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Mitrofan, Claudia-Gabriela; Appleby, Sarah L; Nash, Gerard B; Mallat, Ziad; Chilvers, Edwin R; Upton, Paul D; Morrell, Nicholas W

DOI:
10.1074/jbc.M117.778506

JBC Papers in Press. Published on June 23, 2017 as Manuscript M117.778506
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M117.778506

Bone morphogenetic protein (BMP) 9 and BMP10 enhance tumor necrosis factor-α-induced monocyte recruitment to the vascular endothelium mainly via activin receptor-like kinase 2

Claudia-Gabriela Mitrofan[*Δ], Sarah L Appleby[*Δ], Gerard B Nash[†], Ziad Mallat[*], Edwin R Chilvers[*], Paul D Upton[*ΔΔ], Nicholas W Morrell[*ΔΔ]

From the *Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge, UK and [†]Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, UK. Δ These authors contributed equally. ΔΔ These authors jointly supervised this work.

Running title: *BMP9/BMP10 increases monocyte recruitment to endothelium*

To whom correspondence should be addressed: Professor Nicholas W Morrell, Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge, UK; nwm23@cam.ac.uk; Telephone: +44 1223 331666; Fax: +44 1223 336846

Keywords: endothelial cell, bone morphogenetic protein (BMP), monocyte adhesion, atherosclerosis, Smad, transcription factor

ABSTRACT

Bone morphogenetic proteins 9 and 10 (BMP9/BMP10) are circulating cytokines with important roles in endothelial homeostasis. The aim of this study was to investigate the roles of BMP9 and BMP10 in mediating monocyte-endothelial interactions using an *in vitro* flow adhesion assay. Herein, we report that while BMP9/BMP10 alone had no effect on monocyte recruitment, at higher concentrations both cytokines synergised with Tumor necrosis factor-α (TNFα) to increase recruitment to the vascular endothelium. The BMP9/BMP10-mediated increase in monocyte recruitment in the presence of TNFα was associated with upregulated expression levels of E-selectin, VCAM-1 and ICAM-1 on endothelial cells. Using siRNAs to type I and II BMP receptors and the signaling intermediaries (Smads), we demonstrated a key role for ALK2 in the BMP9/BMP10-induced surface expression of E-selectin, and both ALK1 and ALK2 in the upregulation of VCAM-1 and ICAM-1. The type II receptors, BMPR-II and ACTR-IIA were both required for this response, as was Smad1/5. The upregulation of cell surface adhesion molecules by BMP9/10 in the presence of TNFα was inhibited by LDN193189, which inhibits ALK2 but not ALK1. Furthermore, LDN193189 inhibited monocyte recruitment induced by TNFα and BMP9/10. BMP9/10 increased basal IκB-α protein expression, but did not alter p65/RelA levels. Our findings suggest that higher concentrations of BMP9/BMP10 synergise with TNFα to induce the upregulation of endothelial selectins and adhesion molecules, ultimately resulting in increased monocyte recruitment to the vascular endothelium. This process is mediated mainly via the ALK2 type I receptor, BMPR-II/ACTR-IIA type II receptors, and downstream Smad1/5 signaling.

INTRODUCTION

The vascular endothelium is a key regulator of vascular homeostasis with important roles in regulating blood pressure, coagulation, leukocyte trafficking and angiogenesis (1-3). The normal vascular endothelium regulates the passage of circulating cells into the interstitial space through several mechanisms, including leukocyte recruitment and alterations in permeability. However, endothelial dysfunction initiates a series of events triggering aberrant endothelial activation

that can lead to chronic pathological permeability and leukocyte adherence (4), which contribute to cardiovascular diseases, including atherosclerosis. Chronic systemic inflammation is associated with many cardiovascular, rheumatological and respiratory diseases (5-7), principally through the pathological activation of the vascular endothelium. Inflammatory cytokines including Tumour necrosis factor alpha (TNFα) and interleukin-1β are elevated in atherosclerosis. This promotes the upregulation of endothelial-expressed cell surface proteins that mediate leukocyte adhesion, including P- and E-selectin, which are involved in the initial leukocyte capture, and ICAM- and VCAM-1, which regulate the firm adhesion and transmigration of leukocytes (8-10).

Bone morphogenetic proteins (BMPs) are ligands belonging to the TGFβ superfamily. Aberrant BMP2, BMP4 and BMP6 signaling have been associated with the inflammation, fibrosis, calcification and osteogenesis that are associated with the pathophysiology of atherosclerosis (11-17). Since BMP9 and BMP10 are potent mediators of endothelial function it is likely that they also contribute to the pathobiology of vascular diseases such as atherosclerosis. However, the role played by BMP9 and BMP10 in monocyte transmigration across the endothelium, one of the initiating steps in atherosclerosis, has not been studied. BMP9 is a key regulator of vascular quiescence (18,19), and has been shown to protect the endothelium through the inhibition of vascular permeability (20), endothelial proliferation (18), angiogenesis (21) and lymphangiogenesis (22,23). Whilst BMP9 has been more extensively characterised than BMP10, in cell culture experiments BMP10 regulates a similar set of genes as BMP9 (24) and BMP10 can substitute for BMP9 in a mouse model of postnatal retinal vascular remodelling (21). Moreover, similar to BMP9, BMP10 has been described as a mediator of flow-dependent arterial quiescence (25). These studies suggest an overlapping role and function for BMP9 and BMP10 in the vasculature.

BMP serine-threonine kinase receptors form heterodimeric complexes consisting of type I and type II receptors (26). BMP9 and BMP10 signal through type I and type II receptors expressed on endothelial cells, including the type I receptors, activin-like kinase (ALK)1 and ALK2, and the type II receptors, bone morphogenetic protein receptor 2 (BMPR-II encoded by *BMPR2*), activin receptor 2A (ACTR-IIA encoded by *ACVR2A*) and activin receptor 2B (ACTR-IIB encoded by *ACVR2B*) (24,27,28). Optimal BMP9 and BMP10 signaling requires the type III auxiliary receptor endoglin, also expressed on endothelial cells (27). Mutations in BMP9 and its' receptors underlie vascular diseases, namely hereditary hemorrhagic telangiectasia (ALK1, endoglin and BMP9) (29-31) and pulmonary arterial hypertension (ALK1, BMPR2) (32-34). Furthermore, endothelial deletion of *Bmpr2* in mice enhances the development of atherosclerosis, suggesting an atheroprotective protective role for BMPR-II (35).

Activated BMP receptors transduce their signal primarily through phosphorylation of Smad1, Smad5 and Smad8. Following activation, Smads form heteromeric complexes with the common partner Smad, Smad4 (26). These complexes translocate to the nucleus and regulate the expression of numerous genes through binding to promoter regions, usually in complex with other transcription factors. The best characterised targets of BMP/Smad signaling are the inhibitor of differentiation (*ID*) genes which possess Smad binding elements in their promoters (36).

BMP9 signaling has been implicated previously in neutrophil recruitment to the endothelium, both directly (37) and indirectly (38-40). BMP9 has previously been shown to upregulate E-selectin and VCAM-1 on LPS-stimulated blood outgrowth endothelial cells (37) and endothelial cell surface expressed endoglin enhances leukocyte recruitment through the activation of β1-integrins expressed on the surface of leukocytes (40). Furthermore, *BMPR2* deficient endothelium shows impaired leukocyte recruitment (38,39), thus further implicating BMP9 signaling in the process of leukocyte recruitment.

Monocyte recruitment to the vascular endothelium is a key mediator of the progression of atherosclerotic lesions (41,42). Whilst there is a growing body of evidence associating BMP9 signaling with neutrophil recruitment, the role of BMP9 and BMP10 in monocyte recruitment to the vascular endothelium has yet to be reported. In the current study we show, using an *in vitro* flow adhesion assay that both BMP9 and BMP10, in a concentration-dependent manner, synergistically enhance monocyte recruitment to TNFα-stimulated human aortic endothelial cells (HAECs). This

occurs through the upregulation of E-selectin, VCAM-1 and ICAM-1 on HAECs, and mainly via the type I receptor ALK2, the type II receptors BMPR-II/ACTR-IIA, and the downstream mediators Smad1/5.

RESULTS

BMP9 and BMP10 increase monocyte recruitment to TNFα-treated HAECs in a concentration-dependent manner - First, we investigated the role of BMP9 and BMP10 on monocyte recruitment to the vascular endothelium using an *in vitro* flow adhesion assay, which enables the quantification of real-time interactions between endothelial cells and leukocytes under conditions of physiological flow. As BMP9 has been reported to circulate at concentrations between 2-12ng/ml in humans (18,43), we exposed the endothelium to BMP9 or BMP10 at concentrations ranging from 0-5ng/ml prior to the addition of TNFα, then assessed monocyte recruitment. Negligible monocyte recruitment was observed in HAECs treated with BMP9 (Figure 1A,B) or BMP10 (Figure 1A,C) alone. Whilst TNFα treatment, as previously reported (2,44,45)), induced some monocyte recruitment to HAEC monolayers (Figure 1A-C), a synergistic increase in total monocyte recruitment was observed when TNFα-stimulated HAECs were pre-treated with BMP9 or BMP10 at concentrations equal to or higher than 1.5ng/ml (Figure 1A-C). Pre-treatment of the vascular endothelium with BMP9 or BMP10 did not affect the percentage of rolling, arrested or transmigrated monocytes (Figure 1D,E). Only minimal monocyte rolling was observed in these experiments, suggesting that this process is rapidly followed by arrest and transmigration. Maximal monocyte recruitment was observed when TNFα-stimulated HAECs were pre-treated with BMP9 or BMP10 at a concentration of 5ng/ml, consequently, this concentration was used in all subsequent experiments. To examine whether this response was restricted to aortic cells, we also assessed the influence of BMP9 and BMP10 on TNFα–dependent recruitment of monocytes to blood outgrowth endothelial cell (BOEC) monolayers (46). Similar to HAECs, BMP9 and BMP10 did not influence monocyte adhesion to BOECs, but enhanced the recruitment observed in response to TNFα (Figure 1F,G). Taken together, these data show that both BMP9 and BMP10 synergise with TNFα to enhance monocyte recruitment to the vascular endothelium in a concentration-dependent manner, at or above 1.5ng/ml.

BMP9 and BMP10 increase expression of adhesion molecules and BMP2 in TNFα-treated HAECs - Next, we used qPCR and flow cytometry to identify whether pre-treatment with BMP9 or BMP10 increased expression of the endothelial selectins and adhesion molecules involved in monocyte recruitment in TNFα-stimulated HAECs. In accordance with previous studies (47-50), TNFα induced gene and surface protein expression of E-selectin, VCAM-1 and ICAM-1, which were synergistically increased in HAECs (Figure 2A-F and Figure S1) or BOECs (Figure S2A-C) pre-treated with either BMP9 or BMP10 (5ng/ml). BMP9 and BMP10 alone had no effect on the expression of these adhesion molecules. P-selectin was not detected on HAECs with any of the treatments (data not shown).

Since BMP2, BMP4 and BMP6 have been previously implicated in inflammation, fibrosis and osteogenesis (11-13), we next investigated whether treatment with BMP9 or BMP10 increased expression of these ligands in HAECs. BMP9 and BMP10 alone induced the expression of *BMP2* by 3-4-fold in HAECs, whereas TNFα exerted a weak induction (Figure 2G). However, pre-treatment with either BMP9 or BMP10 prior to TNFα-stimulation accentuated *BMP2* expression in HAECs (Figure 2G). *BMP4* was slightly repressed by BMP9, BMP10 and TNFα, whereas *BMP6* expression did not change with any of the conditions tested (Figure 2H, I). Taken together, these data reveal that both BMP9 and BMP10 synergise with TNFα to up-regulate endothelial-expressed molecules involved in leukocyte recruitment, in addition to *BMP2*, a factor previously implicated in endothelial inflammation.

BMP6 increases the surface expression of adhesion molecules on TNFα-treated HAECs - BMP6 has been previously described as a factor that induces endothelial inflammation (11). BMP6 transduces signaling predominantly via the type I receptor ALK2, and not ALK1 (11,51). Therefore, we investigated the potential role of BMP6/ALK2 in inducing E-selectin, VCAM-1 and ICAM-1 surface expression in HAECs. BMP6 pre-treatment induced a marked upregulation of the surface

expression levels of E-selectin, VCAM-1 and ICAM-1 in TNFα-treated HAECs (Figure 3A, B, C). The upregulation in E-selectin and VCAM-1 in response to BMP6 was completely abrogated by the use of a neutralising anti-BMP6 antibody (Figure 3A, B). Treatment with the BMP6 targeted antibody did not cause any further reduction in ICAM-1 (Figure 3C), indicating that ICAM-1 surface protein expression is regulated through a different mechanism to E-selectin and VCAM-1.

We next determined whether the BMP9/BMP10-induced upregulation of adhesion molecules was mediated by BMP6. Treatment with the anti-BMP6 neutralising antibody likewise had no effect on the surface expression levels of adhesion molecules induced by BMP9, BMP10 and TNFα treatments, indicating that this process was not mediated by BMP9 or BMP10 (Figure 3D-H). Collectively, these data imply a dominant role for ALK2 mediated effects of BMPs in the upregulation of surface expression levels of endothelial adhesion molecules.

The role of type I receptors in BMP9- and BMP10-induced expression of adhesion molecules – Expression analysis for the BMP type I receptors in HAECs revealed that BMP9 and BMP10 induced the expression of *ALK1* and *ALK2*, with little effect on ALK3 (Fig S3A-C). *ALK6* was not expressed. Addition of TNFα slightly reduced the expression of *ALK1*, but not *ALK2*. To determine the BMP type I receptors mediating the BMP9- and BMP10-induced upregulation in adhesion molecules in response to TNFα, we performed siRNA knockdown of *ALK1* and *ALK2* and assessed surface expression of adhesion molecules. The dependence of each adhesion molecule on ALK1 or ALK2 was different. The increase in cell surface E-selectin expression observed in HAECs pre-treated with BMP9 or BMP10 prior to TNFα was inhibited by *ALK2* knockdown, but not *ALK1* knockdown (Figure 4A), suggesting a marked ALK2 dependence of E-selectin regulation by BMP9 or BMP10 in these experiments. Knockdown of *ALK1* together with *ALK2* did not impact further on E-selectin expression. For VCAM-1 individual siRNA knockdown of *ALK1* and *ALK2* substantially impaired BMP9- and BMP10- induced VCAM-1 expression, and their combined knockdown further inhibited surface VCAM-1 expression (Figure 4B). For ICAM-1, only combined *ALK1* and *ALK2* knockdown resulted in impaired BMP9- and BMP10-induced surface ICAM-1 expression (Figure 4C). The knockdown efficiency of si*ALK1* and si*ALK2* in HAECs confirmed >85% reduction in mRNA levels of the corresponding target gene (Figure S3D,E). We also confirmed that si*ALK1*, but not si*ALK2*, reduced the *ID1* induction by BMP9 and BMP10 in HAECs (Fig S4). These data show that ALK2 is essential for BMP9- and BMP10-induced E-selectin expression, whilst either ALK1 or ALK2 can increase VCAM-1 expression. ICAM-1 requires both ALK1 and ALK2 for upregulation in TNFα-stimulated HAECs.

To explore further the role of type I receptors in the BMP9/BMP10-induced expression of adhesion molecules in TNFα-stimulated HAECs, we employed LDN193189, a cell permeable small molecule inhibitor of BMP type I receptors. LDN193189 inhibits ALK2 with an IC_{50} of 5nM, and ALK3 with an IC_{50} of 30nM, but has no effect on ALK1 in cells (52). LDN193189 also inhibits ACTR-IIA and ACTR-IIB (53). LDN193189 did not affect basal responses. However, pre-treatment of HAECs with LDN193189 decreased the BMP9 or BMP10-induced upregulation of E-selectin and VCAM-1 (Figure 5A-B). ICAM-1 surface expression levels were only slightly decreased after LDN193189 treatment (Figure 5C). Furthermore, LDN193189 reduced monocyte recruitment induced by BMP9 or BMP10 treatment to the level of TNFα-only stimulation (Figure 5D, E).

The role of BMP type II receptors in the BMP9- and BMP10-induced expression of adhesion molecules - As *BMPR2* deficiency is associated with pulmonary arterial hypertension (PAH) (32,33) and more recently with atherosclerosis (35), we investigated the role of the BMP Type-II receptors in mediating the expression of adhesion molecules. Expression analysis revealed that BMP9 and BMP10 induced the expression of *BMPR2*, but not *ACVR2A* or *ACVR2B* (Fig S5A-C). TNFα slightly, but non-significantly reduced the expression of *BMPR2* (Fig S5A). Transfection of si*BMPR2* and si*ACVR2A*, both individually and in combination, attenuated the BMP9- and BMP10-induced expression of E-selectin (Figure 6A) and VCAM-1 (Figure 6B) in TNFα-stimulated HAECs. In contrast, individual knockdown of

BMPR2 and *ACVR2A* had no impact on ICAM-1 expression, whereas combined knockdown of these receptors did impair ICAM-1 expression (Figure 6A-C). The knockdown efficiency for si*BMPR2* and si*ACVR2A* in HAECs were again >85% (Figure S5D,E).

Smad1 and Smad5 mediate the BMP9- and BMP10-induced expression of adhesion molecules on TNFα-treated HAECs - To investigate the involvement of Smad1 and Smad5 (26,54) in BMP9/BMP10-induced up-regulation of adhesion molecules we employed siRNA knockdown. Unexpectedly, the TNFα–induced expression of VCAM-1, ICAM-1, and E-selectin was inhibited by *SMAD1/5* knockdown, in keeping with the possibility that induction of BMP2 by TNFα was contributing to increased expression of these adhesion molecules (Figure 7A-C). BMP9- and BMP10-induced expression of E-selectin (Figure 7A) and VCAM-1 (Figure 7B) in TNFα-stimulated HAECs was markedly impaired upon *SMAD1* and *SMAD5* knockdown, both individually and in combination. ICAM-1 expression was only inhibited when *SMAD1* and *SMAD5* were knocked-down in combination (Figure 7C). *SMAD1* and *SMAD5* siRNA knockdown efficiency was confirmed by qPCR and showed an 85% reduction of the target gene (Figure S6A,B).

Since Smad2 and Smad3 have also been described as mediators of BMP9 signaling (28,55), we also employed siRNAs targeting *SMAD2* and *SMAD3* (Figure S6C,D). Knockdown of *SMAD2* and *SMAD3* individually or in combination did not alter the BMP9- or BMP10-induced surface expression of adhesion molecules in HAECs (Figure S3). Collectively, these data show that Smad1/5, but not Smad2/3, are essential to the BMP9- and BMP10-induced expression of E-selectin, VCAM-1 and ICAM-1 in TNFα-stimulated HAECs.

BMP9 and BMP10 increase IκB-α protein levels, but do not alter p65/RelA levels or phosphorylation. TNFα is known to mediate the expression of cell surface adhesion receptors via the NF-κB pathway (10). We examined whether BMP9 or BMP10 mediated changes in the levels or phosphorylation of the canonical signalling proteins, IκB-α and p65. Both BMP9 9and BMP10 increased basal IκB-α protein levels (Fig 8A,B), without any impact on IκB-α phosphorylation or on levels or Serine-536 phosphorylation of p65. These data suggest that BMP9/10 prime endothelial cells for TNFα responsiveness by increasing IκB-α levels.

DISCUSSION

The present study investigated whether BMP9 or BMP10, important circulating regulators of vascular quiescence, play a role in monocyte recruitment to the vascular endothelium. Although BMP9 or BMP10 alone had no effect on monocyte recruitment, in the presence of TNFα both BMPs synergistically and in a concentration-dependent manner increased monocyte recruitment and transmigration. Using siRNA knockdown of type I receptors and a small molecule inhibitor, we show that these effects are predominantly mediated by ALK2 and also involve BMPR-II, ACT-RIIA, and the downstream signaling intermediaries, Smad1/5.

The potentiation of TNFα–mediated monocyte recruitment was observed only at higher concentrations of BMP9 or BMP10, but was also readily induced by BMP6, a ligand with high affinity for ALK2. Knockdown of *ALK1* and *ALK2* in HAECs demonstrated that the BMP9/10-dependent potentiation of the TNFα-stimulated E-selectin expression was entirely ALK2-dependent, whereas VCAM-1 was partially dependent on each receptor and ICAM-1 was only altered when both *ALK1* and *ALK2* were knocked down. Our data suggest a contribution from both ALK1 and ALK2 receptors in mediating the overall response but a dominance of the ALK2 receptor in the potentiation of the BMP9 or BMP10-induced monocyte recruitment to the TNFα-stimulated HAECs. BMP9 and BMP10 both induced the expression of *ALK1* and *ALK2*. The role of these receptors in atherosclerosis is intriguing, as the expression of both *ALK1* and *ALK2* is induced by HDL (56). Moreover, ALK1 mediates endothelial uptake of LDL, but not oxidised LDL in LDLR-deficient mice, suggesting a role for ALK1 in normal endothelial lipid metabolism rather than the pathogenesis of atherosclerosis (57).

To further investigate the role of type I receptors we employed a small molecule inhibitor of ALK2 and ALK3, LDN193189. LDN193189 weakly inhibits ALK4, ALK5, ALK7, ACTR-IIA and ACTR-IIB (53) at the concentration used in this study but does not inhibit ALK1 (52). The

results confirm that the monocyte recruitment and induction of E-selectin, VCAM-1 and ICAM-1 induced by BMP9 or BMP10 in TNFα-treated HAECs is largely independent of ALK1. Our findings also show that BMP9 and BMP10 synergise with TNFα to induce expression of BMP2, which is a known regulator of endothelial inflammation and plays a role in atherosclerosis. Increased levels of BMP2 and BMP4 have been observed in atherosclerotic plaques (14-17,58). Once upregulated, BMP2 and BMP4 induce an inflammatory phenotype in endothelial cells, which results in leukocyte adhesion *in vitro*. BMP4 is increased in response to a high fat diet (a risk factor for atherosclerosis), which then up-regulates BMP2 levels (15). Furthermore, enhanced BMP2 activity has been implicated in triggering and accelerating vascular calcification (14,15). We and others have shown previously that TNFα increases endothelial expression of *BMP2* but not *BMP4* (12,59) and that *BMP2* expression in endothelial cells can be activated by inflammatory stimuli in a NF-κB-dependent manner (59,60). In our current study neither TNFα, BMP9, nor BMP10 alone impacted on the expression of *BMP2*, *BMP4* or *BMP6* in HAECs. However, HAECs that were treated with BMP9 or BMP10 in the presence of TNFα showed a synergistic increase in *BMP2* expression, providing further evidence that BMP9 and BMP10 are not themselves pro-inflammatory, but instead, might prime the vascular endothelium to mount a more intense response upon stimulation with an inflammatory cytokine such as TNFα.

Chronic TNFα exposure reduces *BMPR2* expression in endothelial cells and can alter BMP signalling (59). Even in this acute study, low concentrations of TNFα reduced basal *BMPR2* expression, whilst cell surface E-selectin, ICAM-1 and VCAM-1 were enhanced. Moreover, both type II receptors, BMPR-II and ACT-RIIA, are essential for BMP9- and BMP10-induced expression of E-selectin and VCAM-1 in TNFα-stimulated HAECs. BMPR-II has been implicated previously in leukocyte-endothelial interactions (38,39) and *BMPR2* deficiency is associated with several inflammatory vascular pathologies including PAH (32,33) and atherosclerosis (35). However, there is limited previous information on the role of ACT-RIIA in the regulation of endothelial-expressed selectins or adhesions molecules or the process of leukocyte recruitment (28). These findings provide further insight into the role of endothelial-expressed BMP type II receptors in maintaining endothelial homeostasis.

We questioned whether the mechanism of the enhanced TNFα response in the presence of BMP9/10 might be due to their effect on the NF-κB pathway, the main pathway known to induce endothelial adhesion molecules (10). We identified that BMP9/10 increased IκB-α protein levels, but that the rate of IκB-α phosphorylation and degradation are not altered. This implies that the cells are primed for the TNF-α response by BMP9/10. Although we did not observe changes in p65/RelA levels or Ser-536 phosphorylation, the NF-κB family members are activated by phosphorylation at several Serine residues, so the IκB-α priming may be associated with a different family member and/or different phosphorylation sites (61).

In the present study, we have shown that BMP9- and BMP10-induced E-selectin, VCAM-1 and ICAM-1 expression in TNFα-stimulated HAECs is regulated through the canonical BMP mediators, Smad1/5 and not Smad2/3. This correlates with our previous study which reported that Smad1/5 activation was required for BMP9-induced expression of E-selectin and VCAM-1 in LPS-stimulated endothelial cells (37). Smad1/5 has also been reported to mediate the expression of pro-atherogenic genes that promote atherosclerotic plaque stability in monocyte-derived monocytes (62). Moreover, inhibition of BMP signaling using LDN193189 attenuated Smad1/5 activation and reduced endothelial inflammation and calcification in atherosclerosis mouse models (63,64), thus further supporting our findings that Smad1/5 plays a key role in regulating endothelial homeostasis through the expression of selectins and adhesion molecules.

Whilst BMP9 has been more extensively characterised than BMP10, there is evidence to suggest that BMP9 and BMP10 can perform overlapping roles. This has been seen *in vitro* whereby BMP9 and BMP10 regulate the expression of a similar set of genes in human microvascular endothelial cells (24). Further, both BMP9 and BMP10 are required for complete closure of the ductus arteriosus (65), and BMP10 can compensate for the absence of BMP9, in BMP9 knockout mice during retinal vascularisation (21). However, Chen and

colleagues have shown that BMP9 is not able to substitute for BMP10 during cardiac development in mice (66), indicating a distinct role for BMP10 in cardiogenesis.

Monocyte transmigration across the endothelium is a normal physiological process but this process can lead to vascular pathologies and promote atherosclerosis if exaggerated. Here we show that treatment alone with either BMP9 or BMP10 (even at concentrations ≥1.5ng/ml) had no impact on monocyte recruitment in a flow adhesion assay. However, at concentrations ≥1.5ng/ml BMP9 and BMP10 behave in a near identical manner to synergise with TNFα to upregulate BMP2 expression and to enhance monocyte adhesion and transmigration in HAECs predominantly through ALK2, BMPR-II/ACT-RIIA and Smad1/5 signaling. We propose that the beneficial effects of BMP9 or BMP10 as vascular quiescent factors could be subverted in the presence of inflammatory mediators such as TNFα (59), contributing to pathological levels of monocyte recruitment; this in turn might stimulate foam cell development, inflammatory cytokine production and atherosclerotic plaque development and calcification. Our findings provide further insight into how BMP signaling mediates endothelial homeostasis and the mechanisms by which BMPs impact on cardiovascular disease.

EXPERIMENTAL PROCEDURES

Reagents, primers and antibodies-Cell culture reagents**:** BMP6, BMP9, BMP10 (R&D Systems), LDN193189 used at a working concentration of 250 nM (stock resuspended in DMSO at 5mM, a kind gift from Professor Paul Yu, Department of Medicine, Harvard University) EGM-2 BulletKit (Lonza), fetal bovine serum (FBS) (Sigma-Aldrich), trypsin (Sigma-Aldrich), Histopaque 1077 and 1119 (Sigma-Aldrich), Dulbecco's phosphate buffered saline (PBS) with Ca^{2+}, Mg^{2+} (Sigma-Aldrich), albumin bovine fraction (BSA) V solution 7.5% (Sigma-Aldrich), MACS separation system (Miltenyi Biotec), LS columns (Milteny Biotec) and CD14 microbeads (Milteny Biotec). siRNA transfection reagents: DharmaFECT1™ (Dharmacon), ON-TARGETplus™ siRNA Pools (Dharmacon), namely si*ALK1*, si*ALK2*, si*SMAD1*, si*SMAD2*, si*SMAD3*, si*SMAD5*, si*BMPR2*, si*ACVR2A*, si*ACVR2B* and non-targeting siRNA Pool (siCP). Flow cytometry reagents: anti-hE-selectin fluorescein conjugated mouse IgG1 (anti-human E-selectin-FITC, R&D Systems), allophycocyanin (APC) mouse anti-human CD54 (anti-human ICAM-1-APC, BD Pharmingen) and PE/Cy5 anti-human CD106 (anti-human VCAM-1-PECy5, BioLegend). Flow cytometry isotype control antibodies: mouse IgG1 isotype control fluorescein (R&D Systems), APC-mouse IgG1 (BD Pharmingen) and PE/Cy5 mouse IgG1 isotype control (BioLegend). Western blotting antibodies: IκB-α mouse mAb, phospho-Ser32/36-IκB-α rabbit Ab, p65/RelA rabbit mAb or phospho-Ser536-p65 rabbit Ab (Cell Signaling Technologies). Quantitative polymerase chain reaction (qPCR) reagents: QuantiTect Primer Assays (Qiagen) namely Hs-*ACVRL1*_1_SG (ALK1), Hs_*ACVR1*_1_SG (ALK2), *SMAD2*, *SMAD3*, *ACVR2A*. Primer sequences: *BMPR2* forward 5'-CAAATCTGTGAGCCCAACAGTCAA-3', *BMPR2* reverse 5'-GAGGAAGAATAATCTGGA TAAGGACCAAT-3', *SMAD1* forward 5'-TAGAAAGCCCTGTACTTCCTC-3', *SMAD1* reverse 5'-GGTTGCTGGAAAGAATCTGG-3', *SMAD5* forward 5'-GAGAGTCCAGTCTTACCT CC-3', *SMAD5* reverse 5'-GGAAAGAATCTGGA AACGTG-3', *PBGD* forward 5'-AGCTATGAAGGATGGGCAAC-3', *PBGD* reverse 5'-TTGTATGCTATCTGAGCCGTCTA-3, *B2M* forward 5'-CTCGCGCTACTCTCTCTTT C-3', *B2M* reverse 5'-CATTCTCTGCTGGATGA CGTG-3, *HPRT* forward 5'- GCTATAAATTCTT TGCTGACCTGCTG-3', *HPRT* reverse 5'-AATTACTTTATGTCCCCTGTTGACTGG-3. ROX reference dye (Invitrogen), SYBRGreen JumpStart Taq ReadyMix (Sigma-Aldrich)

Endothelial cell culture-Human aortic endothelial cells (HAECs) were purchased from PromoCell and maintained in EGM2-mv (Lonza) with 5% FBS. HAECs were cultured at 37°C in a 5% CO_2 humidified atmosphere and used in experiments at passages 4-6. HAECs were treated with BMP6, BMP9, BMP10, or with LDN193189 with the indicated concentrations for 16 h prior stimulation with TNFα (0.05ng/ml; 4 h). Blood outgrowth endothelial cells (BOECs) were generated from peripheral blood of control volunteers as described previously (46). Full informed written consent was obtained under ethical approval from the Huntington Local Research Ethics Committee.

Monocyte isolation-Blood samples were derived from healthy volunteers after giving informed consent, according to the protocol approved by the Cambridge Research Ethics Committee (06/Q018/218). Two-step density gradients of Histopaque 1119 and 1077 (Sigma) were used to isolate peripheral blood mononuclear cells (PBMCs). $CD14^+$ monocytes were isolated from PBMCs through positive selection using magnetic-activated cell sorting (MACS) as per the manufacturer's instructions. $CD14^+$ monocytes were resuspended at a cell density of 1 x 10^6 cells/ml in 0.15% BSA in PBS (with Ca^{2+} and Mg^{2+}).

siRNA transfection - HAECs were transfected with siRNA at 10 nM final concentration, using DharmaFECT1™ transfection reagent, following the manufacturer's instructions, 48 hours prior to their use in cell culture experiments.

Monocyte-endothelial interactions under flow-An *in vitro* flow adhesion assay was used to assess endothelial-monocyte interactions as previously described (67). The microslide (μ-Slide $VI^{0.4}$; Ibidi), containing the HAEC monolayer was connected to cell and wash reservoirs by silicon tubing and a valve enabled switching between the two reservoirs with continuous flow. The flow rate of 1 x 10^6 monocytes/ml for 4 minutes, equivalent to a wall shear stress of 0.1 Pascals, was controlled using a glass syringe attached to a withdrawal pump. Monocyte-endothelial interactions were visualised using time lapse imaging at 6 min post the initial monocyte bolus using a phase contrast microscope, placed within a Perspex environmental chamber at 37°C. Quantification of monocyte behaviour including rolling, arrest and transmigration was performed offline using ImagePro software.

Flow cytometric analysis of surface proteins-Flow cytometric analysis of endothelial cell surface adhesion proteins was performed as previously described (34) using anti-human E-selectin-FITC, anti-human VCAM-1-PE-Cy5 and anti-human ICAM-APC with corresponding conjugated isotype controls. Analysis was performed using a BD FACSCanto™ II (BD Biosciences) and quantification was performed using FlowJo software.

Quantitative polymerase chain reaction (qPCR)-An RNAeasy Mini kit (Qiagen) was used to extract the total RNA extracted from HAECs. mRNA expression of the genes of interest was assessed using SYBRGreen Jumpstart Taq ReadyMix, ROX reference dye and primers (Quantitect Primer Assays or in-house designed primers) in a 384 well QuantStudio 6 Flex (Applied Biosystems, Life Technologies). The ΔΔCt method was used for quantification.

Western Blotting – HAECs were seeded in 6cm dishes and grown to confluence. Cells were then incubated in EBM2 (Lonza) with 0.1% FBS (0.1% FBS) for 2 hours and then treated with BMP9, BMP10 or 0.1% FBS for 16h. Cells were then treated with TNFα (0.05ng/ml) or 0.1% FBS for 15min or 30min. Cells were snap-frozen and lysed in 250 mM Tris-HCl, pH 6.8, 4% SDS, 20% v/v glycerol containing an EDTA-free protease inhibitor cocktail (Roche, West Sussex, UK). Lysates were immunoblotted for the relevant proteins.

Statistical analysis-Comparisons between two groups were made using an unpaired student t-test. Comparisons between three or more groups were performed using one-way ANOVA with Tukey's multiple comparisons. A probability (p value) smaller than 0.05 was considered statistically significant. Normality of data distribution was assessed using a D'Agostino & Pearson omnibus normality test. Data are presented as the mean ± the standard error of the mean (SEM).

Acknowledgements: A PhD Fellowship from the Wellcome Trust to C-GM, a British Heart Foundation Programme Grant (NWM), and an MRC Experimental Challenge Award (NWM) supported this work. The NIHR Cambridge Biomedical Research Centre and the Cambridge NIHR Cell Phenotyping Hub provided infrastructure support.

Conflict of interest: NWM is a founder and director of Morphogen-IX. PDU is a founder of Morphogen-IX.

Author contributions: C.G.M designed and performed the research, analysed the results and wrote the paper. S.L.A. designed the research and wrote the paper. G.B.N. wrote the paper. Z.M. wrote the paper. E.R.C wrote the paper. P.D.U designed the research and wrote the paper. N.W.M. designed the research, analysed the data and wrote the paper.

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FIGURES

FIGURE 1**. BMP9 and BMP10 increase monocyte recruitment to TNFα-stimulated human aortic endothelial cells (HAECs) in a concentration-dependent manner.** HAECs were treated with BMP9 and BMP10 16h prior to TNFα treatment (0.05ng/ml, 4 h). Monocytes were perfused over TNFα-stimulated HAECs in a flow adhesion assay in the presence of media alone, BMP9 or BMP10. **(A)** Representative images of HAEC monolayers that were untreated, or treated with BMP9 (5ng/ml), BMP10 (5ng/ml), TNFα, BMP9+TNFα or BMP10+TNFα. Adherent monocytes are the bright phase cells (white arrow) and transmigrated monocytes shown as the smaller dark phase cells (black arrow). Experiments were performed in triplicate and the data are representative of n=3 biological repeats. **(B-C)** Concentration-response analysis of the recruitment of monocytes to HAEC monolayers, in the presence or absence of TNFα, with increasing concentrations of **(B)** BMP9 (0-5ng/ml) and **(C)** BMP10 (0-5ng/ml). **(D-E)** Monocyte behaviour (rolling – clear bar, adherence –grey bar and transmigration – black bar) was expressed as a percentage of total recruitment to TNFα-stimulated HAECs in the presence of **(D)** BMP9 and **(E)** BMP10. **(F)** Analysis of the recruitment of monocytes to BOEC monolayers, treated with 5ng/ml of BMP9 or BMP10, in the presence or absence of TNFα. (**G**) Monocyte behaviour (rolling – clear bar, adherence –grey bar and transmigration – black bar) was expressed as a percentage of total recruitment to

TNFα-stimulated BOECs in the presence of BMP9 or BMP10. Error bars represent ± S.E.M. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

FIGURE 2. **BMP9 and BMP10 increase the expression of E-selectin, VCAM-1, ICAM-1 and BMP2 in TNFα-stimulated human aortic endothelial cells (HAECs).** HAECS were treated with BMP9 or BMP10 (5ng/ml, 16 h) prior to TNFα treatment (0.05ng/ml, 4 hours). Expression of **(A)** *SELE* (E-selectin), **(B)** *VCAM1* and **(C)** *ICAM1* mRNA assessed using qRT-PCR. Surface expression of **(D)** E-selectin (FITC-conjugated anti-human E-selectin), **(E)** VCAM-1 (PE-Cy5-conjugated anti-human VCAM-1) and **(F)** ICAM-1 (APC-conjugated anti-human ICAM-1) was assessed using flow cytometry. Data are shown as median fluorescence intensity (MFI) expressed as fold change relative to untreated HAECs. Expression of **(G)** *BMP2*, **(H)** *BMP4* and **(I)** *BMP6* mRNA assessed using qRT-PCR. Experiments were performed in triplicate and the data are representative of n=3 biological repeats. Error bars represent ± S.E.M. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

FIGURE 3. **BMP6 increases the expression of E-selectin, VCAM-1 and ICAM-1 in TNFα-stimulated human aortic endothelial cells (HAECs).** HAECS were treated with a BMP6 neutralising antibody (BMP6 Ab) 60 min prior to the addition of BMP6 (25ng/ml, 16 h) followed by TNFα (0.05ng/ml; 4 h), Surface expression of **(A)** E-selectin (FITC-conjugated anti-human E-selectin), **(B)** VCAM-1 (PE-Cy5-conjugated anti-human VCAM-1) and **(C)** ICAM-1 (APC-conjugated anti-human ICAM-1) was assessed using flow cytometry. HAECs were treated with a BMP6 neutralising antibody (BMP6 Ab) 60 min prior to the addition of BMP9 (5ng/ml, 16h) followed by TNFα (0.05ng/ml, 4 hours). Surface expression of **(D)** E-selectin (FITC-conjugated anti-human E-selectin), **(E)** VCAM-1 (PE-Cy5-conjugated anti-human VCAM-1) and **(F)** ICAM-1 (APC-conjugated anti-human ICAM-1) was assessed using flow cytometry. HAECs were treated with a BMP6 neutralising antibody (BMP6 Ab) 60 min prior to the addition of BMP10 (5ng/ml, 16h) followed by TNFα (0.05ng/ml, 4 h). Surface expression of **(G)** E-selectin (FITC-conjugated anti-human E-selectin), **(H)** VCAM-1 (PE-Cy5-conjugated anti-human VCAM-1) and **(I)** ICAM-1 (APC-conjugated anti-human ICAM-1) was assessed using flow cytometry. Forward scatter and side scatter gating was applied to the HAEC population. Data are shown as median fluorescence intensity (MFI) expressed as fold change relative to untreated HAECs Experiments were performed in triplicate and the data are representative of n=3 biological repeats. Error bars represent ± S.E.M. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ns=not significant.

FIGURE 4**. Effect of ALK1 and ALK2 siRNA on BMP9- and BMP10-induced upregulation of E-selectin, VCAM-1 and ICAM-1 in TNFα-stimulated human aortic endothelial cells (HAECs).** HAECs were siRNA transfected, then treated with BMP9 or BMP10 (5ng/ml; 16 h) prior to TNFα treatment (0.05ng/ml for 4 hours). Surface expression of **(A)** E-selectin (FITC-conjugated anti-human E-selectin), **(B)** VCAM-1 (PE-Cy5-conjugated anti-human VCAM-1) and **(C)** ICAM-1 (APC-conjugated anti-human ICAM-1) was assessed using flow cytometry. Forward scatter and side scatter gating was applied to the HAEC population. Data is shown as median fluorescence intensity (MFI) expressed as fold change relative to HAECs transfected with siRNA control pool (siCP). Experiments were performed in triplicate and the data are representative of n=3 biological repeats. Error bars represent ± S.E.M. ***P ≤ 0.001.

FIGURE 5**. LDN193189 reduces the BMP9- and BMP10-induced upregulation of E-selectin, VCAM-1 and ICAM-1 and monocyte recruitment in TNFα-stimulated human aortic endothelial cells (HAECs).** HAECs were pre-treated with LDN193189 (250 nM resuspended in DMSO), DMSO, then stimulated with BMP9 or BMP10 (5ng/ml; 16 h) prior to TNFα treatment (0.05ng/ml for 4 hours). Surface expression of **(A)** E-selectin (FITC-conjugated anti-human E-selectin), **(B)** VCAM-1 (PE-Cy5-conjugated anti-human VCAM-1) and **(C)** ICAM-1 (APC-conjugated anti-human ICAM-1) was assessed using flow cytometry. Forward scatter and side scatter gating was applied to the HAEC population. Data are shown as median fluorescence intensity (MFI) expressed as fold change relative to HAECs transfected with siRNA control pool (siCP). **(D)** HAECs were treated as described above and then monocytes were perfused in a flow adhesion assay. **(E)** Monocyte behaviour (rolling – clear bar, adherence –grey bar and transmigration – black bar) was expressed as a percentage of total recruitment. Experiments were performed in triplicate and the data are representative of n=3 biological repeats. Error bars represent ± S.E.M. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

FIGURE 6. **Knockdown of BMPR2 and ACVR2A inhibits the BMP9- and BMP10-induced upregulation of E-selectin, VCAM-1 and ICAM-1 in TNFα-stimulated human aortic endothelial cells (HAECs).** HAECs were siRNA transfected, then treated with BMP9 or BMP10 (5ng/ml; 16 h) prior to TNFα treatment (0.05ng/ml; 4 h). Surface expression of **(A)** E-selectin (FITC-conjugated anti-human E-selectin), **(B)** VCAM-1 (PE-Cy5-conjugated anti-human VCAM-1) and **(C)** ICAM-1 (APC-conjugated anti-human ICAM-1) was assessed using flow cytometry. Forward scatter and side scatter gating was applied to the HAEC population. Data are expressed as median fluorescence intensity (MFI) expressed as fold change relative to HAECs transfected with siRNA control pool (siCP). Experiments were performed in triplicate and the data are representative of n=3 biological repeats. Error bars represent ± S.E.M. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

FIGURE 7. **Smad1 and Smad5 mediate the BMP9- and BMP10-induced upregulation of E-selectin, VCAM-1 and ICAM-1 in TNFα-stimulated human aortic endothelial cells (HAECs).** HAECs were siRNA transfected, then treated with BMP9 or BMP10 (5ng/ml, 16 h) prior to TNFα treatment (0.05ng/ml, 4 h). Surface expression of **(A)** E-selectin (FITC-conjugated anti-human E-selectin), **(B)** VCAM-1 (PE-Cy5-conjugated anti-human VCAM-1) and **(C)** ICAM-1 (APC-conjugated anti-human ICAM-1) was assessed using flow cytometry. Forward scatter and side scatter gating was applied to the HAEC population. Data are shown as median fluorescence intensity (MFI) expressed as fold change relative to HAECs transfected with siRNA control pool (siCP). Experiments were performed in triplicate and the data are representative of n=3 biological repeats. Error bars represent ± S.E.M. *P ≤ 0.05, **P ≤ 0.01, ***P≤ 0.001.

FIGURE 8. **BMP9 and BMP10 increase IκB-α protein levels in human aortic endothelial cells (HAECs).** HAECs were treated with BMP9 or BMP10 (5ng/ml, 16 h) prior to TNFα treatment (0.05ng/ml) for 15 min or 30 min. (A) Protein lysates were immunoblotted for IκB-α, phospho-Ser32/36-

IκB-α (*P-IκB-α*), p65/RelA (*p65*) or phospho-Ser536-p65 (*P-p65*). All blots were reprobed for α-tubulin to confirm equal loading. Blots are representative of n=3 separate experiments. (B) Densitometry was determined using ImageJ for the three IκB-α blots, each band being expressed as a ratio of IκB-α/α-tubulin. These ratios were then normalised to the 0.1% control for the relevant time point.

FOOTNOTES

The abbreviations used are: BMP9, BMP10, TGFβ, TNFα, HAECs, NF-κB

FIGURE1

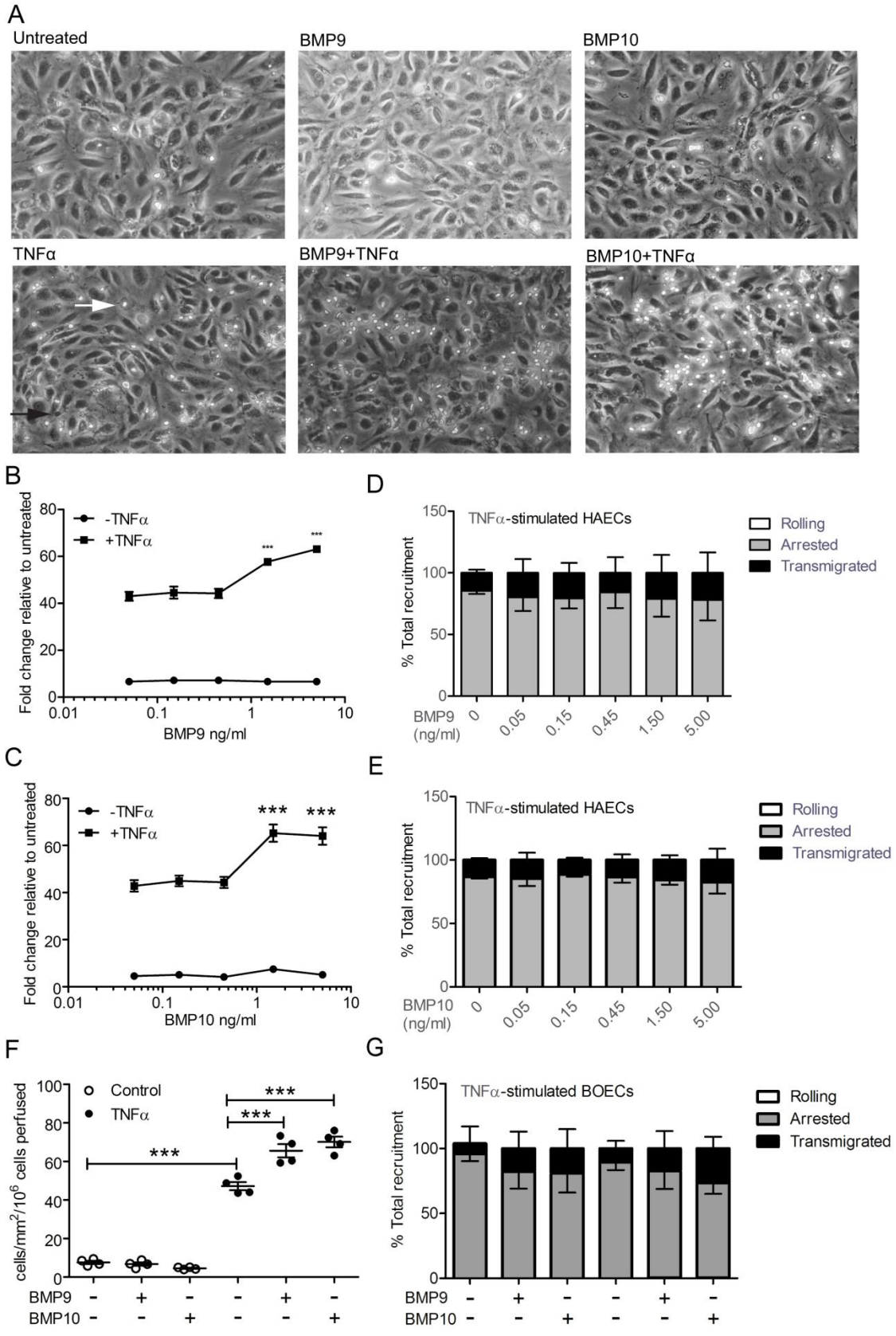


FIGURE 2

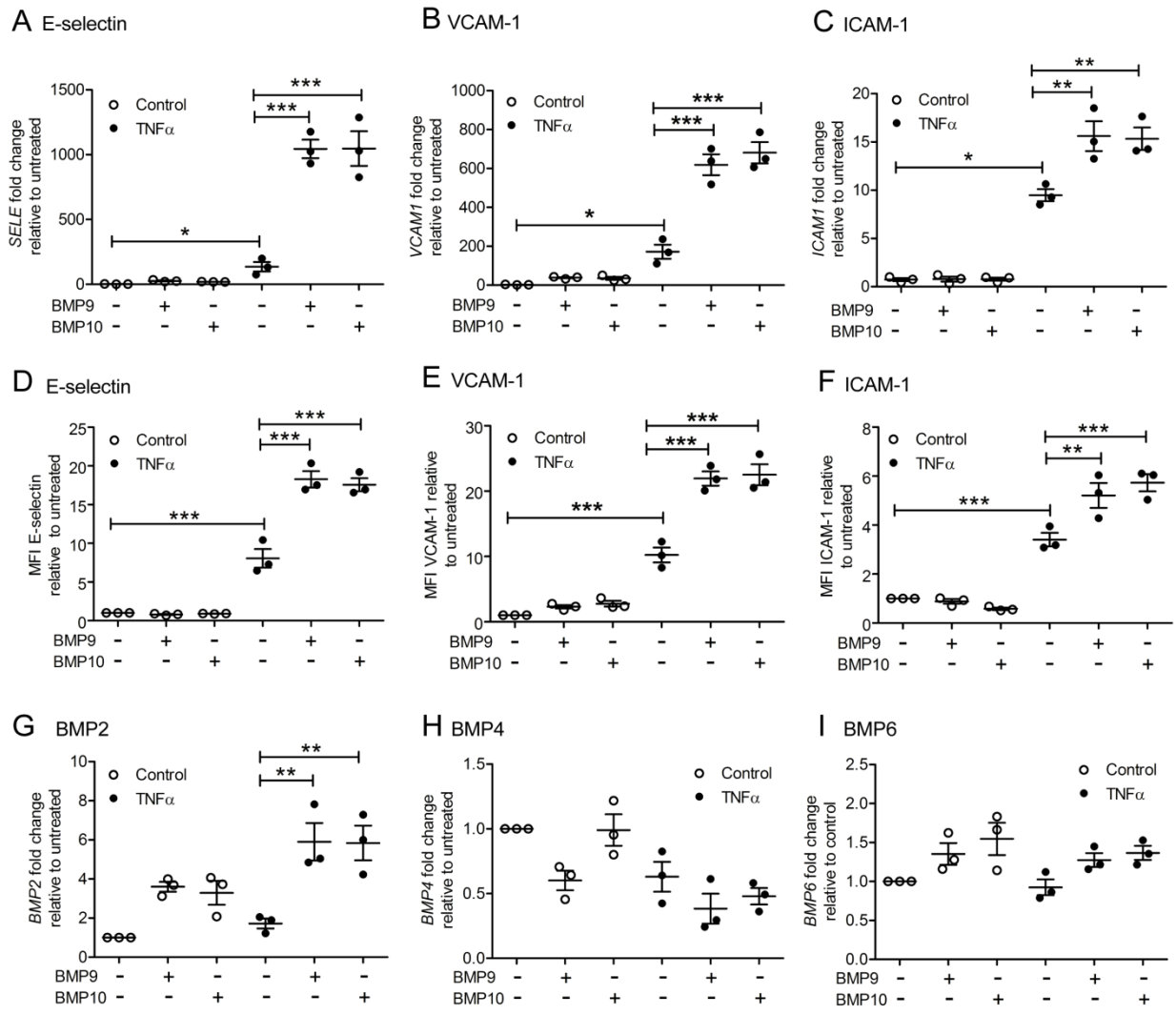


FIGURE 3

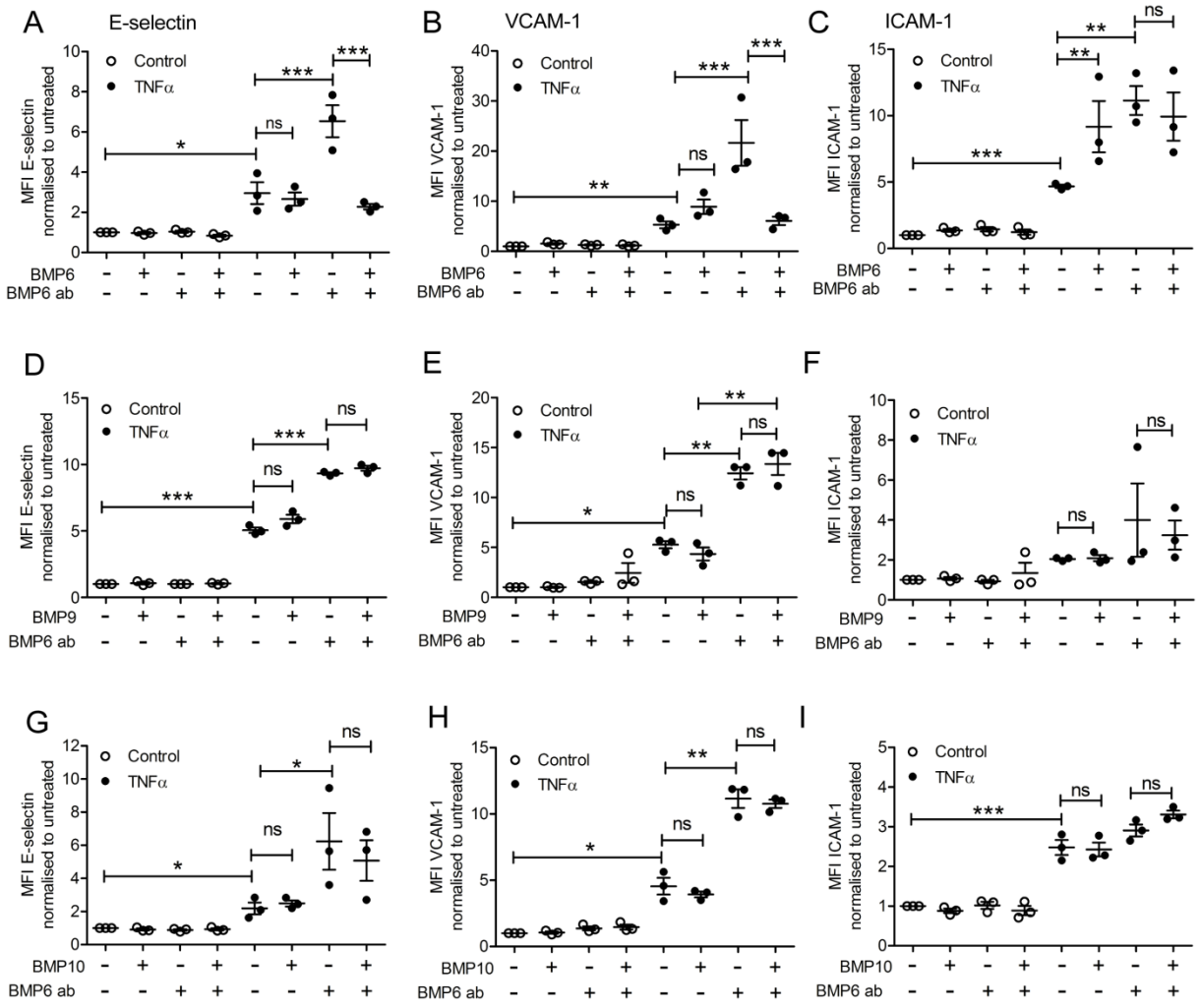


FIGURE 4

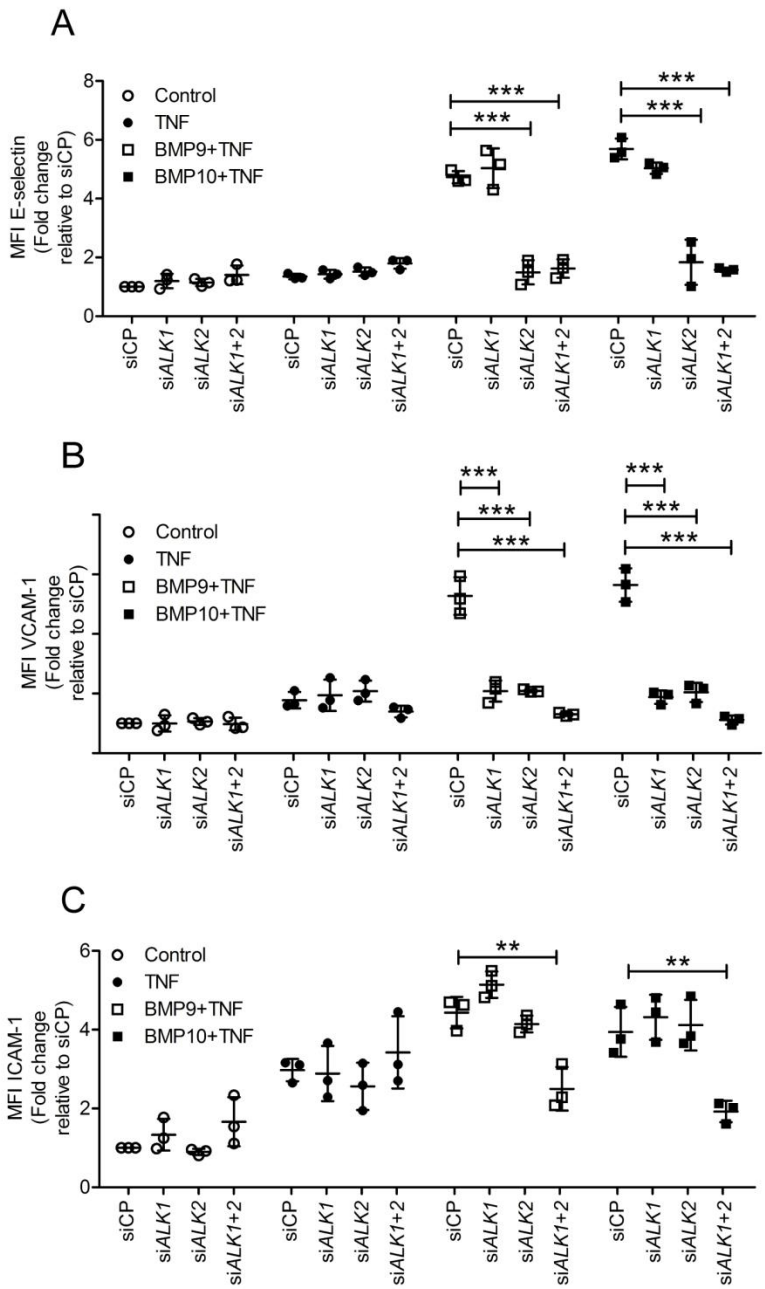


FIGURE 5

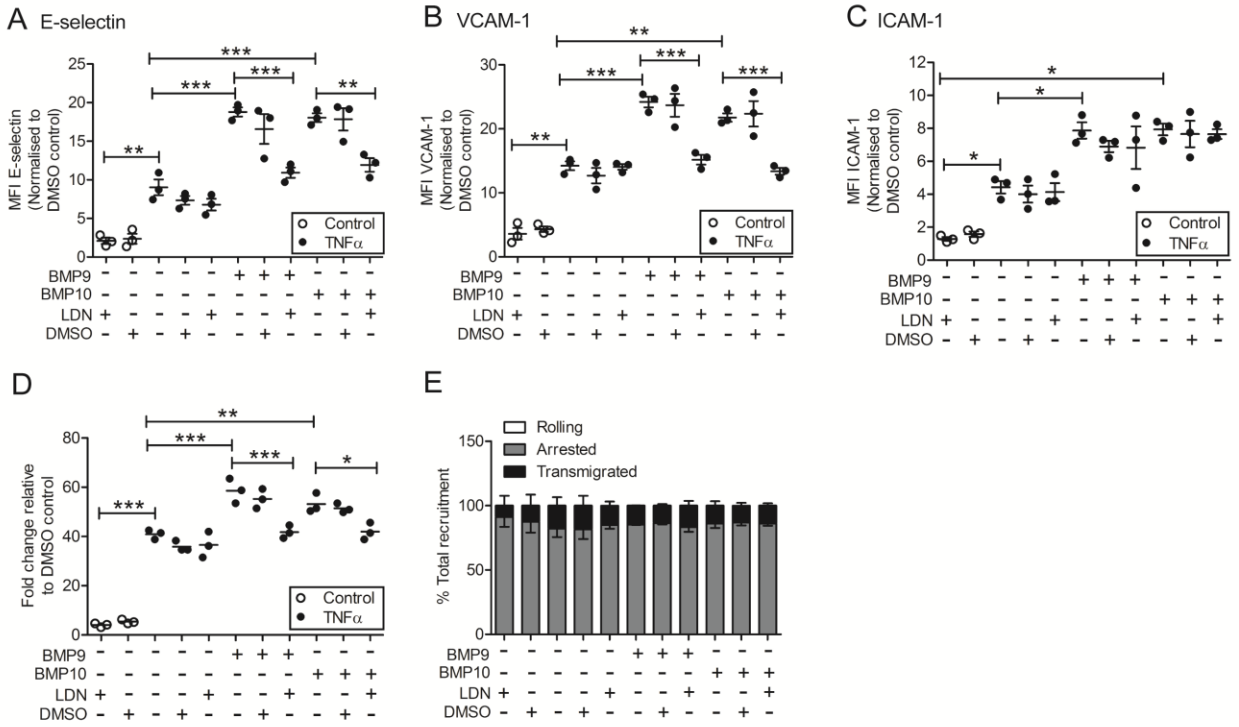


FIGURE 6

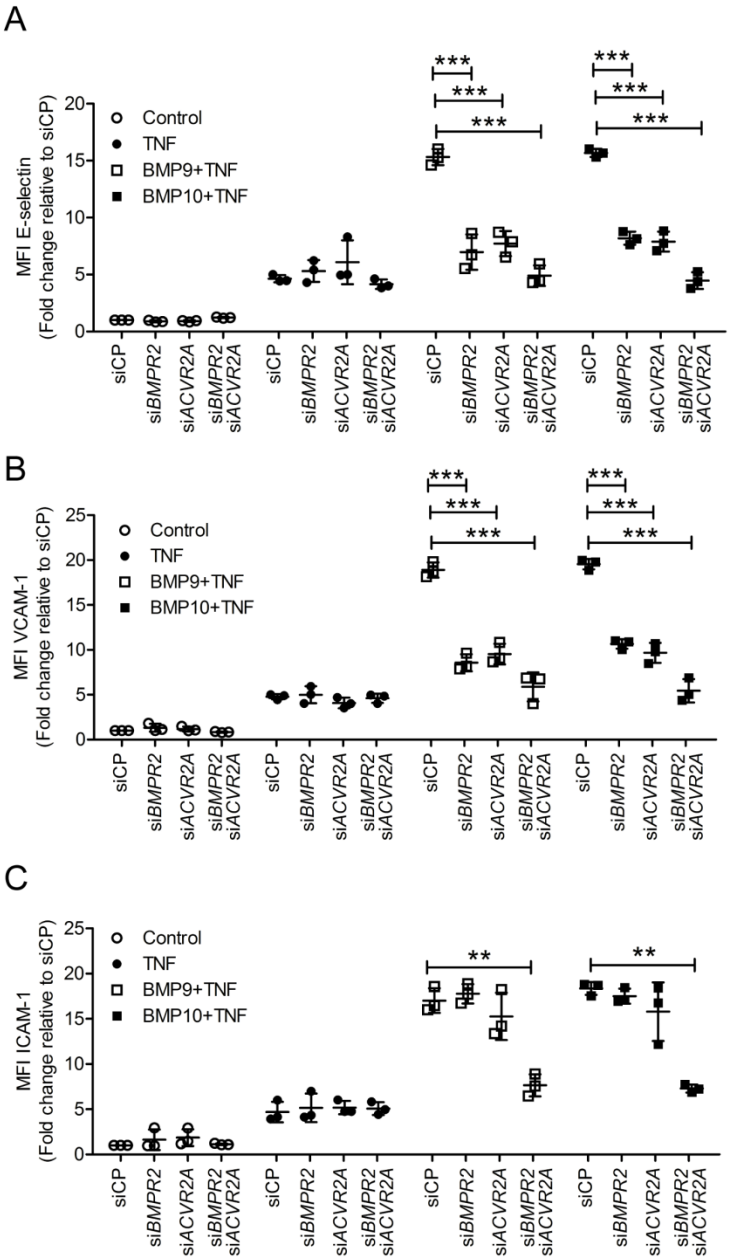


FIGURE 7

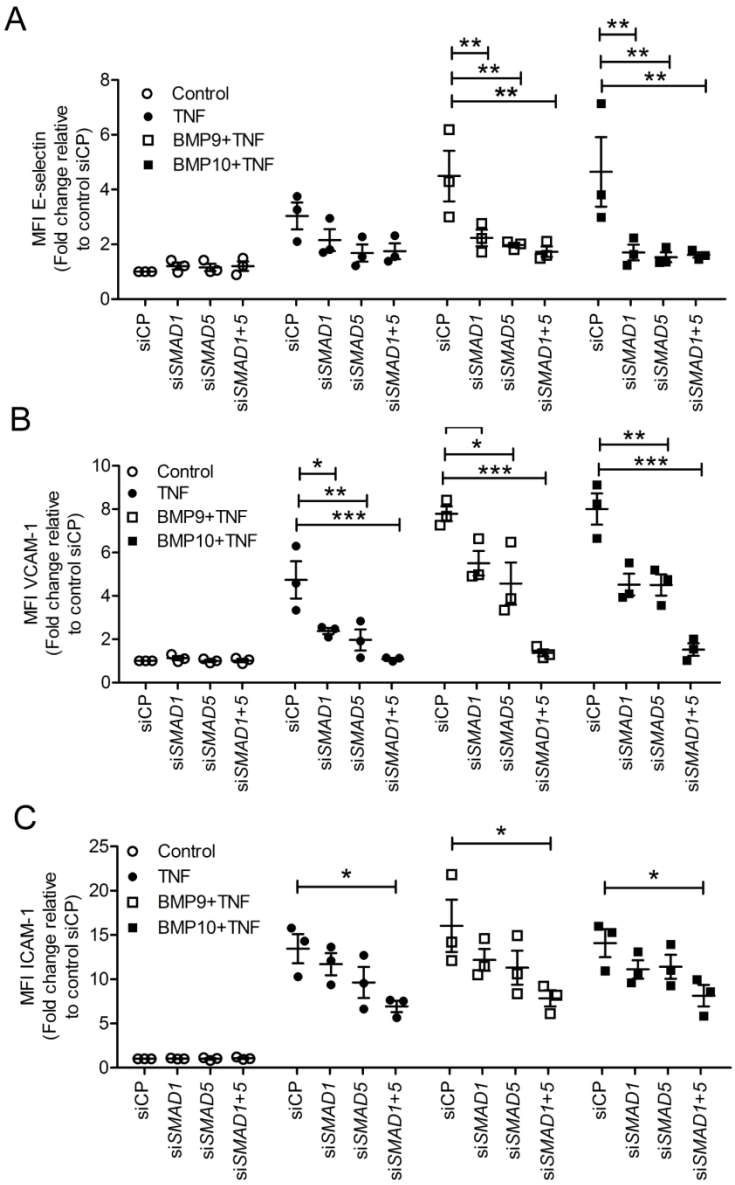
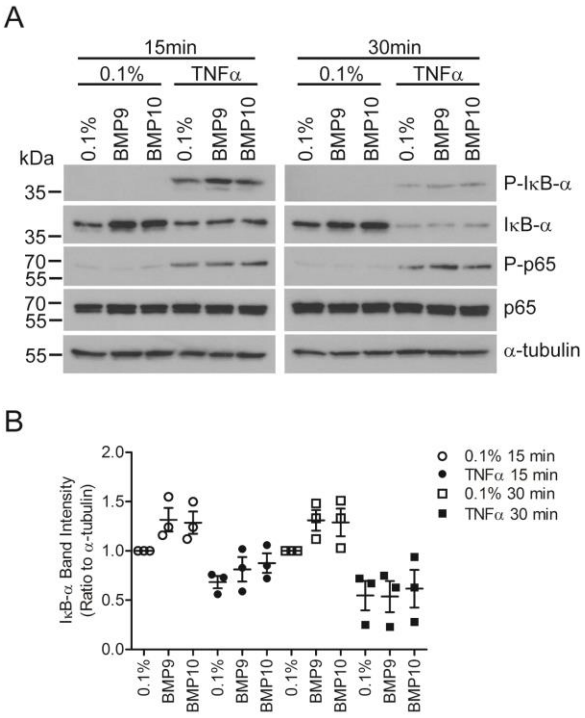


FIGURE 8



Bone morphogenetic protein (BMP) 9 and BMP10 enhance tumor necrosis factor-α -induced monocyte recruitment to the vascular endothelium mainly via activin receptor-like kinase 2.

Claudia-Gabriela Mitrofan, Sarah L Appleby, Gerard B Nash, Ziad Mallat, Edwin R Chilvers, Paul D. Upton and Nicholas W. Morrell

J. Biol. Chem. published online June 23, 2017

Access the most updated version of this article at doi: 10.1074/jbc.M117.778506