

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 (TNF α) to increase recruitment to the vascular endothelium. The BMP9/BMP10-mediated increase in monocyte recruitment in the presence of TNF α 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

TNF α was inhibited by LDN193189, which inhibits ALK2 but not ALK1. Furthermore, LDN193189 inhibited monocyte recruitment induced by TNF α and BMP9/10. BMP9/10 increased basal I κ B- α protein expression, but did not alter p65/RelA levels. Our findings suggest that higher concentrations of BMP9/BMP10 synergise with TNF α 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 (TNF α) and interleukin-1 β 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 TGF β 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 β 1-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 TNF α -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 TNF α -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 TNF α , then assessed monocyte recruitment. Negligible monocyte recruitment was observed in HAECs treated with BMP9 (Figure 1A,B) or BMP10 (Figure 1A,C) alone. Whilst TNF α 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 TNF α -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 TNF α -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 TNF α -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 TNF α (Figure 1F,G). Taken together, these data show that both BMP9 and BMP10 synergise with

TNF α 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 TNF α -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 TNF α -stimulated HAECs. In accordance with previous studies (47-50), TNF α 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 TNF α exerted a weak induction (Figure 2G). However, pre-treatment with either BMP9 or BMP10 prior to TNF α -stimulation accentuated *BMP2* expression in HAECs (Figure 2G). *BMP4* was slightly repressed by BMP9, BMP10 and TNF α , 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 TNF α 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 TNF α -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 TNF α -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 TNF α 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 TNF α 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 TNF α , 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 TNF α 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 TNF α -stimulated HAECs.

To explore further the role of type I receptors in the BMP9/BMP10-induced expression of adhesion molecules in TNF α -stimulated HAECs, we employed LDN193189, a cell permeable small molecule inhibitor of BMP type I receptors. LDN193189 inhibits *ALK2* with an IC₅₀ of 5nM, and *ALK3* with an IC₅₀ 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 TNF α -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). TNF α 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 TNF α -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 TNF α -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 TNF α -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 TNF α 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 TNF α -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 TNF α -stimulated HAECs.

BMP9 and BMP10 increase I κ B- α protein levels, but do not alter p65/RelA levels or phosphorylation. TNF α is known to mediate the expression of cell surface adhesion receptors via the NF- κ B pathway (10). We examined whether BMP9 or BMP10 mediated changes in the levels or phosphorylation of the canonical signalling proteins, I κ B- α and p65. Both BMP9 and BMP10 increased basal I κ B- α protein levels (Fig 8A,B),

without any impact on I κ B- α phosphorylation or on levels or Serine-536 phosphorylation of p65. These data suggest that BMP9/10 prime endothelial cells for TNF α responsiveness by increasing I κ B- 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 TNF α 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, ACTR-IIA, and the downstream signaling intermediaries, Smad1/5.

The potentiation of TNF α -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 TNF α -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 TNF α -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 TNF α -treated HAECs is largely independent of ALK1. Our findings also show that BMP9 and BMP10 synergise with TNF α 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 TNF α 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- κ B-dependent manner (59,60). In our current study neither TNF α , 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 TNF α 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 TNF α .

Chronic TNF α exposure reduces *BMPR2* expression in endothelial cells and can alter BMP signalling (59). Even in this acute study, low concentrations of TNF α 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 TNF α -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 TNF α response in the presence of BMP9/10 might be due to their effect on the NF- κ B pathway, the main pathway known to induce endothelial adhesion molecules (10). We identified that BMP9/10 increased I κ B- α protein levels, but that the rate of I κ B- 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- κ B family members are activated by phosphorylation at several Serine residues, so the I κ B- 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 TNF α -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 ≥ 1.5 ng/ml) had no impact on monocyte recruitment in a flow adhesion assay. However, at concentrations ≥ 1.5 ng/ml BMP9 and BMP10 behave in a near identical manner to synergise with TNF α to upregulate BMP2 expression and to enhance monocyte adhesion and transmigration in HAECs predominantly through ALK2, BMPR-II/ACTRIIA 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 TNF α (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 Ca²⁺, Mg²⁺ (Sigma-Aldrich), albumin bovine fraction (BSA) V solution 7.5% (Sigma-Aldrich), MACS separation system (Miltenyi Biotec), LS columns (Miltenyi Biotec) and CD14 microbeads (Miltenyi Biotec). siRNA transfection reagents: DharmaFECT1™ (Dharmacon), ON-TARGETplus™ siRNA Pools (Dharmacon), namely siALK1, siALK2, siSMAD1, siSMAD2, siSMAD3, siSMAD5, siBMPR2, siACVR2A, siACVR2B 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: I κ B- α mouse mAb, phospho-Ser32/36-I κ B- 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-ACVRL1_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% CO₂ 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 TNF α (0.05 ng/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 1×10^6 cells/ml in 0.15% BSA in PBS (with Ca²⁺ and Mg²⁺).

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 1×10^6 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 $\Delta\Delta C_t$ 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 TNF α (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 \pm 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.

REFERENCES

1. Rajendran, P., Rengarajan, T., Thangavel, J., Nishigaki, Y., Sakthisekaran, D., Sethi, G., and Nishigaki, I. (2013) The Vascular Endothelium and Human Diseases. *International journal of biological sciences* **9**, 1057-1069
2. Cybulsky, M. I., and Gimbrone, M. A. (1991) Endothelial Expression of a Mononuclear Leukocyte Adhesion Molecule during Atherogenesis. *Science* **251**, 788-791
3. Poole, J. C. F., and Florey, H. W. (1958) Changes in the Endothelium of the Aorta and the Behaviour of Macrophages in Experimental Atheroma of Rabbits. *J Pathol Bacteriol* **75**, 245-251
4. Esper, R. J., Nordaby, R. A., Vilarino, J. O., Paragano, A., Cacharron, J. L., and Machado, R. A. (2006) Endothelial dysfunction: a comprehensive appraisal. *Cardiovasc Diabetol* **5**
5. Libby, P. (2002) Inflammation in atherosclerosis. *Nature* **420**, 868-874
6. Ross, R. (1999) Atherosclerosis - an inflammatory disease. *N.Engl.J.Med.* **340**, 115-126
7. Matsumori, A., Yamada, T., Suzuki, H., Matoba, Y., and Sasayama, S. (1994) Increased Circulating Cytokines in Patients with Myocarditis and Cardiomyopathy. *Brit Heart J* **72**, 561-566
8. Stolpe van de, A., and Saag van der, P. T. (1996) Intercellular adhesion molecule-1. *J Mol Med-Jmm* **74**, 13-33
9. Gerhard, T., and Ley, K. (2015) Monocyte trafficking across the vessel wall. *Cardiovascular research* **107**, 321-330
10. Sprague, A. H., and Khalil, R. A. (2009) Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochemical pharmacology* **78**, 539-552
11. Yung, L. M., Sanchez-Duffhues, G., ten Dijke, P., and Yu, P. B. (2015) Bone morphogenetic protein 6 and oxidized low-density lipoprotein synergistically recruit osteogenic differentiation in endothelial cells. *Cardiovascular research* **108**, 278-287
12. Csiszar, A., Ahmad, M., Smith, K. E., Labinskyy, N., Gao, Q., Kaley, G., Edwards, J. G., Wolin, M. S., and Ungvari, Z. (2006) Bone morphogenetic protein-2 induces proinflammatory endothelial phenotype. *Am.J.Pathol.* **168**, 629-638
13. Csiszar, A., Labinskyy, N., Jo, H. J., Ballabh, P., and Ungvari, Z. (2008) Differential proinflammatory and prooxidant effects of bone morphogenetic protein-4 in coronary and pulmonary arterial endothelial cells. *Am J Physiol-Heart C* **295**, H569-H577
14. Li, X., Yang, H. Y., and Giachelli, C. M. (2008) BMP-2 promotes phosphate uptake, phenotypic modulation, and calcification of human vascular smooth muscle cells. *Atherosclerosis* **199**, 271-277
15. Nakagawa, Y., Ikeda, K., Akakabe, Y., Koide, M., Uraoka, M., Yutaka, K., Kurimoto-Nakano, R., Takahashi, T., Matoba, S., Yamada, H., Okigaki, M., and Matsubara, H. (2010) Paracrine Osteogenic Signals via Bone Morphogenetic Protein-2 Accelerate the Atherosclerotic Intimal Calcification In Vivo. *Arterioscl Throm Vas* **30**, 1908-U1986
16. Zhang, M., Sara, J. D., Wang, F. L., Liu, L. P., Su, L. X., Zhe, J., Wu, X., and Liu, J. H. (2015) Increased plasma BMP-2 levels are associated with atherosclerosis burden and coronary calcification in type 2 diabetic patients. *Cardiovasc Diabetol* **14**
17. Buendia, P., de Oca, A., Madueno, J. A., Merino, A., Martin-Malo, A., Aljama, P., Ramirez, R., Rodriguez, M., and Carracedo, J. (2015) Endothelial microparticles mediate inflammation-induced vascular calcification. *Faseb Journal* **29**, 173-181

18. David, L., Mallet, C., Keramidas, M., Lamande, N., Gasc, J. M., Dupuis-Girod, S., Plauchu, H., Feige, J. J., and Bailly, S. (2008) Bone Morphogenetic Protein-9 Is a Circulating Vascular Quiescence Factor. *Circ.Res.* **102**, 914-922
19. Herrera, B., Dooley, S., and Breitkopf-Heinlein, K. (2014) Potential Roles of Bone Morphogenetic Protein (BMP)-9 in Human Liver Diseases. *Int J Mol Sci* **15**, 5199-5220
20. Long, L., Ormiston, M. L., Yang, X., Southwood, M., Graf, S., Machado, R. D., Mueller, M., Kinzel, B., Yung, L. M., Wilkinson, J. M., Moore, S. D., Drake, K. M., Aldred, M. A., Yu, P. B., Upton, P. D., and Morrell, N. W. (2015) Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension. *Nature medicine* **21**, 777-785
21. Ricard, N., Ciais, D., Levet, S., Subileau, M., Mallet, C., Zimmers, T. A., Lee, S. J., Bidart, M., Feige, J. J., and Bailly, S. (2012) BMP9 and BMP10 are critical for postnatal retinal vascular remodeling. *Blood* **119**, 6162-6171
22. Yoshimatsu, Y., Lee, Y. G., Akatsu, Y., Taguchi, L., Suzuki, H. I., Cunha, S. I., Maruyama, K., Suzuki, Y., Yamazaki, T., Katsura, A., Oh, S. P., Zimmers, T. A., Lee, S. J., Pietras, K., Koh, G. Y., Miyazono, K., and Watabe, T. (2013) Bone morphogenetic protein-9 inhibits lymphatic vessel formation via activin receptor-like kinase 1 during development and cancer progression. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 18940-18945
23. Levet, S., Ciais, D., Merdzhanova, G., Mallet, C., Zimmers, T. A., Lee, S. J., Navarro, F. P., Texier, I., Feige, J. J., Bailly, S., and Vittet, D. (2013) Bone morphogenetic protein 9 (BMP9) controls lymphatic vessel maturation and valve formation. *Blood* **122**, 598-607
24. David, L., Mallet, C., Mazerbourg, S., Feige, J. J., and Bailly, S. (2007) Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood* **109**, 1953-1961
25. Laux, D. W., Young, S., Donovan, J. P., Mansfield, C. J., Upton, P. D., and Roman, B. L. (2013) Circulating Bmp10 acts through endothelial Alk1 to mediate flow-dependent arterial quiescence. *Development* **140**, 3403-3412
26. Heldin, C. H., Miyazono, K., and P., t. D. (1997) TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390**, 465-471
27. Scharpfenecker, M., van, D. M., Liu, Z., van Bezooijen, R. L., Zhao, Q., Pukac, L., Lowik, C. W., and P., t. D. (2007) BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. *J.Cell Sci.* **120**, 964-972
28. Upton, P. D., Davies, R. J., Trembath, R. C., and Morrell, N. W. (2009) Bone Morphogenetic Protein (BMP) and Activin Type II Receptors Balance BMP9 Signals Mediated by Activin Receptor-like Kinase-1 in Human Pulmonary Artery Endothelial Cells. *J.Biol.Chem.* **284**, 15794-15804
29. Johnson, D. W., Berg, J. N., Baldwin, M. A., Gallione, C. J., Marondel, I., Yoon, S. J., Stenzel, T. T., Speer, M., Pericak-Vance, M. A., Diamond, A., Gutmacher, A. E., Jackson, C. E., Attisano, L., Kucherlapati, R., Porteous, M. E., and Marchuk, D. A. (1996) Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. *Nat.Genet.* **13**, 189-195
30. McAllister, K. A., Grogg, K. M., Johnson, D. W., Gallione, C. J., Baldwin, M. A., Jackson, C. E., Helmbold, E. A., Markel, D. S., McKinnon, W. C., and Murrell, J. (1994) Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat.Genet.* **8**, 345-351
31. Wooderchak-Donahue, W. L., McDonald, J., O'Fallon, B., Upton, P. D., Li, W., Roman, B. L., Young, S., Plant, P., Fulop, G. T., Langa, C., Morrell, N. W., Botella, L. M., Bernabeu, C., Stevenson, D. A., Runo, J. R., and Bayrak-Toydemir, P. (2013) BMP9 Mutations Cause a Vascular-Anomaly Syndrome with Phenotypic Overlap with Hereditary Hemorrhagic Telangiectasia. *Am.J.Hum.Genet.* **93**, 530-537

32. Lane, K. B., Machado, R. D., Pauciulo, M. W., Thomson, J. R., Phillips, J. A., III, Loyd, J. E., Nichols, W. C., and Trembath, R. C. (2000) Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. *Nat.Genet.* **26**, 81-84
33. Deng, Z., Morse, J. H., Slager, S. L., Cuervo, N., Moore, K. J., Venetos, G., Kalachikov, S., Cayanis, E., Fischer, S. G., Barst, R. J., Hodge, S. E., and Knowles, J. A. (2000) Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am.J.Hum.Genet.* **67**, 737-744
34. Harrison, R. E., Flanagan, J. A., Sankelo, M., Abdalla, S. A., Rowell, J., Machado, R. D., Elliott, C. G., Robbins, I. M., Olschewski, H., McLaughlin, V., Gruenig, E., Kermeen, F., Halme, M., Raisanen-Sokolowski, A., Laitinen, T., Morrell, N. W., and Trembath, R. C. (2003) Molecular and functional analysis identifies ALK-1 as the predominant cause of pulmonary hypertension related to hereditary haemorrhagic telangiectasia. *J.Med.Genet.* **40**, 865-871
35. Kim, C. W., Song, H., Kumar, S., Nam, D., Kwon, H. S., Chang, K. H., Son, D. J., Kang, D. W., Brodie, S. A., Weiss, D., Vega, J. D., Alberts-Grill, N., Griendling, K., Taylor, W. R., and Jo, H. (2013) Anti-inflammatory and antiatherogenic role of BMP receptor II in endothelial cells. *Arteriosclerosis, thrombosis, and vascular biology* **33**, 1350-1359
36. Katagiri, T., Imada, M., Yanai, T., Suda, T., Takahashi, N., and Kamijo, R. (2002) Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. *Genes Cells* **7**, 949-960
37. Appleby, S. L., Mitrofan, C. G., Crosby, A., Hoenderdos, K., Lodge, K., Upton, P. D., Yates, C. M., Nash, G. B., Chilvers, E. R., and Morrell, N. W. (2016) Bone Morphogenetic Protein 9 Enhances Lipopolysaccharide-Induced Leukocyte Recruitment to the Vascular Endothelium. *Journal of immunology* **197**, 3302-3314
38. Burton, V. J., Ciucan, L. I., Holmes, A. M., Rodman, D. M., Walker, C., and Budd, D. C. (2011) Bone morphogenetic protein receptor II regulates pulmonary artery endothelial cell barrier function. *Blood* **117**, 333-341
39. Burton, V. J., Holmes, A. M., Ciucan, L. I., Robinson, A., Roger, J. S., Jarai, G., Pearce, A. C., and Budd, D. C. (2011) Attenuation of leukocyte recruitment via CXCR1/2 inhibition stops the progression of PAH in mice with genetic ablation of endothelial BMPR-II. *Blood* **118**, 4750-4758
40. Rossi, E., Sanz-Rodriguez, F., Eleno, N., Duwell, A., Blanco, F. J., Langa, C., Botella, L. M., Cabanas, C., Lopez-Novoa, J. M., and Bernabeu, C. (2013) Endothelial endoglin is involved in inflammation: role in leukocyte adhesion and transmigration. *Blood* **121**, 403-415
41. Tacke, F., Alvarez, D., Kaplan, T. J., Jakubzick, C., Spanbroek, R., Llodra, J., Garin, A., Liu, J. H., Mack, M., van Rooijen, N., Lira, S. A., Habenicht, A. J., and Randolph, G. J. (2007) Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *Journal of Clinical Investigation* **117**, 185-194
42. Hansson, G. K., and Hermansson, A. (2011) The immune system in atherosclerosis. *Nature immunology* **12**, 204-212
43. Herrera, B., and Inman, G. J. (2009) A rapid and sensitive bioassay for the simultaneous measurement of multiple bone morphogenetic proteins. Identification and quantification of BMP4, BMP6 and BMP9 in bovine and human serum. *BMC.Cell Biol.* **10**, 20
44. Venkatesh, D., Hernandez, T., Rosetti, F., Batal, I., Cullere, X., Luscinskas, F. W., Zhang, Y. Z., Stavrakis, G., Garcia-Cardena, G., Horwitz, B. H., and Mayadas, T. N. (2013) Endothelial TNF Receptor 2 Induces IRF1 Transcription Factor-Dependent Interferon-beta Autocrine Signaling to Promote Monocyte Recruitment. *Immunity* **38**, 1025-1037
45. Gerszten, R. E., Garcia-Zepeda, E. A., Lim, Y. C., Yoshida, M., Ding, H. A., Gimbrone, M. A., Luster, A. D., Luscinskas, F. W., and Rosenzweig, A. (1999) MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature* **398**, 718-723
46. Toshner, M., Dunmore, B. J., McKinney, E. F., Southwood, M., Caruso, P., Upton, P. D., Waters, J. P., Ormiston, M. L., Skepper, J. N., Nash, G., Rana, A. A., and Morrell, N. W. (2014)

- Transcript analysis reveals a specific HOX signature associated with positional identity of human endothelial cells. *PLoS one* **9**, e91334
47. Wolle, J., Hill, R. R., Ferguson, E., Devall, L. J., Trivedi, B. K., Newton, R. S., and Saxena, U. (1996) Selective inhibition of tumor necrosis factor-induced vascular cell adhesion molecule-1 gene expression by a novel flavonoid - Lack of effect on transcription factor NF-kappa B. *Arterioscl Throm Vas* **16**, 1501-1508
 48. D'Alessio, P., Moutet, M., Coudrier, E., Darquenne, S., and Chaudiere, J. (1998) ICAM-1 and VCAM-1 expression induced by TNF-alpha are inhibited by a glutathione peroxidase mimic. *Free Radical Bio Med* **24**, 979-987
 49. Jiang, J., Fu, W. P., Wang, X. W., Lin, P. H., Yao, Q. Z., and Chen, C. Y. (2010) HIV gp120 induces endothelial dysfunction in tumour necrosis factor-alpha-activated porcine and human endothelial cells. *Cardiovascular research* **87**, 366-374
 50. Zhang, F., Yu, W., Hargrove, J. L., Greenspan, P., Dean, R. G., Taylor, E. W., and Hartle, D. K. (2002) Inhibition of TNF-alpha induced ICAM-1, VCAM-1 and E-selectin expression by selenium. *Atherosclerosis* **161**, 381-386
 51. Fujii, M., Takeda, K., Imamura, T., Aoki, H., Sampath, T. K., Enomoto, S., Kawabata, M., Kato, M., Ichijo, H., and Miyazono, K. (1999) Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. *Mol.Biol.Cell* **10**, 3801-3813
 52. Cuny, G. D., Yu, P. B., Laha, J. K., Xing, X., Liu, J. F., Lai, C. S., Deng, D. Y., Sachidanandan, C., Bloch, K. D., and Peterson, R. T. (2008) Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. *Bioorg.Med.Chem.Lett.* **18**, 4388-4392
 53. Horbelt, D., Boergemann, J. H., Chaikuad, A., Alfano, I., Williams, E., Lukonin, I., Timmel, T., Bullock, A. N., and Knaus, P. (2014) Small Molecules Dorsomorphin and LDN-193189 Inhibit Myostatin/GDF8 Signaling and Promote Functional Myoblast Differentiation. *The Journal of biological chemistry* **290**, 3390-3404
 54. Wang, R. N., Green, J., Wang, Z., Deng, Y., Qiao, M., Peabody, M., Zhang, Q., Ye, J., Yan, Z., Denduluri, S., Idowu, O., Li, M., Shen, C., Hu, A., Haydon, R. C., Kang, R., Mok, J., Lee, M. J., Luu, H. L., and Shi, L. L. (2014) Bone morphogenetic protein (BMP) signaling in development and human diseases. *Genes Dis* **1**, 87-105
 55. Holtzhausen, A., Golzio, C., How, T., Lee, Y. H., Schiemann, W. P., Katsanis, N., and Blobe, G. C. (2014) Novel bone morphogenetic protein signaling through Smad2 and Smad3 to regulate cancer progression and development. *Faseb Journal* **28**, 1248-1267
 56. Yao, Y., Shao, E. S., Jumabay, M., Shahbazian, A., Ji, S., and Bostrom, K. I. (2008) High-density lipoproteins affect endothelial BMP-signaling by modulating expression of the activin-like kinase receptor 1 and 2. *Arterioscler.Thromb.Vasc.Biol.* **28**, 2266-2274
 57. Kraehling, J. R., Chidlow, J. H., Rajagopal, C., Sugiyama, M. G., Fowler, J. W., Lee, M. Y., Zhang, X. B., Ramirez, C. M., Park, E. J., Tao, B., Chen, K. Y., Kuruvilla, L., Larrivee, B., Foltz-Stogniew, E., Ola, R., Rotllan, N., Zhou, W. P., Nagle, M. W., Herz, J., Williams, K. J., Eichmann, A., Lee, W. L., Fernandez-Hernando, C., and Sessa, W. C. (2016) Genome-wide RNAi screen reveals ALK1 mediates LDL uptake and transcytosis in endothelial cells. *Nature communications* **7**
 58. Pachori, A. S., Custer, L., Hansen, D., Clapp, S., Kemppa, E., and Klingensmith, J. (2010) Bone morphogenetic protein 4 mediates myocardial ischemic injury through JNK-dependent signaling pathway. *Journal of molecular and cellular cardiology* **48**, 1255-1265
 59. Hurst, L. A., Dunmore, B. J., Long, L., Crosby, A., Al-Lamki, R., Deighton, J., Southwood, M., Yang, X. D., Nikolic, M. Z., Herrera, B., Inman, G. J., Bradley, J. R., Rana, A. A., Upton, P. D., and Morrell, N. W. (2017) TNF alpha drives pulmonary arterial hypertension by suppressing the BMP type-II receptor and altering NOTCH signalling. *Nature communications* **8**
 60. Csiszar, A., Smith, K. E., Koller, A., Kaley, G., Edwards, J. G., and Ungvari, Z. (2005) Regulation of bone morphogenetic protein-2 expression in endothelial cells: role of nuclear

- factor-kappaB activation by tumor necrosis factor-alpha, H2O2, and high intravascular pressure. *Circulation* **111**, 2364-2372
61. Christian, F., Smith, E. L., and Carmody, R. J. (2016) The regulation of NF-κB subunits by phosphorylation. *Cells* **5**
 62. Nurgazieva, D., Mickley, A., Moganti, K., Ming, W., Ovsyi, I., Popova, A., Sachindra, Awad, K., Wang, N., Bieback, K., Goerd, S., Kzhyshkowska, J., and Gratchev, A. (2015) TGF-beta 1, but Not Bone Morphogenetic Proteins, Activates Smad1/5 Pathway in Primary Human Macrophages and Induces Expression of Proatherogenic Genes. *Journal of immunology* **194**, 709-718
 63. Derwall, M., Malhotra, R., Lai, C. S., Beppu, Y., Aikawa, E., Seehra, J. S., Zapol, W. M., Bloch, K. D., and Yu, P. B. (2012) Inhibition of Bone Morphogenetic Protein Signaling Reduces Vascular Calcification and Atherosclerosis. *Arterioscl Throm Vas* **32**, 613-U168
 64. Saeed, O., Otsuka, F., Polavarapu, R., Karmali, V., Weiss, D., Davis, T., Rostad, B., Pachura, K., Adams, L., Elliott, J., Taylor, W. R., Narula, J., Kolodgie, F., Virmani, R., Hong, C. C., and Finn, A. V. (2012) Pharmacological Suppression of Hepcidin Increases Macrophage Cholesterol Efflux and Reduces Foam Cell Formation and Atherosclerosis. *Arterioscl Throm Vas* **32**, 299-U297
 65. Levet, S., Ouarne, M., Ciais, D., Coutton, C., Subileau, M., Mallet, C., Ricard, N., Bidart, M., Debillon, T., Faravelli, F., Rooryck, C., Feige, J. J., Tillet, E., and Bailly, S. (2015) BMP9 and BMP10 are necessary for proper closure of the ductus arteriosus. *Proceedings of the National Academy of Sciences of the United States of America* **112**, E3207-E3215
 66. Chen, H., Ridgway, J. B., Sai, T., Lai, J., Warming, S., Chen, H. Y., Roose-Girma, M., Zhang, G., Shou, W., and Yan, M. H. (2013) Context-dependent signaling defines roles of BMP9 and BMP10 in embryonic and postnatal development. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 11887-11892
 67. Butler, L. M., Jeffery, H. C., Wheat, R. L., Rae, P. C., Townsend, K., Alkharsah, K. R., Schulz, T. F., Nash, G. B., and Blackburn, D. J. (2011) Kaposi's Sarcoma-Associated Herpesvirus Infection of Endothelial Cells Inhibits Neutrophil Recruitment through an Interleukin-6-Dependent Mechanism: a New Paradigm for Viral Immune Evasion. *Journal of virology* **85**, 7321-7332

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

TNF α -stimulated BOECs in the presence of BMP9 or BMP10. Error bars represent \pm S.E.M. *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001.

FIGURE 2. BMP9 and BMP10 increase the expression of E-selectin, VCAM-1, ICAM-1 and BMP2 in TNF α -stimulated human aortic endothelial cells (HAECs). HAECs were treated with BMP9 or BMP10 (5ng/ml, 16 h) prior to TNF α 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 \pm S.E.M. *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001.

FIGURE 3. BMP6 increases the expression of E-selectin, VCAM-1 and ICAM-1 in TNF α -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 TNF α (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 TNF α (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 TNF α (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 \pm S.E.M. *P \leq 0.05, **P \leq 0.01, ***P \leq 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 TNF α -stimulated human aortic endothelial cells (HAECs). HAECs were siRNA transfected, then treated with BMP9 or BMP10 (5ng/ml; 16 h) prior to TNF α 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 \pm S.E.M. ***P \leq 0.001.

FIGURE 5. LDN193189 reduces the BMP9- and BMP10-induced upregulation of E-selectin, VCAM-1 and ICAM-1 and monocyte recruitment in TNF α -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 TNF α 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 \pm S.E.M. *P \leq 0.05, **P \leq 0.01, ***P \leq 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 TNF α -stimulated human aortic endothelial cells (HAECs). HAECs were siRNA transfected, then treated with BMP9 or BMP10 (5ng/ml; 16 h) prior to TNF α 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 \pm S.E.M. *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001.

FIGURE 7. Smad1 and Smad5 mediate the BMP9- and BMP10-induced upregulation of E-selectin, VCAM-1 and ICAM-1 in TNF α -stimulated human aortic endothelial cells (HAECs). HAECs were siRNA transfected, then treated with BMP9 or BMP10 (5ng/ml, 16 h) prior to TNF α 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 \pm S.E.M. *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001.

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

I κ B- α (*P-I κ B- 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 I κ B- α blots, each band being expressed as a ratio of I κ B- 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, TGF β , TNF α , HAECs, NF- κ B

FIGURE 1

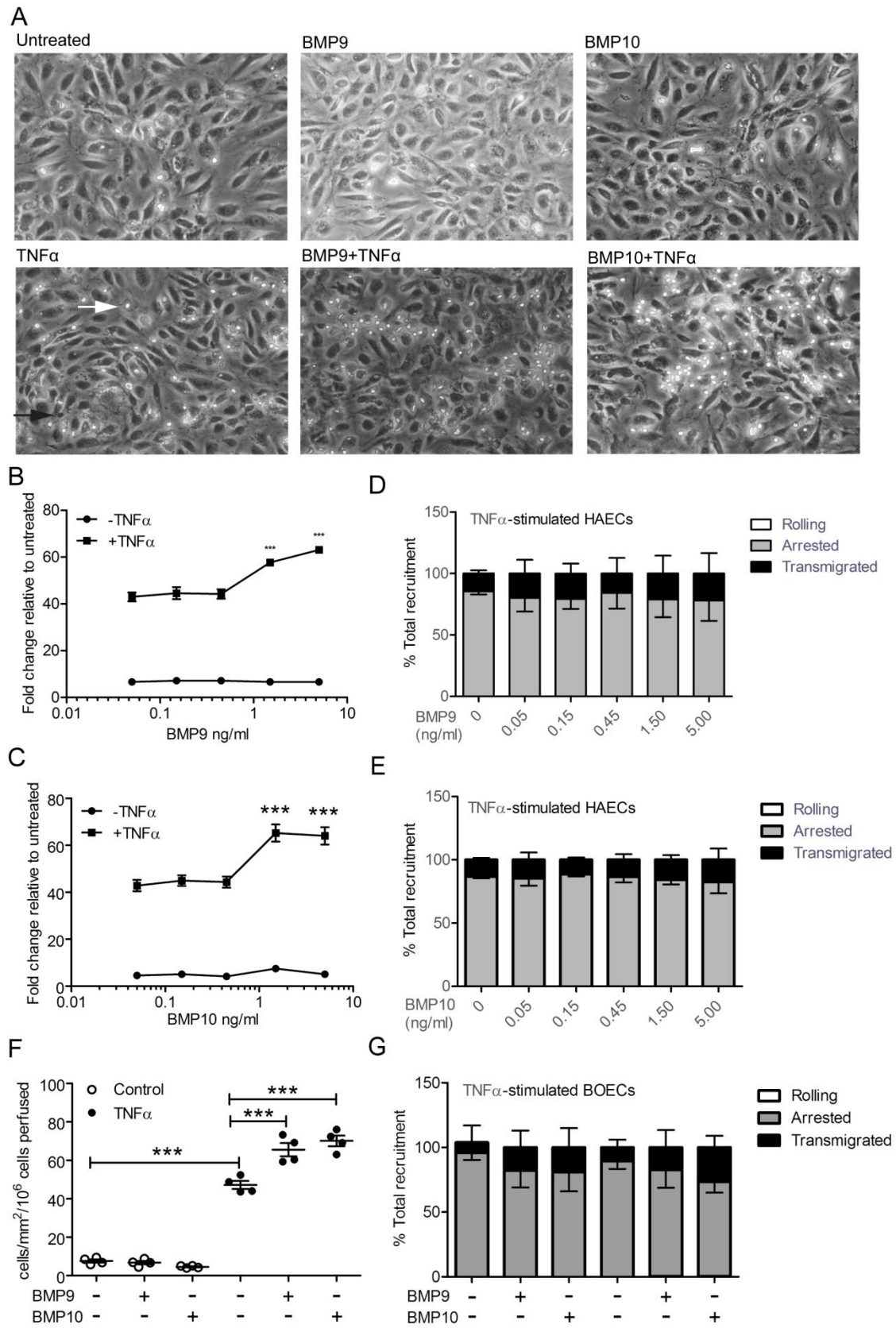
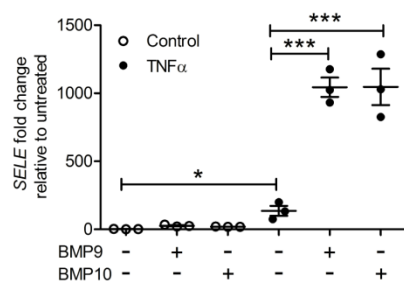
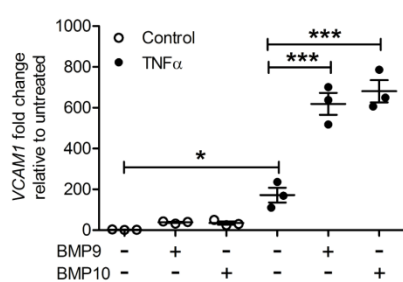


FIGURE 2

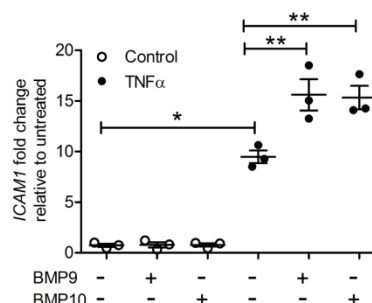
A E-selectin



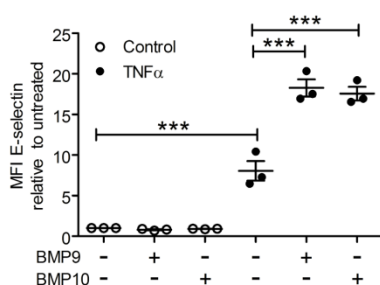
B VCAM-1



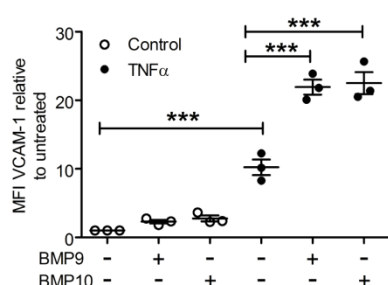
C ICAM-1



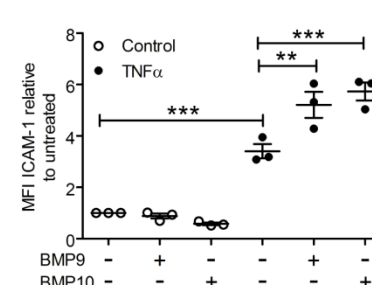
D E-selectin



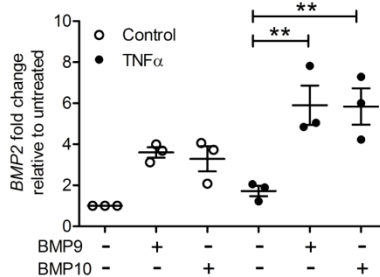
E VCAM-1



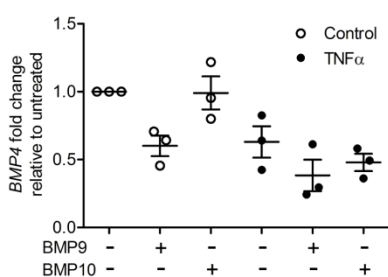
F ICAM-1



G BMP2



H BMP4



I BMP6

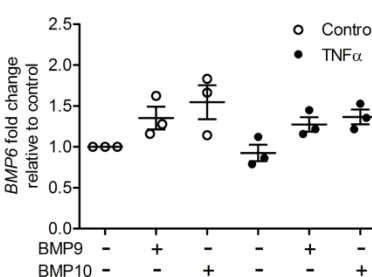


FIGURE 3

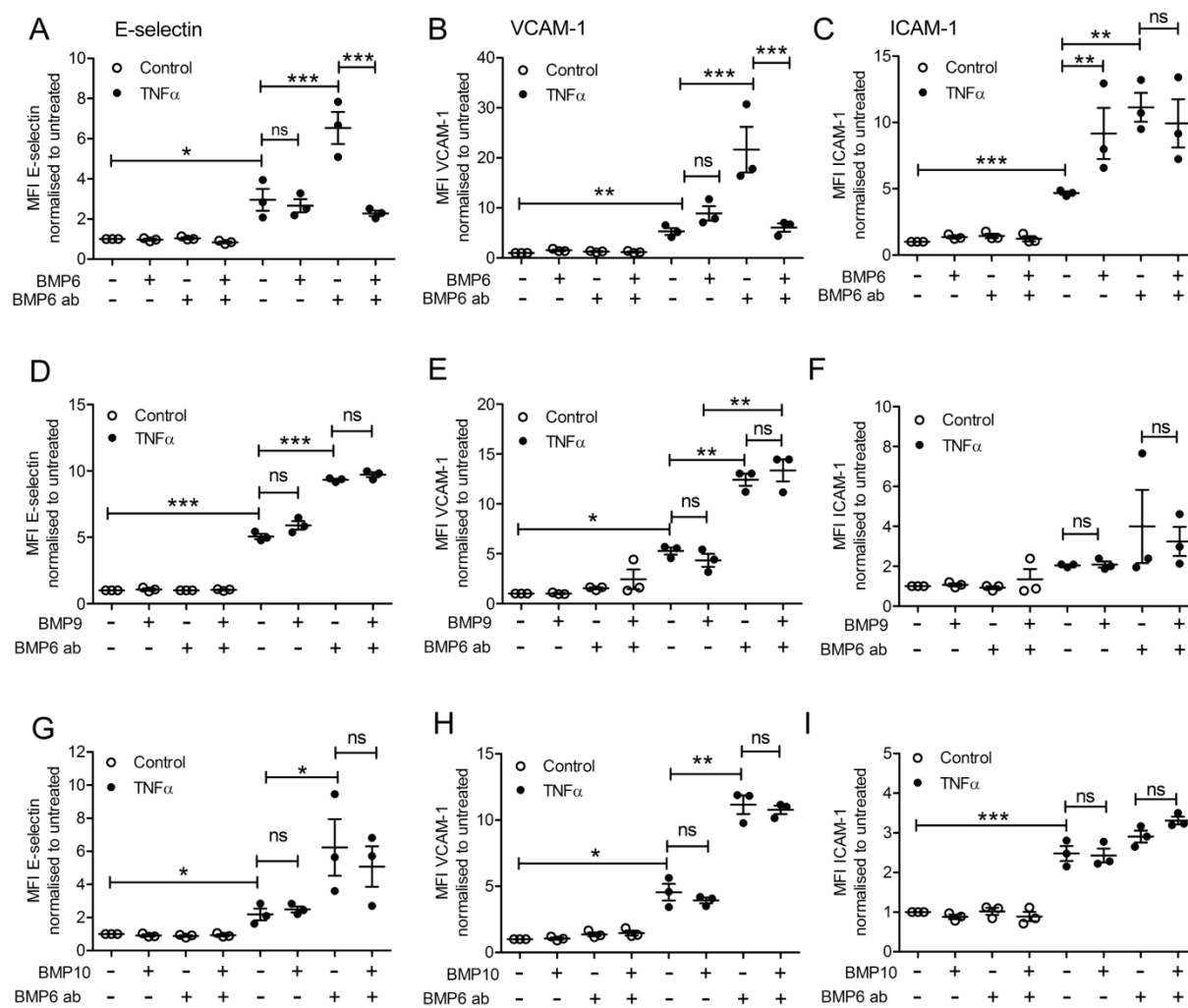


FIGURE 4

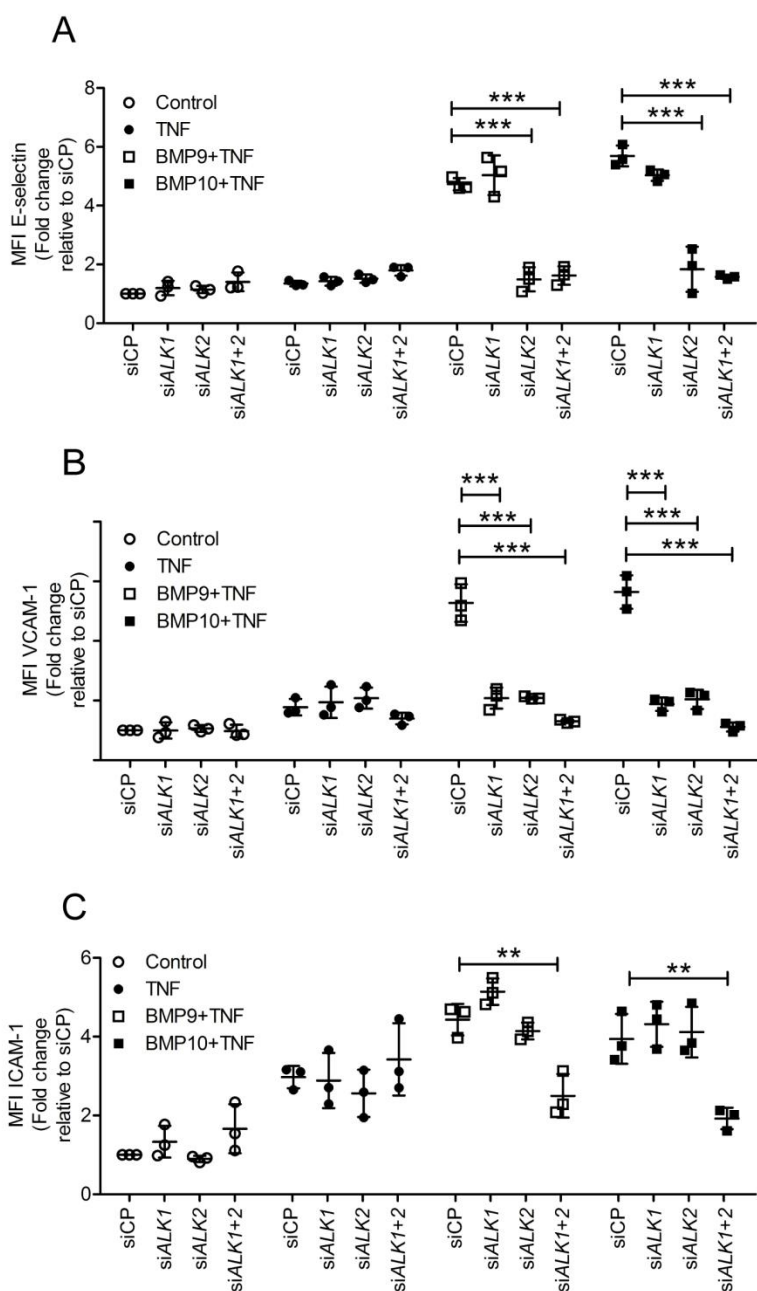


FIGURE 5

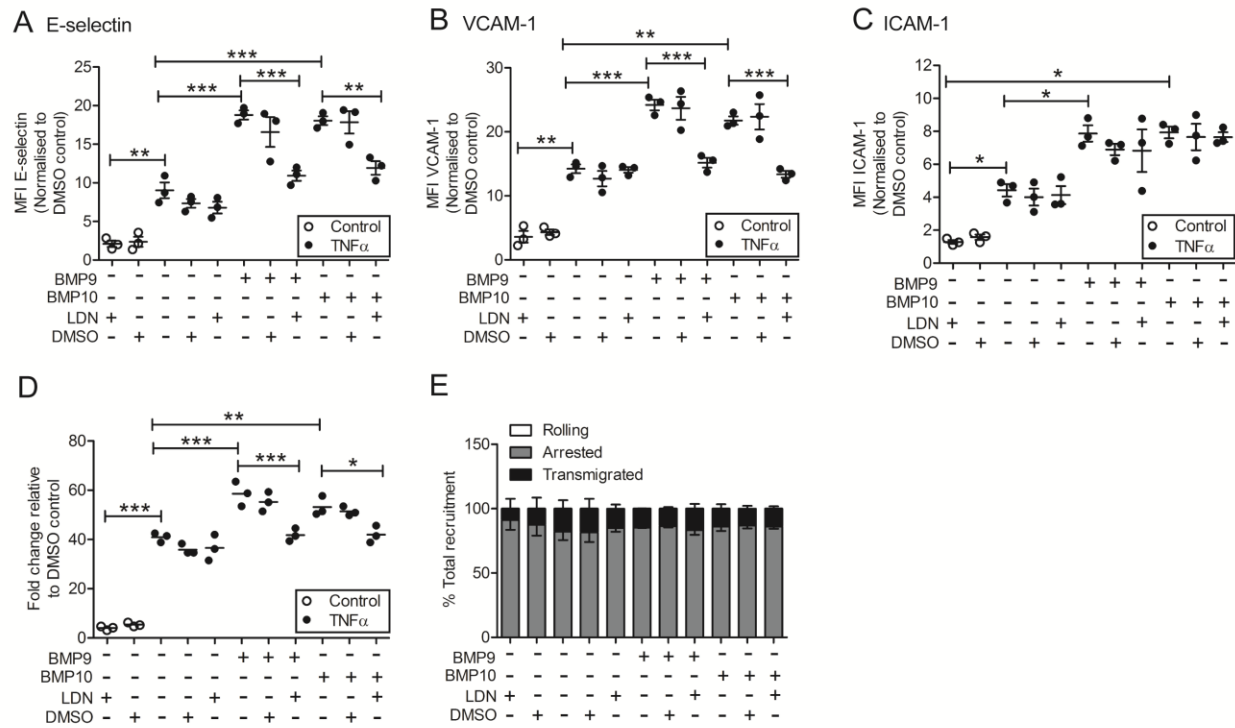


FIGURE 6

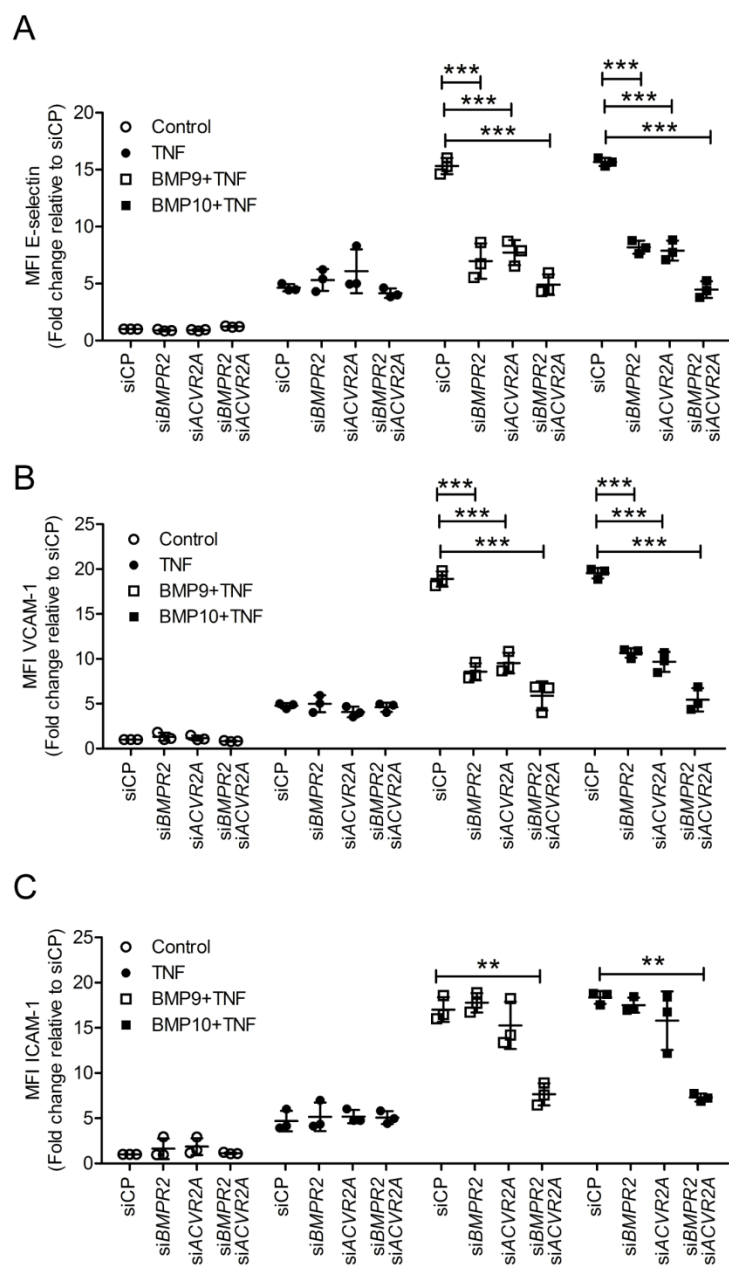


FIGURE 7

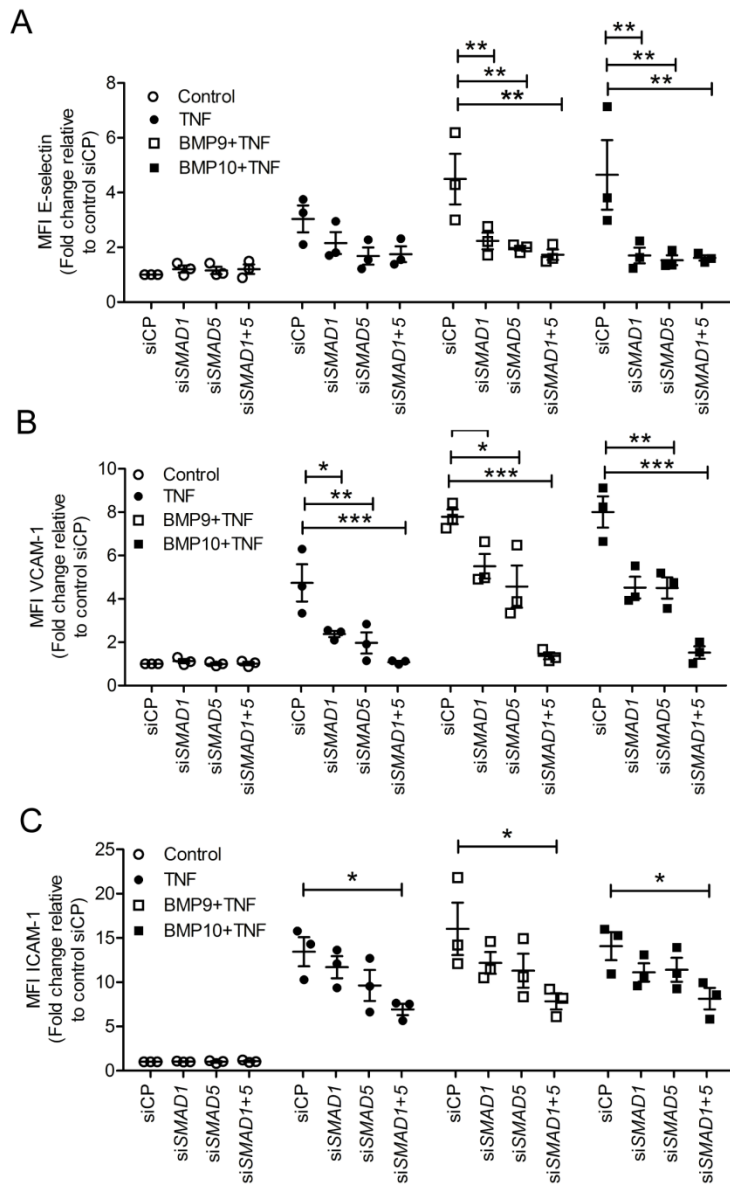
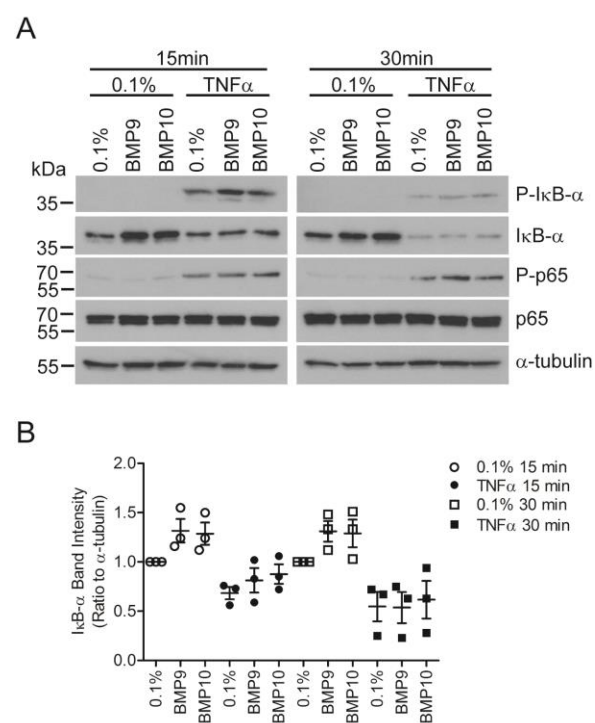


FIGURE 8



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](https://doi.org/10.1074/jbc.M117.778506)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

Supplemental material:

<http://www.jbc.org/content/suppl/2017/06/23/M117.778506.DC1>

This article cites 0 references, 0 of which can be accessed free at

<http://www.jbc.org/content/early/2017/06/23/jbc.M117.778506.full.html#ref-list-1>