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## EVIDENCE FOR CONSTITUTIVE BONE MORPHOGENETIC PROTEIN-2 SECRETION BY M1 MACROPHAGES:

### Constitutive auto/paracrine osteogenic signaling by BMP-2 in M1 macrophages

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### Abstract

Mechanisms mediating vascular calcification recapitulate osteogenic processes encompassing bone formation and imply participation of bone related proteins such as bone morphogenetic protein-2 (BMP-2). Macrophages are amongst the cells that contribute to vascular ossification by releasing cytokines that induce an osteogenic program in vascular smooth muscle cells, and also by becoming themselves osteoclast-like cells. In inflammatory vascular disease, the macrophage population in the vascular wall is diverse, with the M1 or inflammatory, and the M2 or anti-inflammatory macrophage types being dominant. Yet, the osteogenic potential of M1 and M2 macrophages remains unknown. Prompted by recent studies from our laboratory showing that in macrophages the Transient Receptor Potential Canonical 3 (TRPC3) channel contributes to endoplasmic reticulum (ER) stress-induced apoptosis in M1, but not in M2 macrophages, and given the strong relationship between ER stress and vascular calcification, we wished to examine whether TRPC3 would play a role in the osteogenic signaling of polarized macrophages. The findings reported here indicate that a constitutive BMP-2-dependent signaling operates in M1 macrophages, which is not affected by deletion of *Trpc3* and is not subject to regulation by ER stress. Our studies suggest operation of an auto/paracrine mechanism by which BMP-2 secreted by M1 macrophages maintains constitutive activation of a BMP-2 receptor/SMAD1/5 signaling axis.

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### DISCLOSURES

None.

## Keywords

TRPC3; macrophages; osteogenic signaling; BMP-2; vascular calcification

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## INTRODUCTION

Vascular calcification is a frequent complication of a number of vascular diseases that course with a clear inflammatory component, such as atherosclerosis or vascular pathology associated to chronic kidney disease and certain metabolic disorders (7). Mechanisms of vascular calcification are somewhat reminiscent of osteogenic processes encompassing bone formation, and involve the participation of a number of bone related proteins, among these, bone morphogenetic protein-2 (BMP-2), a key regulator of osteogenesis (21). BMP-2 belongs to the family of bone morphogenetic proteins (BMPs) within the transforming growth factor (TGF)- $\beta$  superfamily (13). By selectively acting on BMP-2 receptors type I and type II, BMP-2 promotes osteogenesis through regulation of key osteogenic transcription factors such as Runt-related transcription factor 2 (Runx-2) (5, 13). At the cellular level vascular calcification involves an intricate interplay between the endothelium, smooth muscle and immune cells recruited to the vessel wall, most importantly monocyte-derived macrophages (1, 4, 14). One mechanism by which macrophages promote calcification is by activating the osteogenic program in smooth muscle cells through the release of cytokines such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and/or interleukin-6 (IL-6) (1, 4). Macrophages can also contribute osteoclast-like cells to the osteogenic process in the vessel (1). Despite these roles being well-recognized, the macrophage population in the vascular wall is diverse, and there have not been systematic efforts to explore the specific roles of macrophage subsets in osteogenic signaling within the context of their potential impact on vascular calcification. This is of particular relevance in calcification related to atherosclerosis, as the M1 or inflammatory, and the M2 or anti-inflammatory macrophage types are dominant throughout all stages of plaque development (11, 19).

Transient Receptor Potential Canonical 3 (TRPC3) is a non-selective Ca<sup>2+</sup>-permeable channel belonging to the TRPC family (TRPC1-7) and the larger TRP superfamily of cation channels (10, 16). TRPC3 exhibits both receptor-dependent, and receptor-independent or constitutive functions (20). Recent studies from our laboratory have shown that in macrophages the Transient Receptor Potential Canonical 3 channel (TRPC3) plays a key role in signaling associated with endoplasmic reticulum (ER) stress-induced apoptosis (17). Remarkably, this effect is selective for M1 macrophages, as genetic or pharmacological inhibition of TRPC3 reduces unfolded protein response signaling and ER stress-induced apoptosis in M1, but not M2 macrophages (17). *In vivo*, this effect seems to have a beneficial impact on atherosclerosis, as the advanced aortic plaques in hyperlipidemic mice with bone marrow-selective or with macrophage-selective deletion of *Trpc3* have less necrosis and number of apoptotic macrophages than control animals, indicative of more stable plaques (18). Based on this information and considering the strong relationship between ER stress and vascular calcification, we wished to examine whether or not TRPC3 expression would have an impact on osteogenic signaling in polarized macrophages. Surprisingly, the findings in the present work show that whereas *Trpc3* deletion does not

affect the BMP-2 osteogenic signaling pathway in polarized macrophages, a constitutive BMP-2-dependent signaling operates in M1 macrophages, which is not further regulated by a number of stimuli. These studies suggest the existence of an auto/paracrine signaling mechanism by which BMP-2 secreted by M1 macrophages suffices to evoke constitutive activation of a BMP-2 receptor/SMAD1/5 signaling axis that may control osteogenic genes. We discuss our findings in the context of the potential implications of this novel mechanism in vascular calcification, such as that present in atherosclerosis.

## MATERIALS AND METHODS

### Experimental animals

All animal use for experimentation in this work conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and have been approved by University of Toledo IACUC. Generation and characterization of LysMCre<sup>+/-</sup>/Trpc3<sup>lox/lox</sup> mice (for simplicity, MacTrpc3KO) was described in detail by us in (17).

### Bone marrow-derived macrophages

Preparation of bone marrow-derived macrophages (BMDMs) and *in vitro* differentiation to the M1 phenotype was performed essentially as we described in (17). Differentiation to an M1 phenotype was confirmed by qRT-PCR with primers for typical phenotypic markers of M1 as we described in (17).

### Cell lysis and immunoblotting

**Cell lysis and immunoblotting:** essentially as we described in (17). Briefly, after cell lysis solubilized proteins were subjected to polyacrylamide gel electrophoresis (10% acrylamide), electrotransferred to PVDF membranes and immunoblotted with the indicated primary antibody. Following incubation with HRP-conjugated secondary antibodies, immunoreactive bands were visualized by ECL (Amersham, PA). Primary antibodies used were: phospho-Smad1/5 (Ser463/465; clone 41D10 rabbit monoclonal, cat. #9516, Cell Signaling (MA), Runx-2 (Abcam, cat. #102711) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

### Real-time PCR (qRT-PCR)

**Real-time PCR (qRT-PCR):** essentially as we described in (17). cDNA prepared from M1 or M2 macrophages was evaluated by semi-quantitative real-time PCR (qRT-PCR) with TrueAmp SYBR green qPCR supermix (Applied Biosystems, CA). The relative amount of mRNA was estimated by comparing with corresponding standards and normalized relative to glyceraldehyde 3-phosphate dehydrogenase (Gapdh). Results are expressed as mean  $\pm$  SEM relative to controls (relative quantitation, RQ). Sequences of primers are: Bmp-2 (F: AACTGGCTAGAAATATTAAGCACTGCA; R: AGTGATTTCCTAACTGCCAGG); Runx-2 (F: GGGCACAAGTTCTATCTGGAAAA; R: CGGTGTCAGTGCCTGAA); Gapdh (F: AGGTCCGGTGTGAACGGATTTG; R: TGTAGACCATGTAGTTGAGGTCA).

## Enzyme linked immunosorbent assay (ELISA)

The concentration of BMP-2 in the supernatants of M1 macrophages was measured using BMP2 Mouse ELISA kit (ab119582, Abcam), following manufacturer's instructions. Briefly, aliquots from supernatants or BMP-2 standards were added to a pre-coated anti-mouse BMP-2 antibody microplate (90 min, 37°C) followed by addition of biotinylated anti-mouse BMP-2 antibody (60 min, 37°C) and the avidin-biotin-peroxidase complex (30 min, 37°C). After color development with TMB reagent (20 min, 37°C), a stop solution was added and final O.D. absorbance was measured at 450 nm using a microplate reader.

## Statistical analysis

**Statistical analysis:** values are shown as mean  $\pm$  SEM and corresponding "n" number indicated in figure legends or text. Comparison of mean values between groups was performed with a two-tail Student's *t* test using Prism Graph Pad version 6 for Windows 2007 (Graph Pad Software, San Diego CA). P values of less than 0.05 were considered significant.

## RESULTS

Based on the recognized role of ER stress in mechanisms of vascular calcification, and considering recent work from our laboratory showing that TRPC3 exerts a selective effect on ER stress-associated signaling in M1 macrophages (17), we first examined the impact of Trpc3 deletion on the expression of the two key osteogenic regulators BMP-2 and Runx-2 in M1 and M2 macrophages. To that end, bone marrow derived macrophages prepared from Trpc3<sup>+/+</sup> and MacTrpc3KO mice were induced to differentiate *in vitro* to the M1 or M2 types as we described in (17). Following RNA isolation, the mRNA levels of Bmp-2 and Runx-2 were evaluated by qRT-PCR and found to be markedly decreased (>2-fold) in Trpc3-deficient-M1 macrophages as compared to Trpc3-expressing cells (Bmp-2:  $1.21 \pm 0.04$  vs.  $0.50 \pm 0.05$ ,  $P=0.001$ ; Runx-2:  $1.10 \pm 0.02$  vs.  $0.55 \pm 0.03$ ,  $P=0.003$ ;  $n=3$ , for normalized mRNA levels in Trpc3<sup>+/+</sup> vs. MacTrpc3KO macrophages, respectively). In M2 macrophages however, neither Bmp-2 nor Runx-2 mRNA levels were affected by Trpc3 deletion (Bmp-2:  $1.90 \pm 0.01$  vs.  $1.91 \pm 0.03$ ,  $P=0.80$ ; Runx-2:  $1.44 \pm 0.06$  vs.  $1.50 \pm 0.09$ ,  $P=0.66$ ;  $n=3$ , for normalized mRNA levels in Trpc3<sup>+/+</sup> vs. MacTrpc3KO macrophages, respectively). Prompted by these findings, we next focused on the M1 macrophages to examine if the reduced gene expression of Runx-2 and Bmp-2 observed in Trpc3-deficient M1 macrophages was also manifested at the protein level. Macrophages were maintained for 24 hours in complete medium (RPMI+10% fetal bovine serum) in the absence or presence of thapsigargin (1  $\mu$ M) or tunicamycin (5 $\mu$ g/ml) to induce chronic ER stress by depletion of ER Ca<sup>2+</sup> stores or inhibition of protein glycosylation, respectively, as we previously described in (17). As shown in Figure 1, not only Runx-2 protein levels were similar between MacTrpc3KO and control M1 macrophages, but its basal expression levels were not significantly affected by any of the ER stress treatment conditions (Figure 1).

Since BMP-2 is secreted to the immediate cell environment, we next examined BMP-2 levels by enzyme-linked immunosorbent assay (ELISA) in the medium of M1 macrophages that have been maintained for 24 hours in complete medium with or without tunicamycin.

The results in Figure 2A show that under basal conditions M1 macrophages secrete significant amounts of BMP-2 ( $213.50 \pm 15.30$  pg/ml). Most notable, secreted BMP-2 levels were neither affected by ER stress, nor by the TRPC3 expression status (Figure 2A) but were markedly reduced (~40%, Figure 2B) when cells were pre-treated with dorsomorphin (5  $\mu$ M), a general inhibitor of BMP-2 type I receptors (8).

One of the earliest events downstream BMP-2-dependent activation of BMP-2 receptors is the phosphorylation of SMAD1/5 proteins (12). To gather insight on the functional status of the BMP-2 signaling pathway in M1 macrophages, cells were kept for 15 minutes (Figure 3A) or 24 hours (Figure 3B) in complete medium in the absence or presence of the ER stressors thapsigargin (1  $\mu$ M) or tunicamycin (5  $\mu$ g/ml), or the BMP-2 receptor ligand bone morphogenetic protein 4 (BMP-4, 10 ng/ml). Following treatments the activation status of SMAD1/5 was indirectly evaluated from the extent of phosphorylation of Ser463/465 by western blot (Figure 3). In line with our findings with BMP-2 secretion, the amount of phospho-SMAD1/5 was not different between *Trpc3*-deficient and *Trpc3*-expressing M1 macrophages (Fig. 3). Remarkably, the constitutive activation of SMAD1/5, as evidenced by the phosphorylation status under control conditions, was not significantly altered by ER stress or exogenous activation of the BMP-2 receptor (Fig. 3). Of note, phospho-SMAD1/5 was not detected in M2 macrophages, neither under basal nor under ER stress or BMP-4 conditions (not shown).

The findings above suggested that BMP-2 secreted by the M1 macrophages was acting in an autocrine manner to maintain, to a certain extent, constitutive activation of the BMP-2 receptor/SMAD1/5 signaling axis. To test this possibility, M1 macrophages (*Trpc3*<sup>+/+</sup>) were subjected to ER stress or BMP-4 treatment for 15 minutes or 24 hours – incubation in RPMI alone was included as an alternative chronic ER stress condition to simulate serum starving conditions that macrophages may undergo in an inflammatory setting *in vivo*, as we did in (17)-, but in the absence or presence of dorsomorphin (5  $\mu$ M), and the phosphorylation status of SMAD1/5 was evaluated at the end of the treatments. As shown in Figure 4, treatment with the BMP-2 receptor inhibitor almost completely prevented phosphorylation of SMAD1/5, irrespectively of the treatment condition.

## DISCUSSION

Calcification is a frequent vascular complication in a number of metabolic disorders and chronic inflammatory vascular diseases such as atherosclerosis (2, 7). A major mechanism underlying vascular calcification involves BMP-2-dependent osteogenesis, in a manner reminiscent of mechanisms that mediate endochondral ossification (21). Whereas vascular smooth muscle cells are traditionally envisaged as the culprit of the osteogenic program leading to vessel calcification, existing evidence also supports important roles of endothelial cells and macrophages (1, 4, 14). Macrophages can promote calcification by contributing to activation of the osteogenic program in vascular smooth muscle cells, effect that has been attributed for the most part to the macrophage released cytokines TNF $\alpha$  and IL-6 (4). An important aspect that has been systematically ignored in most of these studies is the fact that macrophages in the vasculature are a heterogenous population. Indeed, the presence of distinctive macrophage subsets is well documented in human pathology and in

animal models of disease (11, 19). This is particularly important in atherosclerosis, where the M1 and the M2 types dominate throughout all stages of the disease (19). Despite this and the importance of calcification in atherosclerosis, whether M1 and M2 macrophages have different osteogenic capability remained unknown. The present studies were conceived to determine whether genetic deletion of macrophage TRPC3, a cation channel with a selective pro-apoptotic and anti-migration effect in M1 macrophages (9, 17), had an impact on the osteogenic signaling of polarized macrophages.

Against our expectations, whereas the initial evaluation of mRNA levels for the osteogenic regulators BMP-2 and Runx-2 demonstrated downregulation of these genes in Trpc3-deficient M1, but not M2 macrophages, these differences were not reflected at the protein level. Not only were Runx-2 protein and BMP-2 secretion unaffected in M1 macrophages lacking Trpc3, but their relatively high basal levels remained unaltered when cells were subjected to ER stress or to stimulation of the BMP-2 receptor. Importantly, BMP-2 secretion was significantly reduced in the presence of the BMP-2 receptor inhibitor dorsomorphin, strongly suggesting that BMP-2 constitutive secretion is, at least in part, self-driven through a BMP-2/BMP-2 receptor axis.

The observation that the phosphorylation status of SMAD1/5, a very early indicator of BMP-2 receptor-dependent signaling, was neither different between Trpc3-deficient and Trpc3-expressing M1 macrophages, nor altered by ER stress or exogenous activation of the BMP-2 receptor, is in line with constitutive secretion of BMP-2 being responsible for a sustained activation of SMAD1/5. This interpretation is further supported by the almost complete abrogation of SMAD1/5 activation upon conditions entailing inhibition of the BMP-2 type I receptors. Altogether, these findings suggest that a mechanism exists in M1 macrophages by which constitutive secretion of BMP-2 results in an autocrine action that maintains the activation of a BMP-2 receptor/SMAD1/5 signaling axis, which in turn accounts for constitutive expression of both BMP-2 and Runx-2.

That macrophages from human and murine origin produce BMP-2 has been known for some time (3). Yet, their ability to promote ossification has been traditionally attributed to the release of cytokines such as TNF $\alpha$  and IL-6 (4, 6). A recent study examined whether different subsets of macrophages differ in their ability to regulate the osteoblastic differentiation of mesenchymal stem cells *in vitro* (6). Although a more pronounced osteogenic effect was observed for M2 macrophages compared to M1 cells, this was credited to pro-regenerative cytokines such as tumor growth factor- $\beta$  or vascular endothelial growth factor, produced by M2 macrophages (6), with no consideration to macrophage-derived bone morphogenetic proteins. To the best of our knowledge, our findings represent the first evidence indicating that BMP-2 released from M1 macrophages exerts an autocrine action on these cells in what constitutes a self-sustainable osteogenic signaling path. Whereas not specifically tested here, it is likely that besides Runx-2 other osteogenic genes regulated by the BMP-2 receptor/SMAD1/5 axis are modulated by this constitutive mechanism. In atherosclerosis the relative abundance of M1 and M2 macrophages varies with the lesion stage and it generally increases as the atherosclerotic plaque burden and complexity augment (15, 19). Although awaiting *in vivo* validation, the speculation can thus be made that in the setting of the atherosclerotic plaque, operation of a constitutive BMP-2 mechanism in M1

macrophages may lead, as the M1/M2 ratio increases, to accumulation of M1-derived osteogenic signals whose paracrine actions on the vascular smooth muscle cell will gain increasing pathological relevance over time.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

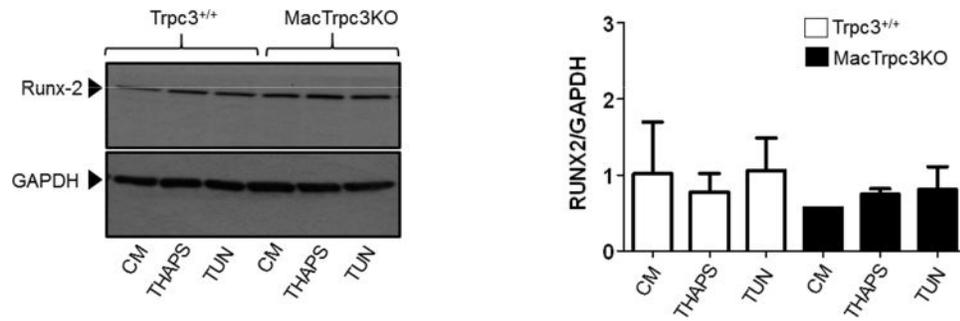
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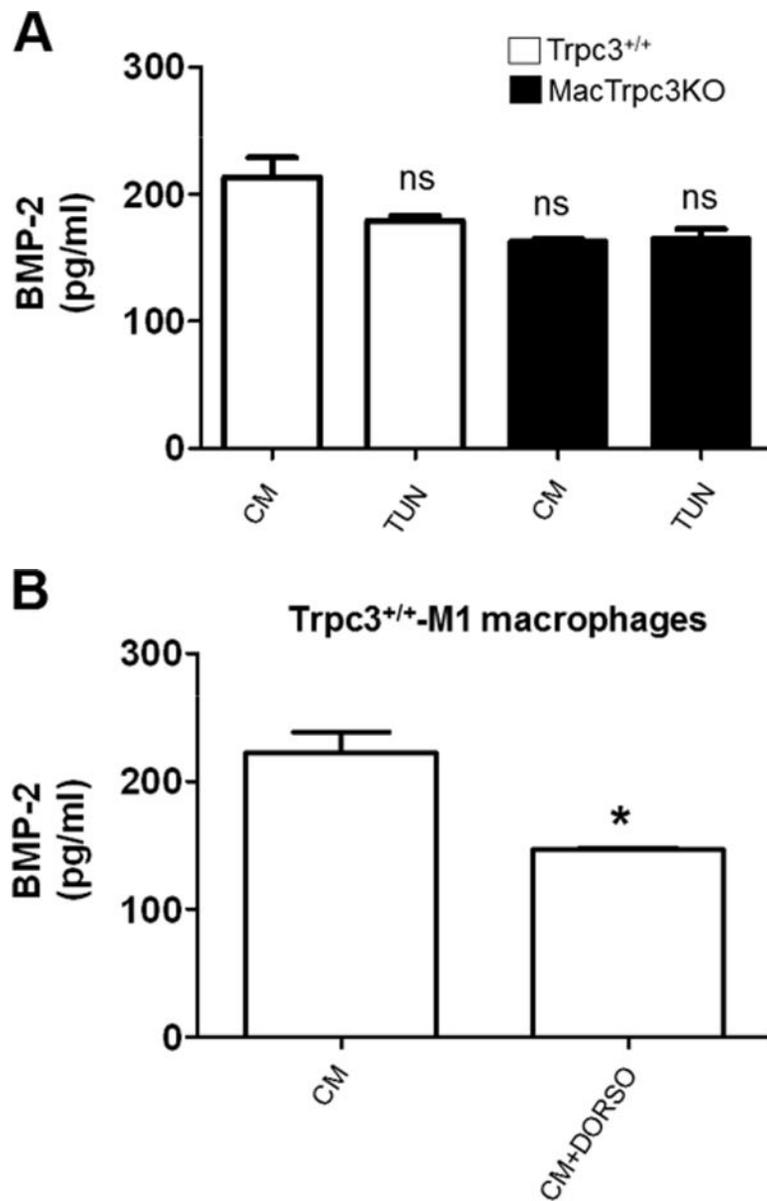
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- A constitutive BMP-2-dependent osteogenic signaling in M1 macrophages is reported.
- The mechanism requires autocrine/paracrine actions of BMP-2 on M1 macrophages.
- This novel mechanism may have implications in vascular calcification in vivo.



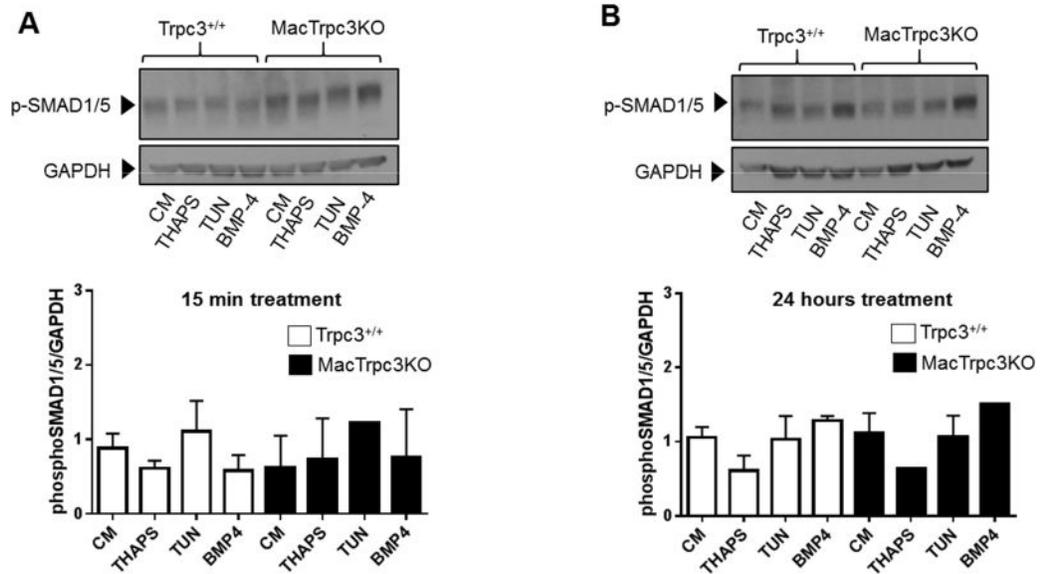
**Figure 1.**

Trpc3<sup>+/+</sup>-M1 or MacTrpc3KO-M1 macrophages were maintained in complete medium (“CM”), or complete medium containing thapsigargin (“THAPS”, 1  $\mu$ M) or tunicamycin (“TUN”, 5 $\mu$ g/ml) for 24 hours. Following treatments cells were processed for immunodetection of Runx-2 in whole cell lysates as described in Methods. Membranes were reprobed for GAPDH to control for protein loading. Shown are representative blots from 3 independent experiments and the corresponding normalized densitometric analysis.

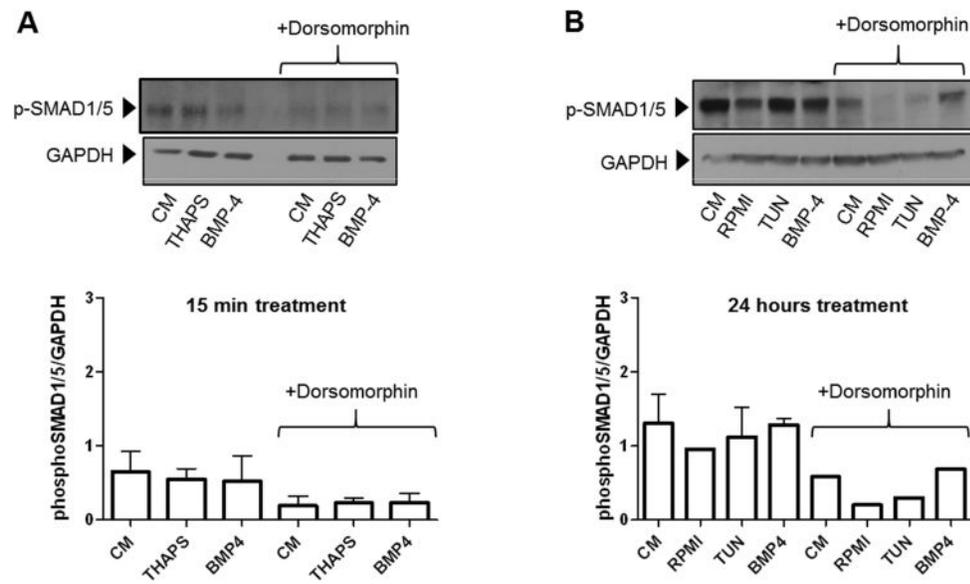


**Figure 2.**

**A)** Trpc3<sup>+/+</sup>-M1 or MacTrpc3KO-M1 macrophages were maintained in complete medium (“CM”), or complete medium containing tunicamycin (“TUN”, 5µg/ml) for 24 hours. In **B)** Trpc3<sup>+/+</sup>-M1 macrophages were maintained in control medium (“CM”) in the absence or presence of the BMP-2 receptor inhibitor dorsomorphin (“CM+DORSO”, 5 µM) as indicated, for 24 hours. In both instances, following treatments culture supernatants were collected and secreted BMP-2 was measured by ELISA as described in Materials and Methods. Shown are average values from 3 independent experiments, each performed in triplicates. ns: not statistically different from CM; \*p=0.03 vs. CM alone.

**Figure 3.**

Trpc3<sup>+/+</sup>-M1 or MacTrpc3KO-M1 macrophages were maintained in complete medium (“CM”), or complete medium containing thapsigargin (“THAPS”, 1 $\mu$ M), tunicamycin (“TUN”, 5 $\mu$ g/ml) or bone morphogenetic protein-4 (“BMP-4”, 10ng/ml) for **A**) 15 minutes, or **B**) 24 hours. Following treatments cells were processed for immunodetection of p-SMAD1/5 (Ser463/465) in whole cell lysates. Membranes were reprobbed for GAPDH to control for protein loading. Shown are blots representative of 3 independent experiments and the corresponding average normalized densitometric quantitation.

**Figure 4.**

Trpc3<sup>+/+</sup>-M1 macrophages were maintained in **A**) complete medium (“CM”), or complete medium containing thapsigargin (“THAPS”, 1 $\mu$ M), or bone morphogenetic protein-4 (“BMP-4”, 10ng/ml) for 15 minutes, in the absence or presence of the BMP-2 receptor inhibitor dorsomorphin (5  $\mu$ M) as indicated. In **B**) Trpc3<sup>+/+</sup>-M1 macrophages were maintained in complete medium (“CM”), RPMI (serum starvation condition), or complete medium containing tunicamycin (“TUN”, 5 $\mu$ g/ml) or bone morphogenetic protein-4 (“BMP-4”, 10ng/ml) for 24 hours, in the absence or presence of dorsomorphin (5  $\mu$ M) as indicated. Following treatments cells were processed for immunodetection of p-SMAD1/5 (Ser463/465) in whole cell lysates. Membranes were reprobbed for GAPDH to control for protein loading. Shown are blots representative of 3 independent experiments and the corresponding average normalized densitometric quantitation.