatment [25–28], and the threat of relapse is likely to contribute to long-term stress and decline in health-related quality of life [29,30]. Prolonged stress is known to have deleterious effects on the physiology of multiple tissues, via the release of norepinephrine (NE) from nerve terminals, which then binds post-synaptic adrenergic receptors (βAR) on target cells, including bone-forming cells [31]. During normal bone remodeling in mice, osteoblasts respond to the synthetic βAR agonist isoproterenol (ISO) by an increase in RANKL and VEGF production, leading to increased osteoclastogenesis and vascular bone density *in vivo*, respectively [18,32]. We have shown in the context of preclinical bone metastasis models that these SNS-induced changes in the bone marrow environment promote breast cancer bone colonization following intracardiac injection of MDA-MB-231 breast cancer cells, by favoring breast cancer cell migration (in a RANKL-dependent fashion) and by increasing the density of bone marrow blood vessel (in a VEGF-dependent fashion) [18,32]. Independent studies have shown that chronic SNS activation promotes breast primary tumor growth and metastasis via changes in stromal homeostasis as well, which involved an influx of pro-inflammatory macrophages and an increase in primary tumor vasculature density [16,33].

The vasculature is a crucial component for primary tumor growth, but also for the metastatic process in distant organs. Interactions with endothelial cells must occur in order for tumor cells to extravasate from the circulatory system and to establish at a secondary site [34,35]. The bone is a highly vascularized tissue, and osteoblasts are known regulators of angiogenesis during skeletal development and bone regeneration [36,37]. Our previous findings suggest SNS activation effectively increases bone marrow vessel density in adult mice [32]. The extravasation process of cancer cells is very similar to lymphocytes diapedesis after injury or infection [38,39]. During the resulting inflammation, endothelial cells are activated by inflammatory cytokines to express adhesion molecules and synthesize chemokines that are presented on their luminal surface [40]. Similar to the leukocyte extravasation process, tumor cells are recruited to secondary sites by chemotactic gradients of cytokines and growth factors [41]. Inflammatory mediators such as interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and lipopolysaccharide (LPS) for instance have been shown to favor cancer cell interactions with endothelial cells by altering the levels of adhesion proteins present on the endothelium [42]. The activated endothelium expresses inducible adhesion molecules such as E-selectin, P-selectin, ICAM1, VCAM1 and multiple β-integrins, all of which can facilitate arrest, docking, and extravasation of metastatic breast and prostate tumor cells into bone [43–45]. Selectins are members of the carbohydrate-binding proteins family. These molecules are involved in adhesive interactions between endothelium and leukocytes or platelets within the blood circulatory system. There are three members of the selectin family: P-, E-, and L-selectin. The expression of these adhesion molecules on the endothelium is temporally coordinated to ensure efficient inflammatory response [46]: whereas L-selectin mediates fast rolling of leukocytes on the endothelium, P- and E-selectins support rolling at lower velocities [40]. Selectins binds to various classes of molecules (mucins, sulfated glycolipids, glycosaminoglycans) and most of these molecules were shown to be functional selectin ligands *in vivo* [47]. Prostate [48], colorectal [49], pancreatic [50] and breast cancer cells [51] expressing high levels of ligands for selectins have been shown to exhibit more aggressive oncogenic properties *in vitro, in vivo,* and in patient samples.

In this study, we investigated the putative impact of sympathetic nerve activation on the adhesive properties of the activated bone endothelium for metastatic breast cancer cells, via *in vitro* assays designed to probe the communication and interaction between osteoblasts, endothelial cells and breast cancer cells.

2. Materials and methods

2.1. Cell lines

Human GFP^+ MDA-MB-231 and murine GFP^+ 4T1 mammary tumor cells were cultured with 10% FBS DMEM High Glucose (ThermoFisher, #1965118), BMSCs with 10% FBS α-MEM (Fisher scientific, #SH3026501), mouse C166 endothelial cells and BMECs with complete ECM (ScienCell, #1001) at 37 °C and 8% CO_2.

2.2. Primary mouse bone marrow stromal cells

Hindlimbs from WT C57BL/6 mice were used to prepare primary mouse bone marrow stromal cells (BMSCs). Femur and tibia were stripped of skin and muscles, distal and proximal epiphyses were cut off, and each bone was inserted into a punctured 0.5 mL tube placed into a 1.5 mL tube. Tubes were centrifuged for 4 min at 4000 g. Resulting pellets were resuspended in complete α-MEM (Fisher Scientific, #SH3026501), and cells were plated at 1×10^6 cells/mL. Cultures were grown in 10% FBS α-MEM for 7 days and then switched to an osteogenic medium (10% α-MEM containing 50 μg/mL ascorbic acid [Sigma, #A5950] and 10 mM β-glycerophosphate [Sigma, #G9891-25 G]) for 7 more days.

2.3. Primary mouse bone marrow endothelial cells

Primary mouse bone marrow endothelial cells (BMECs) were harvested as described for BMSCs. Flushed cells were resuspended in complete ECM (ScienCell, #1001). Tissue culture dishes were coated for 20 min at 37 °C with 0.8 μg/cm^2 fibronectin (Gibco, #33016015) then cells were plated at 3×10^6 cells/mL. Cultures were then grown in complete ECM for 7 days.

2.4. Gene expression assay

For all gene expression assays, total RNA was extracted from cells using TRIzol (Invitrogen, #15596-026). Following DNAse I treatment (ThermoFisher, #18068015), cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368813). Real-time PCR was performed using SYBR Green Supermix (Biorad, #1708884) gene expression assays on a Biorad CFX96 Real-Time System with appropriated primers (see Supplementary Table 1). Amplification specificity was verified by the presence of a single peak on the melting curve of the amplicon. Gene expression was analyzed by the ΔΔCt method.

2.5. Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, then blocked in 1% bovine serum albumin for 1 h at room temperature. Immunodetection of CD62E, CD31, and endomucin was

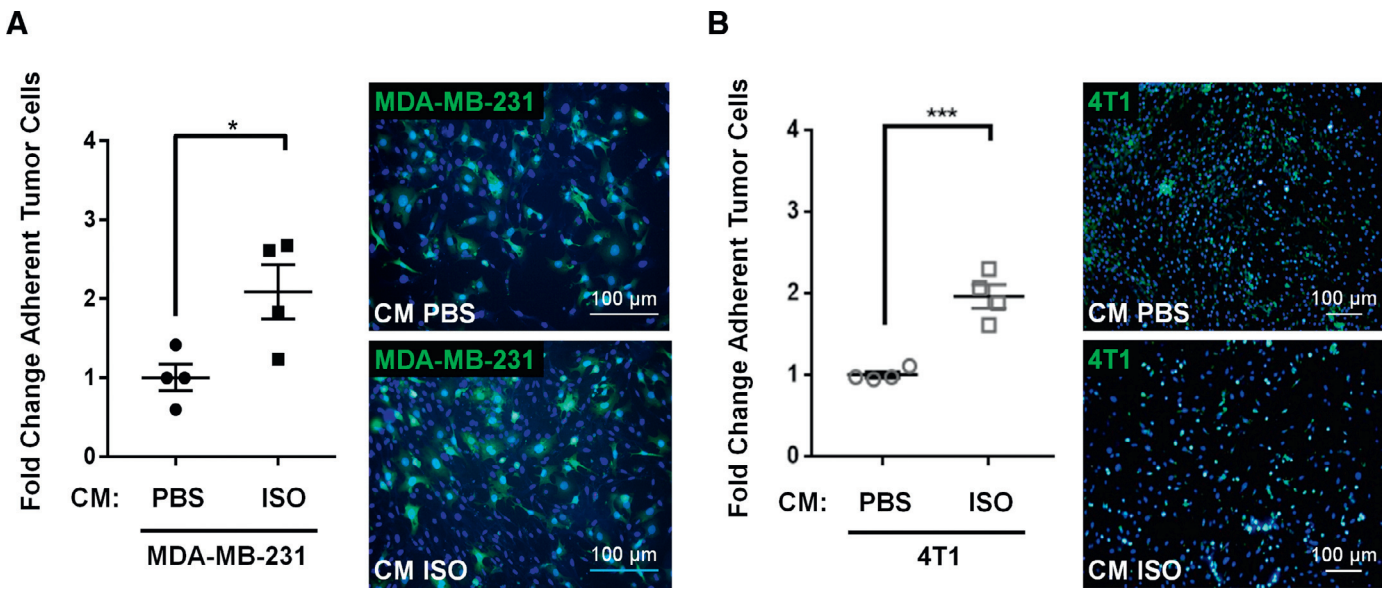

Fig. 1. Confluent and adherent primary mouse bone marrow endothelial cells (BMECs) were treated for 24 h with the conditioned medium (CM) from vehicle or ISO-treated BMSCs prior to addition of GFP-positive (+) MDA-MB-231 (**A**) or GFP + 4T1 (**B**) cells. After 3.5 h, wells were washed in PBS and the relative adhesion of cancer cells to the endothelial monolayer was quantified by fluorescence-labeled cell count assay. Results are graphed as fold change relative to the CM of PBS-treated BMECs (n = 4 independent experiments, * = $p < 0.05$, *** = $p < 0.001$). A and B, right panel: Representative images of tumor cells attached to BMEC monolayers after treatment (blue: DAPI nuclear staining, green: GFP + tumor cells). Scale bar = 100 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

performed using rat anti-CD62E (1:50, BD Pharmingen, #553749), mouse anti-CD31 (1:100, Abcam, ab24590), or rat anti-endomucin (1:100, Santa Cruz, sc65495) antibody at a 1:100 dilution overnight at 4 °C, and donkey anti-rat Alexa594 (Invitrogen, #A21209) or goat anti-mouse Alexa488 (Abcam, ab150113) secondary antibody at a 1:1,000 dilution for 1 h at room temperature. Nucleus staining was performed using 2 µg/mL Hoescht solution (Invitrogen, #H3569), and immunofluorescence was preserved in FluorSave (Millipore, #345789). Images were acquired with a Leica fluorescent microscope (Leica, Germany).

2.6. Adhesion assays

Endothelial cells (C166 or BMEC) were suspended in serum-free medium at 1×10^6 cells/mL and then incubated with 5 µL of Vybrant DiI per mL of cell suspension (Invitrogen, #V22889) for 20 min at 37 °C. The cells were washed of excess dye twice prior to plating.

2.6.1. Coverslip assay

Endothelial cells were grown to confluence in a 24-well plate on a fibronection-coated glass coverslip (VWR, #89167-106). Complete medium was then removed and replaced with growth factor-free medium in control wells or growth factor-free medium supplemented with rIL-1β (10 ng/mL) or BMSC-derived CM for 4 h. GFP^+ tumor cells were resuspended in 10% DMEM High Glucose (Thermo Fisher, #1965118). 3×10^4 cancer cells/well were incubated for 3.5 h at 37 °C on the different endothelial cell monolayers. Non-adherent cells were washed with PBS (VWR, #45000-434), then cells fixed with 4% PFA and counted.

2.6.2. Gravity assay

Endothelial cells were grown to confluence on a 96-well clear bottom black plate. After 4 h of cytokine treatment, tumor cells (3×10^5 cells/mL) were incubated for 1 h on the endothelial cell monolayer, then the plate was turned upside-down. After 1.5 h, adherent tumor cells were fixed with 4% PFA and counted.

2.6.3. Flow chamber assay

Endothelial cells were grown to confluence in endothelial cell medium in a fibronectin-coated flow chamber (Ibidi, µ-Slide I Luer #80176). After incubation with rIL-1β or BMSC-derived CM for 6 h, cancer cells (5×10^5 cells/mL) were infused into a flow chamber at 1 dyn/cm^2 shear stress for 5 min. The unbound cells were washed off for 5 min with DMEM then PBS, and fixed with 4% PFA prior to counting.

For all adhesion assays, pictures were taken using a fluorescence microscope (Leica, Germany) and analyzed by ImageJ by counting GFP + adherent tumor cells on 10 random fields per slide in a blinded fashion by two investigators. The Otsu's method, an automatic clustering-based image thresholding, was used as previously described [52], with slight modifications. Briefly, the GFP filter image was set in greyscale (8-bit), Otsu Threshold was used to highlight all of the individual tumor cells, then the Analyze Particles option was run (between 0.001 - 0.05 inches to ignore regions that were particularly small or large) to detect and measure all the foreground regions as individual objects. Particular care was taken to insure the endothelial cell layers were confluent in all wells/slides, and cell cultures were authenticated by the expression of marker genes (Supplementary Fig. 1). Results are expressed as fold change compared to either PBS or PBS-CM level set up at 1.

2.7. Statistics

All data are presented as means ± the standard error mean (SEM). For experiments comparing two groups a non-parametric Mann-Whitney was used and for experiments comparing more than two groups a Kruskal-Wallis test was used. For all analyses, a p-value ≤ 0.05 was considered significant.

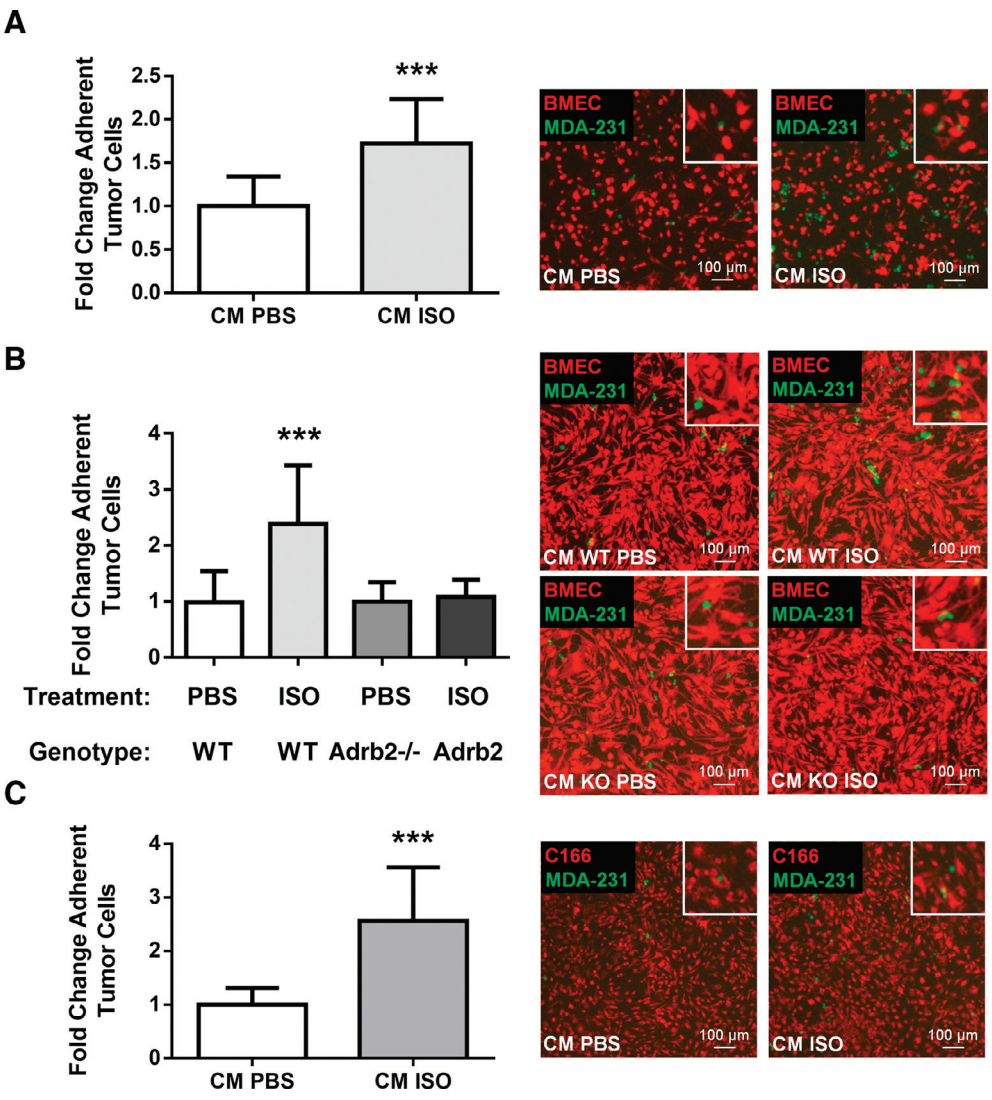

Fig. 2. (**A**) DiI-labeled BMECs (red) were grown on flow chamber pre-coated with fibronectin (0.8 µg/cm^2) until 100% confluent and were then stimulated with the CM from PBS or ISO-treated bone marrow stromal cells (BMSCs) for 6 h prior to perfusion of GFP + MDA-MB-231 cancer cells at 1 dyn/cm^2 shear stress for 5 min. The number of adherent tumor cells was calculated following fluorescence-labeled cell count of ten observation fields. The results are graphed as fold change relative to CM PBS (n = 3 independent experiments, *** = $p < 0.001$). Representative images are shown for each condition. Scale bar = 100 µm. (**B**) The 24 h CM from WT or *Adrb2*-deficient BMSCs following treatment with either PBS or ISO was collected and used for BMECs adhesion assay as described in 2A (n = 3 independent experiments, *** = $p < 0.001$). (**C**) C166 endothelial cells were labeled with Vybrant DiI prior to injection in the flow chamber. Then, adhesion assay was performed as described in 2A (n = 3 independent experiments, *** = $p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. βAR stimulation in bone marrow stromal cells promotes the release of factor(s) that favor adhesion between tumor and endothelial cells

The extravasation of metastatic cancer cells in distant organs requires successive steps of adhesive interactions with endothelial cells, mediated by cell adhesion molecules expressed by the endothelium and cancer cells. Femurs of mice treated with the βAR agonist isoproterenol (ISO) are characterized by higher vascularity than PBS controls [32], thus increasing the likelihood of circulating metastatic tumor cells to arrest in the bone microenvironment. This effect is mediated by the β2AR in osteoblasts and is VEGF-dependent. However, whether ISO alters the adhesive properties of these new vessels in a way that promotes tumor cell extravasation and bone metastasis remains unknown. To address this question, we assessed if the conditioned medium (CM) from β2AR + bone marrow stromal cells (BMSCs) differentiated for 14 days in osteogenic medium, and treated with ISO for 24 h, promoted tumor cell interaction with endothelial cells. Two endothelial cell populations were used in this study: CD31 and endomucin-positive primary bone marrow-derived endothelial cells (BMECs, Supplementary Fig. S1A and S1B) were chosen based on their skeletal origin and direct relevance to the bone metastatic process, and the endothelial cell line C166, which represents a pure population of endothelial cells [53].

In a short-term (3.5 h) adhesion assay under static conditions, the CM from differentiated BMSCs treated with ISO doubled the number of human MDA-MB-231 breast cancer cells attached to a confluent monolayer of BMECs (Fig. 1A), compared to the CM from PBS-treated BMSCs. The same result was observed with mouse 4T1 mammary tumor cells (Fig. 1B), and when using MDA-MB-231 in a more stringent flow-resistance adhesion assay used to better represent physiological flow conditions (Fig. 2A and Supplementary Fig. S2A). Cancer cell adhesion nearly doubled in the CM from ISO-treated BMSCs compared to PBS control, and showed negligible adhesion in the flow chamber when BMECs were omitted (Supplementary Figs. S2B and S2C), thus confirm the existence of true cell-cell interactions stimulated by the CM from ISO treated BMSCs. In this dynamic flow assay, the CM from ISO-treated BMSCs lacking the β2AR did not stimulate the number of adherent MDA-MB-231 cancer cells on BMECs (Fig. 2B), thus demonstrating that the stimulatory effect of ISO, a β1/β2 non-selective βAR agonist, on tumor-endothelial cell adhesion, is mediated by the β2AR expressed in BMSCs, specifically. The use of the C166 mouse endothelial cell line in the flow assay to generate the endothelial layer recapitulated what was observed with the more heterogenous BMEC population (Fig. 2C). These results thus reveal that β2AR activation in osteoblasts stimulates the release of soluble factor(s) that favor the adhesion of tumor cells to bone marrow endothelial cells.

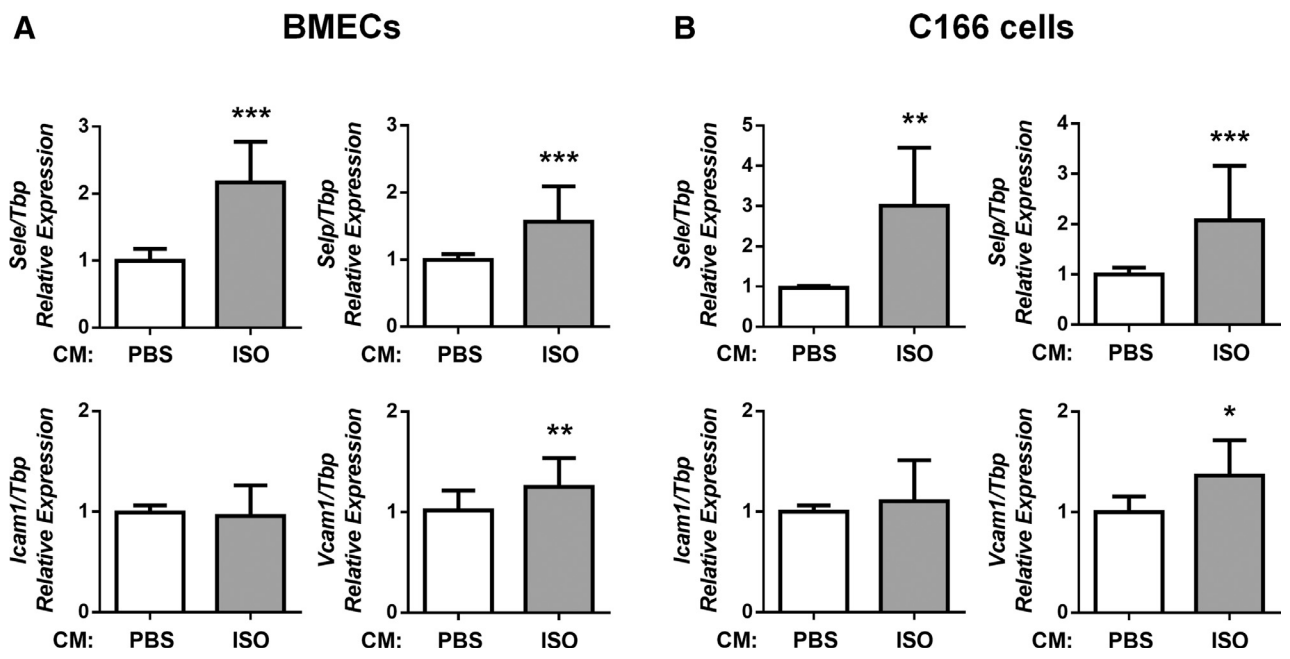

Fig. 3. BMECs (**A**) or C166 cells (**B**) were treated for 1 h with the CM from PBS or ISO-treated BMSCs. The CM from ISO-treated BMSCs induced a 2-fold increase of *Sele* and *Selp* expression compared to the CM from PBS-treated BMSCs, whereas *Vcam*1 level was increased only by 20% and *Icam1* expression did not change significantly in both BMECs and C166 cells (qPCR, n = 3 independent experiments, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

3.2. βAR stimulation in bone marrow stromal cells promotes the expression of adhesion molecules by BMECs

The increased adhesion of cancer cells to endothelial cells treated with the CM from ISO-treated BMSCs suggested that the latter caused the endothelium layer to switch from a basal to activated phenotype [54,55]. To address whether a soluble factor from βAR-stimulated osteoblasts activates endothelial cells, we incubated BMECs in the 24hr-CM from PBS or ISO-treated BMSCs for 1 h and measured the expression of adhesion molecules typical of an activated endothelium [42,56,57]. In response to the CM from ISO-treated BMSCs, we were able to detect a significant increase in the expression of *Sele, Selp* and to a lesser extent *Vcam1* compared to the PBS control, whereas *Icam1* expression was unchanged (Fig. 3A). Very similar results were obtained with the C166 mouse endothelial cell line (Fig. 3B). βAR-stimulated BMSCs thus can trigger an activated phenotype in BMECs *in vitro*, which is associated with increased adhesive properties for breast cancer cells (Figs. 1 and 2).

3.3. ISO treatment increases IL-1β and IL-6 levels in BMSCs in a β2AR-dependant manner

Pro-inflammatory cytokines can switch the endothelium into an activated state [42,56,57], and chronic stress is known to be associated with a pro-inflammatory environment in multiple tissues [58]. We thus focused our attention on pro-inflammatory cytokines expressed in osteoblasts in response to βAR stimulation. Upon ISO treatment, BMSCs displayed a rapid (1–2 h) and robust (40–200 times) induction of *Il1β* and *Il6* expression (Fig. 4A and B), which was blunted in ISO-treated BMSCs prepared from mice lacking the β2AR (Fig. 4C and D). The expression of other inflammatory cytokines such as *Il8 and Il10* was not increased in ISO-treated BMSCs, except for *Mcp1,* which showed a 2-fold induction (Supplementary Fig. S3).

3.4. Recombinant IL-1β increases the expression of Sele and Selp in BMECs

The above results suggested that IL-1β and/or IL-6 secreted by osteoblasts following βAR stimulation activate bone marrow endothelial cells and increase their adhesive properties for breast cancer cells, *via* the induction of E- and P-selectin expression. However, endothelial cells are known to express β1ARs and β2ARs for their control of vasomotor function [59,60]. We, therefore, sought to tease out the relative contribution of IL-1β, IL-6 and βARs stimulation to the increase in adhesion molecules observed in BMECs stimulated by the CM of ISO-treated osteoblasts. For that purpose, BMECs were directly treated with recombinant mouse IL-1β (10 ng/mL), recombinant mouse IL-6 (10 ng/mL) or ISO (10 μM) for 1 h and gene expression was measured. Treatment of BMECs with rIL-1β led to a robust increase in the expression of *Sele, Selp*, and *Icam1*, whereas ISO treatment had no significant effect (Fig. 4E and F and Supplementary Fig. S4A). Recombinant IL-6 had no effect on *Sele* and *Selp* expression across multiple time points (Fig. 4G and Supplementary Fig. S4B). Direct ISO treatment of BMECs, however, increased expression of *Il6*, thus revealing an autocrine activation loop in BMECs in response to βAR stimulation (Supplementary Fig. S4C).

To determine the functional contribution of IL-1β to the increased cancer cell adhesion on endothelial cells, we then tested the effect of this pro-inflammatory cytokine in adhesion assays under static and flow conditions. For these experiments, we used the C166 murine endothelial cell line which responded to rIL-1β by a robust expression of E-selectin surface expression (Fig. 5A). Under dynamic conditions in a flow chamber adhesion assay (Fig. 5B) and in two static adhesion assays (Fig. 5C and D), we observed a 2-3-fold increase in the number of MDA-MB-231 cancer cells attached to an endothelial monolayer of C166 cells following pre-treatment of the endothelial layer with rIL1β (10 ng/mL, 6 h). The increase in *Sele, Selp* and *Icam1* expression induced by rIL-1β in endothelial cells is thus associated with a functional increase in their adhesive properties for cancer cells.

3.5. IL-1β mediates the increase in tumor cell adhesion to endothelial cells induced by the CM from ISO-treated BMSCs

To determine if IL-1β in the CM from ISO-treated osteoblasts increases the activation status of C166 endothelial cells and the adhesion of MDA-MB-231 cancer cells to these cells, we used a loss-of-function strategy to block this cytokine or the increase in selectin expression in

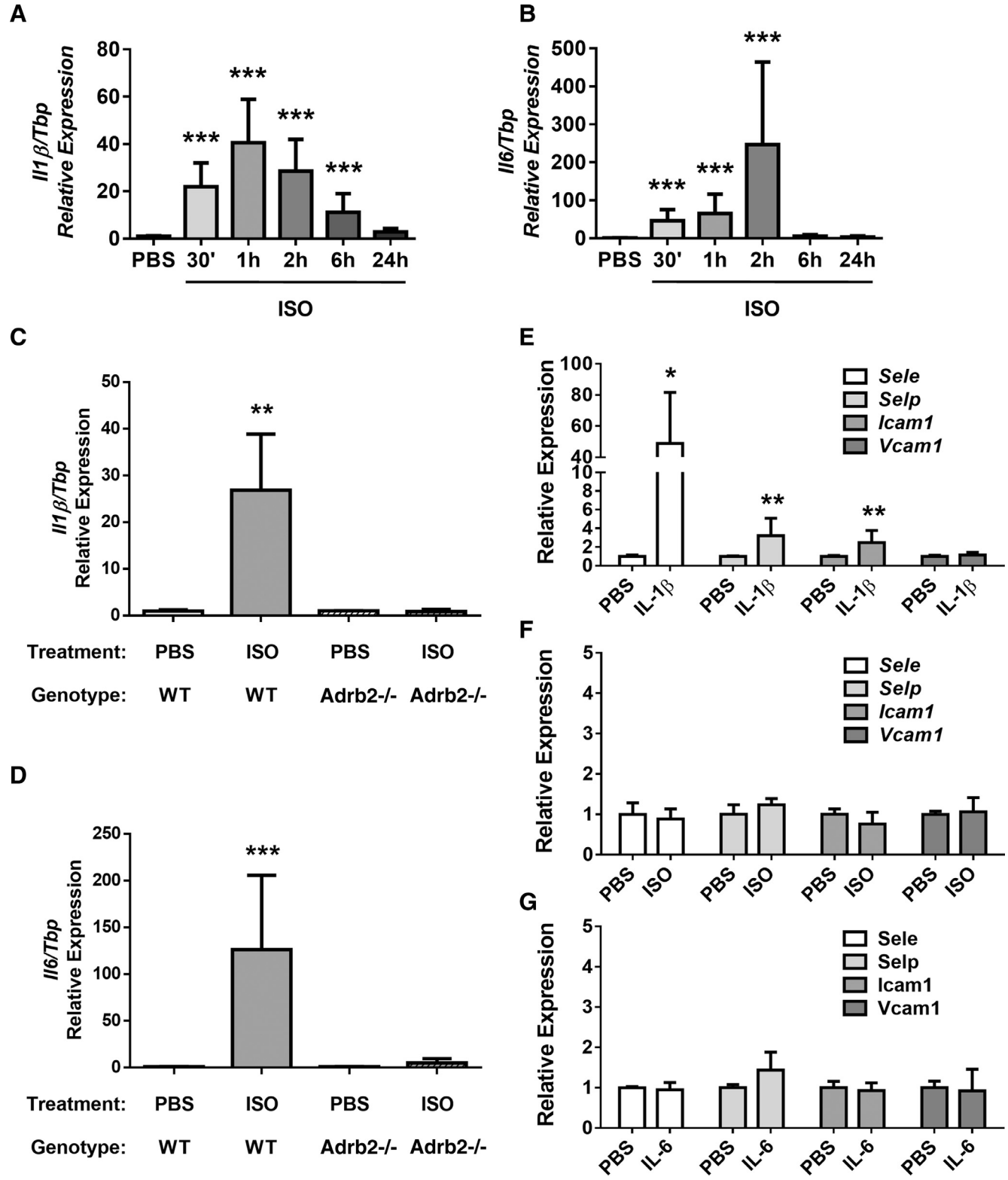

Fig. 4. ISO (10 μM) increased expression of the pro-inflammatory cytokines *Il1β* (**A**) and *Il6* (**B**) in mouse BMSCs within 2 h (qPCR, n = 5 independent experiments, *** = $p < 0.001$). *Il1β* (**C**) and *Il6* (**D**) expression was increased in WT BMSCs but not in BMSCs extracted from *Adrb2*-deficient mice following 2 h of ISO treatment (qPCR, n = 4 independent experiments, ** = $p < 0.01$, *** = $p < 0.001$). One hour treatment with murine rIL-1β (10 ng/mL, **E**) but neither ISO (10 μM, **F**) nor murine rIL-6 (50 ng/mL, **G**) increased expression of *Sele, Selp*, and *Icam1* in BMECs (qPCR, n = 5 independent experiments, * = $p < 0.05$, ** = $p < 0.01$).

endothelial cells induced by exposure to the CM of ISO-treated BMSCs. In this experiment, confluent layers of C166 endothelial cells were pre-incubated in flow chambers with the CM from PBS or ISO-treated osteoblasts for 6 h, in the presence of an IL-1β neutralizing antibodies or an IgG control antibody or cimetidine, an inhibitor of selectin expression [61]. Then, MDA-MB-231 cancer cells were circulated in the flow chambers for 5 min. In this dynamic flow adhesion assay again, the CM from ISO-treated BMSCs (in the presence of an IgG antibody control)

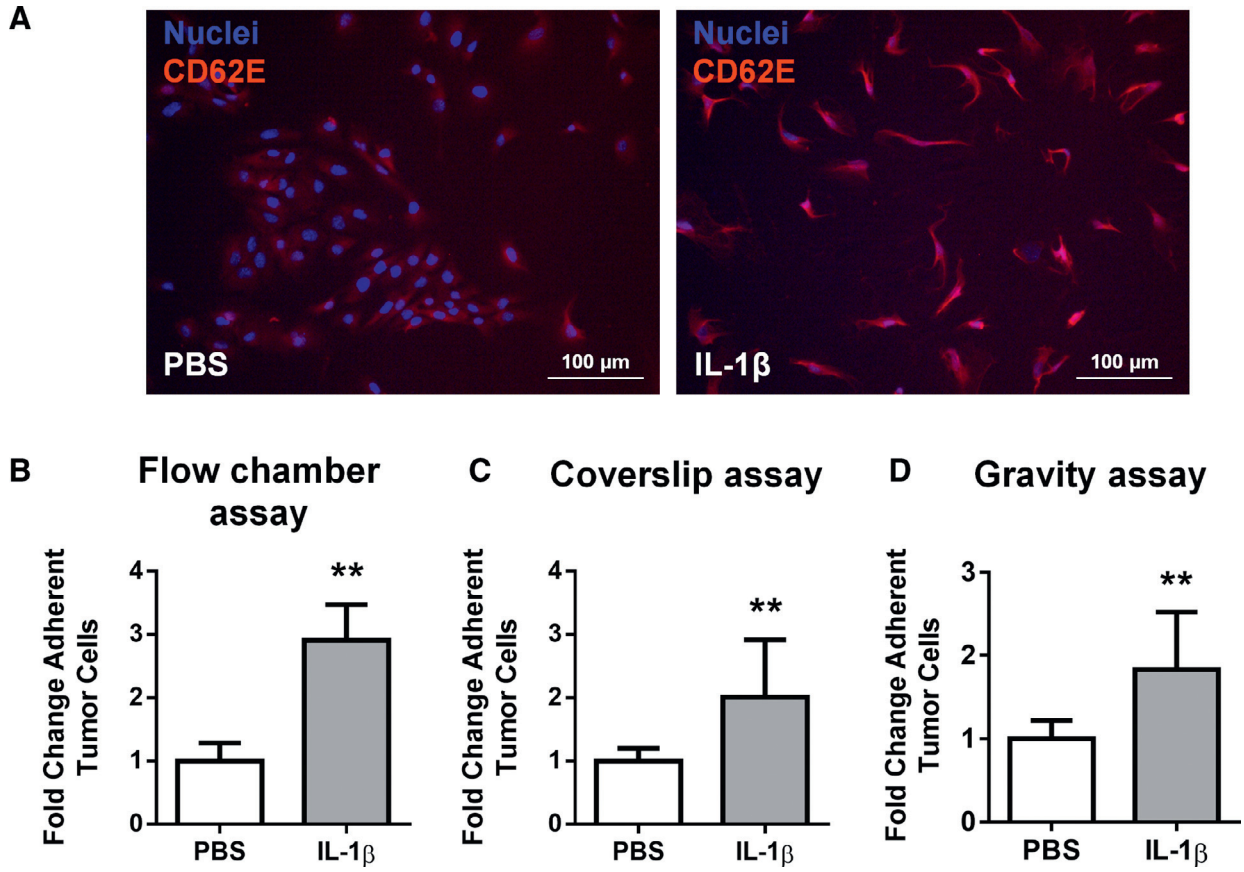

Fig. 5. (**A**) C166 endothelial cells were treated for 6 h with rIL-1β (10 ng/mL) before immunofluorescence staining for E-selectin (blue: Hoechst nuclear staining, red: E-selectin/CD62E). Scale bar = 100 µm. (**B–D**) Murine rIL-1β (10 ng/mL) increased the adhesion of GFP + MDA-MB-231 cancer cells to a C166 monolayer 3-fold compared to vehicle-treated endothelial cells in flow conditions (flow chamber assay, **B**) and 2-fold in static conditions (in a coverslip assay, **C** and in a gravity assay, **D**). Quantification of adherent tumor cells was calculated by fluorescence-labeled cell count assay and graphed as fold change relative to PBS (n = 3 independent experiments, ** = $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

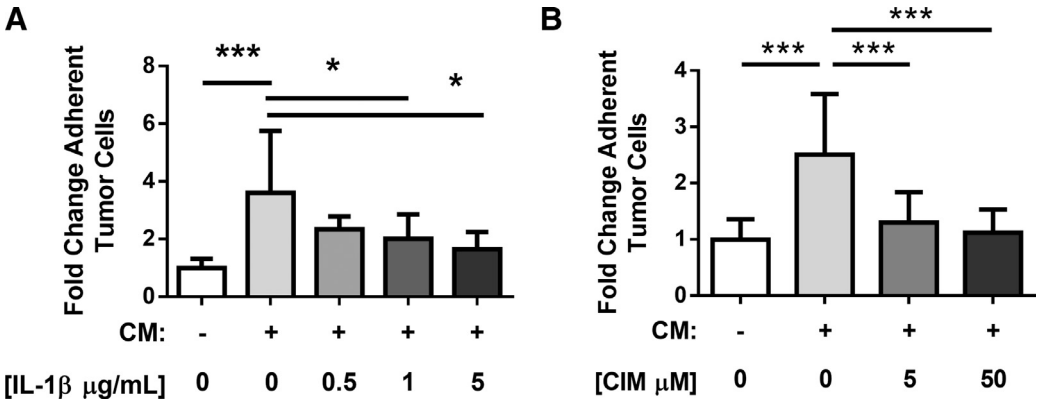

Fig. 6. C166 cells were treated for 6 h with the CM of PBS (-) or ISO (+) treated BMSCs along with an IL-1β neutralizing antibody or an IgG antibody control (**A**) or cimetidine (**B**), prior to addition of GFP + MDA-MB-231 cells in the flow chamber (n = 3 independent experiments, * = $p < 0.05$, *** = $p < 0.001$). Number of adherent tumor cells was calculated by fluorescence-labeled cell count assay and graphed as fold change relative to untreated group.

induced a 3–4 fold induction of MDA-MD-231-cell adhesion, but pre-incubation with an IL-1β-neutralizing antibody or cimetidine led to a significant reduction in the number of adherent MDA-MB-231 cells (Fig. 6A and B).

4. Discussion

Successful dissemination of circulating cancer cells from primary epithelial tumors to distant organs requires arrest into the vasculature and extravasation. This step is dependent on the expression of adhesion ligands that allow cells to roll along the endothelium by interacting with corresponding binding partners present on blood vessels [62]. Cancer cells colonize the bone marrow environment through a distinct E-selectin and SDF1-positive sinusoidal vasculature [63,64]. In this study, we used a series of *in vitro* assays to show that β2AR stimulation in osteoblasts, analogous to an increase in sympathetic outflow to bone *in vivo*, triggers the release of osteoblastic soluble factors that favor the adhesion between breast cancer cells and bone marrow-derived endothelial cells. We show that this effect involves IL-1β released by osteoblasts and the upregulation of E- and P-selectin expression by endothelial cells.

These findings are directly related to previous observations showing that osteoblastic βAR stimulation and chronic immobilization stress in adult mice augment bone vascularity and the likelihood of metastatic

cancer cells to engraft in the skeleton [32]. Although a higher bone vascular density can indeed favor the likelihood of metastatic cancer cells engraftment in this organ, the process is likely to be much more efficient with an activated endothelium promoting capture, rolling, attachment and extravasation of these cells. E-selectin is an adhesion molecule that is thought to play a major role in this process. The observation that metastasis is redirected from lung to liver in mice overexpressing E-selectin in the liver best supports this notion that E-selectin on the activated endothelium can facilitate tumor cell adhesion, extravasation and seeding into distant organs [43]. In cancer patients, an elevated serum level of E-selectin is generally recognized as a biomarker of endothelial cell activation and is associated with clinical metastasis [65], and several studies support the therapeutic potential of inhibiting metastasis by targeting selectins [66,67]. A pro-metastatic function of activated endothelial cells released in the blood circulation has also been observed before distant organ seeding, via chaperoning of circulating tumor cells and protection of cancer cells from anoikis [68]. Therefore, the fact that protection from anoikis and extravasation into distant organs are first, critical and endothelial-dependent steps for successful metastasis suggests that targeting endothelial-tumor cell interactions represents a valid potential therapeutic window for further investigation, especially in the context of chronic stress or inflammation. The observation that βAR stimulation by ISO promotes the colonization of the mouse skeleton by osteotropic MDA-MB-231 cells via a RANKL-mediated increase in cell migration [69] also suggests that high sympathetic outflow in bone might not only increase probability for cancer cell seeding and extravasation in the bone marrow environment, but also contributes to the migration and retention of these cells in this milieu.

IL-1β is a pro-inflammatory cytokine known as “gatekeeper” of inflammation. It is not expressed in normal conditions, hence its expression by primary breast tumors makes it a potential biomarker for breast cancer patients at increased risk for developing bone metastasis [70]. IL-1β expression in human breast primary tumors correlates with disease recurrence and skeletal metastasis, and increased expression is linked with the ability of tumor cells to home to the bone microenvironment in mouse models [70]. In previous studies, IL-1β was shown to increase adherence of tumor cells to endothelial cells *in vitro*, and administration of IL-1β to mice increased tumor growth and the number of melanoma cancer metastatic colonies in lung [71,72] and in bone [73]. Importantly, reducing endogenous IL-1α or IL-1β activity in a mouse model of melanoma reduced both tumor burden as well as metastases [74]. Three recombinant protein-based drugs targeting specifically IL-1β signaling have been approved for clinical use (Anakinra, Rilonacept, and Canakinumab) and other related agents are under study. These drugs are mostly used for the management of rheumatoid arthritis or other inflammatory diseases [75], but multiple clinical trials are ongoing with them in patients with advanced malignancies. One especially is focusing on Anakinra *versus* Denosumab in combination with everolimus (mTOR Inhibitor) for the treatment of advanced or metastatic cancers that are refractory to standard therapy [76]. The *in vitro* data presented herein suggest that such drugs might be beneficial in the context of breast cancer metastasis and for reducing the pro-adhesive properties of the activated bone endothelium for circulating tumor cells, although this will need to be tested experimentally in preclinical models. In another study, the IL-1R1 antagonist Anakinra was shown to reduce the number of mice with bone metastases caused by osteotropic MDA-MB-231 cells, possibly through reduction in angiogenesis and tumor growth within the bone microenvironment [77]. Tumor cell dissemination in bone appeared not to be affected by the treatment, suggesting that the impact of osteoblast-derived IL-1β on endothelial cells described in our *in vitro* study might be most relevant to states of high sympathetic outflow/high βAR stimulation or inflammation, consistent with IL-1β being important mainly in diseased states.

Although expression of both IL-1β and IL-6 was robustly induced by βAR stimulation in BMSCs, only IL-1β significantly stimulated short-term endothelium activation and adhesive properties. Similar findings were reported by Giavazzi and al., who demonstrated that a single injection of human recombinant IL-1β, but not IL-6, before cancer cell inoculation induced an augmentation of experimental lung metastases of A375M melanoma tumor cells in mice [71]. The increase in osteoblast-derived IL-6 induced by ISO may thus not be relevant to endothelial cell activation in the bone marrow, but certainly has the potential to stimulate osteoclast differentiation [78–80], cancer cell survival, proliferation and metabolism [81], and eventually bone destruction at later stages of the metastatic process [82].

Evidence from preclinical studies and the study herein support a model whereby vascular bone density [32], the adhesive properties of the bone endothelium (this study) and the migration of breast cancer cells toward RANKL-secreting cells [83] are under the direct control of β2-adrenergic signaling in osteoblasts. An important implication of these findings is that βAR blockade represents a low cost and potentially safe therapeutic option in the context of a long-term preventative treatment to prevent relapse in women treated for their breast tumors. This is supported by the inhibitory effect of β-blockers on bone metastasis in mice subjected to chronic immobilization stress (aka high sympathetic and HPA activity) [69] and by the longer survival of women that happened to take β-blockers at time of diagnosis [12,84,85].

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Conflict of interest

The authors declare no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jbo.2018.09.002.

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