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BASIC SCIENCE

A novel role for myeloid endothelin-B receptors in hypertension

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Aims Hypertension is common. Recent data suggest that macrophages (Mϕ) contribute to, and protect from, hypertension. Endothelin-1 (ET-1) is the most potent endogenous vasoconstrictor with additional pro-inflammatory properties. We investigated the role of the ET system in experimental and clinical hypertension by modifying Mϕ number and phenotype.

Methods and results *In vitro*, Mϕ ET receptor function was explored using pharmacological, gene silencing, and knockout approaches. Using the CD11b-DTR mouse and novel mice with myeloid cell-specific endothelin-B (ET_B) receptor deficiency (*LysMET*$_B^{-/-}$), we explored the effects of modifying Mϕ number and phenotype on the hypertensive effects of ET-1, angiotensin II (ANG II), a model that is ET-1 dependent, and salt. In patients with small vessel vasculitis, the impacts of Mϕ depleting and non-depleting therapies on blood pressure (BP) and endothelial function were examined. Mouse and human Mϕ expressed *both* endothelin-A and ET_B receptors and displayed chemokinesis to ET-1. However, stimulation of Mϕ with exogenous ET-1 did not polarize Mϕ phenotype. Interestingly, both mouse and human Mϕ cleared ET-1 through ET_B receptor mediated, and dynamin-dependent, endocytosis. Mϕ depletion resulted in an augmented chronic hypertensive response to both ET-1 and salt. *LysMET*$_B^{-/-}$ mice displayed an exaggerated hypertensive response to both ET-1 and ANG II. Finally, in patients who received Mϕ depleting immunotherapy BP was higher and endothelial function worse than in those receiving non-depleting therapies.

Conclusion Mϕ and ET-1 may play an important role in BP control and potentially have a critical role as a therapeutic target in hypertension.

Keywords Myeloid cell • Endothelin • Hypertension

Introduction

Arterial hypertension is a major risk factor for atherosclerosis, coronary artery disease, stroke, and chronic kidney disease, and is a prominent contributor to death worldwide.[1] It is estimated that a quarter of the world's adult population is hypertensive and this number is projected to rise to nearly 30% by 2025.[2] By age 70 years, 70% of the US population have hypertension. However, despite the

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Translational perspective

Hypertension is a costly global health problem, and an important risk factor for the development and progression of chronic kidney disease. Its aetiology remains unclear in most adults. Here, the data provided suggest that the immune and endothelin systems play important roles in blood pressure regulation and provide a rational basis for further investigation into the modulation of these pathways. These studies may encourage industry to take a lead in this relatively orphan area, potentially resulting in a more rational prescribing of endothelin receptor antagonists for hypertension that has developed as part of a multi-system inflammatory disease, with these agents potentially affording broader cardiovascular protection. These studies may also help inform the design of novel antihypertensive therapies.

frequency of hypertension, its cause in the majority of adults is unknown.

Hypertension is complex, with no single mechanism—sodium retention, renin release, and increased vascular tone—entirely explaining the blood pressure (BP) rise. The past 50 years have seen growing evidence implicating the immune system.[3] Recent data suggest that macrophages (Mϕ) contribute to, and protect from, hypertension. Early studies in the spontaneously hypertensive rat[4] found a correlation between the distribution of sub-endothelial Mϕ and endothelial function, and that treatment with an angiotensin converting enzyme inhibitor improved endothelial function and reduced the number of vascular Mϕ. Furthermore, many models of hypertension—angiotensin II (ANG II), high salt—are associated with renal accumulation of Mϕ.[5,6] Despite these and many other observational studies, few have attempted to modify Mϕ phenotype/number to examine their role in hypertension.[7–10]

Endothelin-1 (ET-1) is the most potent endogenous vasoconstrictor.[11] Its production is triggered by multiple stimuli including ANG II and pro-inflammatory cytokines.[12,13] ET-1 acts by binding to two distinct receptors, the endothelin-A (ET_A) and the endothelin-B (ET_B) receptors.[14,15] ET_A and ET_B receptors on vascular smooth muscle cell (VSMC) mediate the vasoconstrictor effects of ET-1.[16] ET_B receptors are also found on vascular endothelial cells (EC) where their activation results in dilation.[17,18] Production of vascular ET-1 is increased in some but not all animal models of hypertension.[19] Interestingly, ET antagonism can blunt BP elevation in ANG II infused rats suggesting that in this model, ET-1 largely mediates the hypertensive effects of ANG II.[20,21] Importantly, a number of clinical studies have gone on to show the effectiveness of both selective ET_A and mixed $ET_{A/B}$ antagonism in lowering BP in hypertension.[22–24]

ET-1 is considered to be pro-inflammatory. ET_A receptor activation is crucial in mediating the ANG II-induced infiltration of renal cortical T cells.[25] In both models of aldosterone-induced hypertension and diabetic nephropathy, selective ET_A receptor antagonism reduced Mϕ infiltration,[26,27] and in the diabetic model also reduced fibrosis.[27] However, the effects of ET-1 on Mϕ biology are not well studied. In the current studies, we explored the role of Mϕ and monocytes in mediating the pro-hypertensive effects of ET-1 and ANG II.

Methods

See Supplementary material online.

Results

Macrophages possess endothelin receptors but are not polarized by endothelin-1

We first found that mouse bone marrow-derived macrophages (BMDM) possess both ET_A and ET_B receptors ($ET_B > ET_A$) although in relatively lower amounts than seen in VSMC and EC, respectively (see Supplementary material online, *Figure S1*). To examine the role of the Mϕ ET_B receptor in greater detail, we generated novel mice deficient in ET_B on myeloid cells alone ($LysMET_B^{-/-}$). These mice showed no differences in baseline vascular function or circulating immune cells compared with littermate controls (see *Figure 1* and Supplementary material online, *Figures S2–S4*).

We then explored the ability of ET-1 to polarize BMDM. Increasing concentrations of ET-1 (10–10^4 pg/mL) were unable to polarize BMDM to a classical (M1) or alternative (M2) phenotype either alone or in combination (see *Figure 2* and Supplementary material online, *Figure S5*). Furthermore, neither co-stimulation with LPS *and* ET-1 nor LPS stimulation following ET-1 priming, in the presence or absence of ET receptor blockade, augmented the BMDM response to LPS (see Supplementary material online, *Figure S6*).

BMDM stimulation with LPS/INFγ (but not IL-4/IL-13) increased the media concentration of ET-1 at 24 h (see Supplementary material online, *Figure S7*), an effect that was blocked by phosphoramidon, an inhibitor of endothelin converting enzyme (ECE) which catalyzes the conversion of big ET-1 to the mature peptide. Thus, this increase in ET-1 likely represents *de novo* production.

Macrophages demonstrate chemokinesis to endothelin-1

Despite the lack of polarization by ET-1, BMDM demonstrated chemokinesis to ET-1. This effect was more apparent at higher concentrations of ET-1 and no different to MCP-1 at ET-1 10^3 pg/mL and 10^4 pg/mL. BMDM chemokinesis to ET-1 was blocked by both

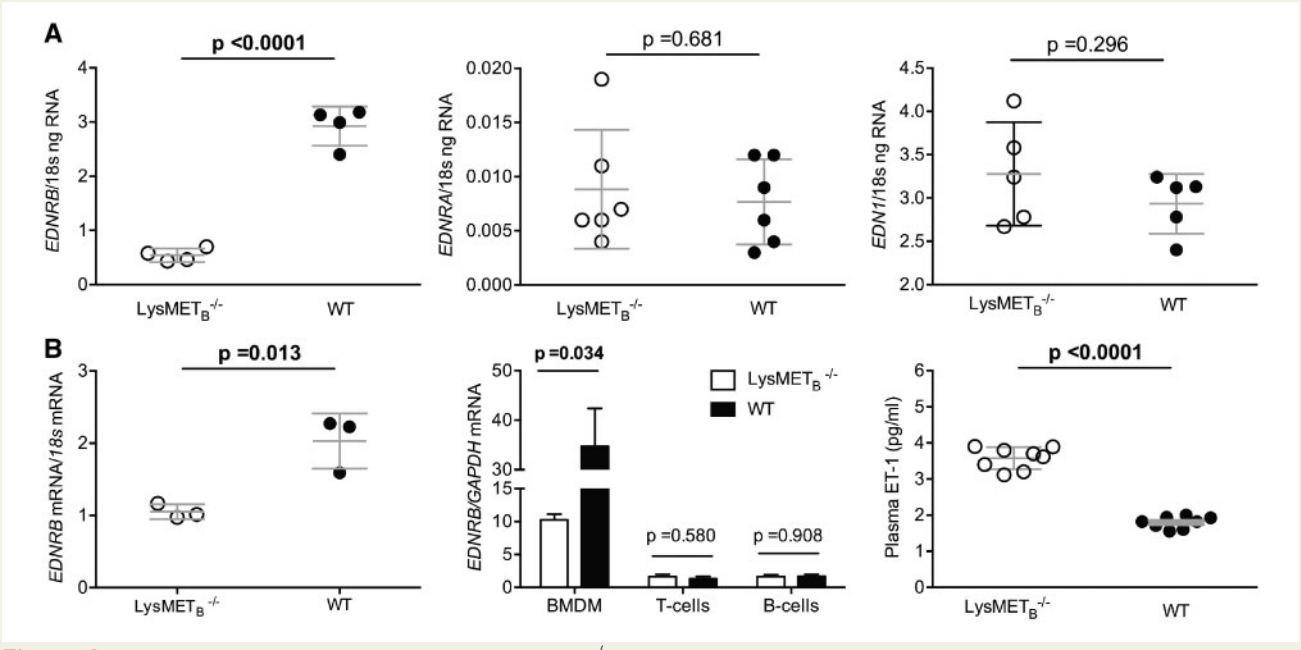

Figure 1 Characterization of the endothelin system in *LysMET$_B^{-/-}$*. (*A*) qRT-PCR analysis of *EDNRB* (endothelin-B), *EDNRA* (endothelin-A), and *EDN1* (endothelin-1) expression in bone marrow-derived Mϕ. (*B*) *EDNRB* (endothelin-B) expression in peritoneal Mϕ (left panel), bone marrow-derived Mϕ, T and B cells (middle panel), and plasma endothelin-1 (right panel). Data are expressed as mean ± standard deviation and comparisons were made with unpaired *t*-tests and for (*B*) middle panel, Holm–Sidak was used to correct for multiple comparisons; adjusted *P*-values are shown.

selective ET_A (BQ123) and selective ET_B (BQ788) receptor antagonism (*Figure 3A*). In terms of chemokinetic ability, *LysMET$_B^{-/-}$* BMDM had a blunted response to incremental concentrations of ET-1 compared with control BMDM, although the response to MCP-1 was maintained (*Figure 3B*).

Macrophages demonstrate endothelin-B-mediated endothelin-1 uptake

BMDM were then exposed to ET-1 10 pg/mL in their media. Serial assay of the media for ET-1 showed a gradual reduction over a 24 h period with the concentration at 24 h being approximately 60% that at baseline (*Figure 4A*). This reduction in media ET-1 was seen when BMDM were exposed to increasing concentrations of ET-1 (*Figure 4B*) and was not explained by degradation of the peptide over the 24 h period (see Supplementary material online, *Figure S8*). The fall in ET-1 was prevented by both selective antagonism of the ET_B receptor (but not ET_A) as well as inhibition of dynamin-dependent endocytosis (see *Figure 4C* and Supplementary material online, *Figure S9*), supporting BMDM uptake of ET-1 through ET_B receptor-mediated endocytosis. Neutral endopeptidase (NEP) inhibition had no effect. Additionally, mixed $ET_{A/B}$ receptor antagonism was no different to selective ET_B blockade alone (see Supplementary material online, *Figure S10*).

Further data to support the role of the Mϕ ET_B receptor in clearing ET-1 were provided using an ET_B gene silencing approach. Mϕ *EDNRB* knockdown again prevented ET-1 uptake by BMDM, an effect that was similar in magnitude to that seen with pharmacological ET_B receptor antagonism (*Figure 4D*). As ET_B receptors are also present on VSMC, we assessed their ability to remove ET-1 *in vitro*. VSMC did not remove ET-1 from their surrounding media (*Figure 4E*). BMDM from *LysMET$_B^{-/-}$* and controls were also exposed to ET-1 for 24 h. As previously seen, control BMDM removed ET-1 from their medium, an effect that was blocked by BQ788. Medium from *LysMET$_B^{-/-}$* BMDM showed no difference in ET-1 concentration at 24 h compared with baseline and there was no effect of pre-treatment with BQ788 (*Figure 4F*). To further support our hypothesis of Mϕ ET-1 uptake, we exposed BMDM to fluorescent ET-1. Our results suggested a statistically significant ($P < 0.01$) increase in BMDM intracellular fluorescence following treatment with ET-1 (*Figure 4G*). Uptake of fluorescence was blocked by selective blockade of the ET_B receptor and absent, as expected, in BMDM from *LysMET$_B^{-/-}$* mice. Furthermore, this uptake process occurred rapidly (*Figure 4H*) and reached a steady state within 60 min.

Macrophages modify vascular contractility

The functional importance of Mϕ ET_B receptor-mediated ET-1 uptake is demonstrated in *Figure 5*. Here, we infused Mϕ into the myography bath 15 min prior to infusing ET-1. Increasing number of Mϕ significantly attenuated the vasoconstrictor actions of ET-1 ($P < 0.001$ vs. control); this effect was lost when the Mϕ were pre-treated with selective ET_B receptor antagonism or when *LysMET$_B^{-/-}$* BMDM were infused. There was no effect of pre-treating Mϕ with a selective ET_A receptor antagonist. Mϕ pre-treatment did not alter the contractile response to KCl. Again, these findings are in keeping with a rapid binding of ET-1 to Mϕ ET_B that blunts its functional effects. Additionally, and as expected, ET-1 increased oxidative stress in mesenteric vessels; this effect was attenuated by Mϕ and attenuation was dependent on an unblocked ET_B receptor.

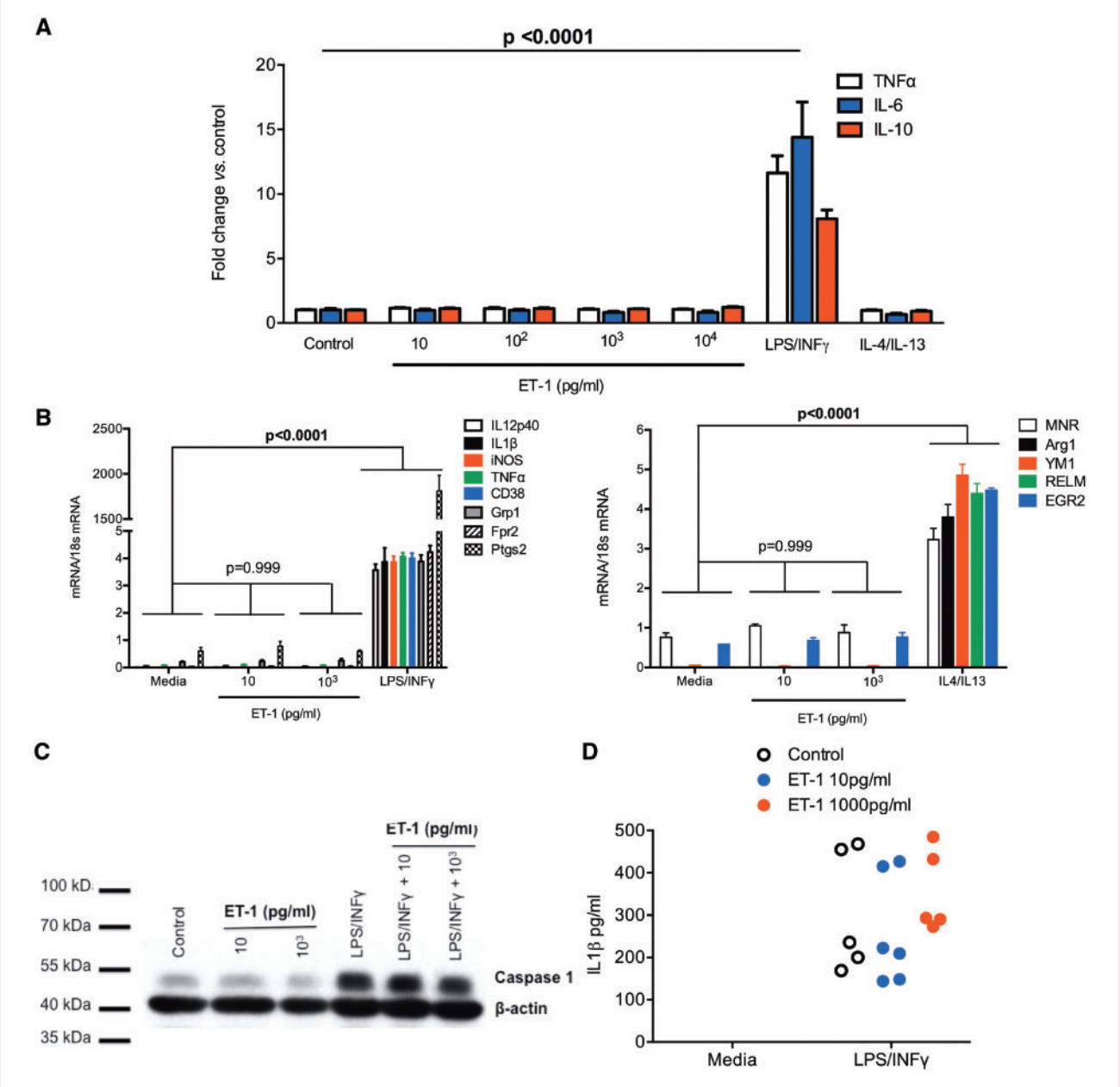

Figure 2 *In vitro* stimulation with endothelin-1 does not polarize Mϕ to a classical or alternative phenotype. (*A*) Bone marrow-derived Mϕ production of TNFα, IL-6, and IL-10 after 24 h stimulation with endothelin-1 (10–10^4 pg/mL), LPS/INFγ, IL-4/IL-13, and in untreated controls. Data (mean ± standard deviation; $n = 4$ per group) were compared by two-way analysis of variance, with main effects of treatment ($P < 0.0001$), production ($P = 0.186$), and the interaction ($P = 0.0006$). Multiple comparisons were made to compare the effect of each treatment against control (media alone), with a family *P*-value of 0.01; an adjusted *P*-value is shown. (*B*) Bone marrow-derived Mϕ mRNA production of a range of M1 (left) and M2 (right) markers following 24 h stimulation with endothelin-1 (10 pg/mL and 10^4 pg/mL). Data (mean ± standard deviation; $n = 4$ per group) were compared by two-way analysis of variance (main effects of treatment, of gene product and the interaction were all $P < 0.0001$). Multiple comparisons were made to compare the effect of each treatment on mRNA production against control (media), with a family *P*-value of 0.01. Adjusted *P*-values are shown. (*C*) Western blot analysis demonstrating endothelin-1, either alone or in combination with LPS/INFγ, did not stimulate production of caspase 1 in mouse Mϕ. (*D*) Endothelin-1 alone (media) or in combination with LPS/INFγ, did not stimulate production of IL-1β by mouse Mϕ.

Human and mouse macrophages show similar responses to endothelin-1

As in mouse BMDM, human Mϕ showed expression of both the ET_A and ET_B receptor (see Supplementary material online, *Figure 11A*). Similarly, human Mϕ were not polarized to a classical or alternative phenotype by ET-1 (see Supplementary material online, *Table S1* and *Figure S11B*) but showed evidence of ET_B receptor-mediated ET-1 uptake and

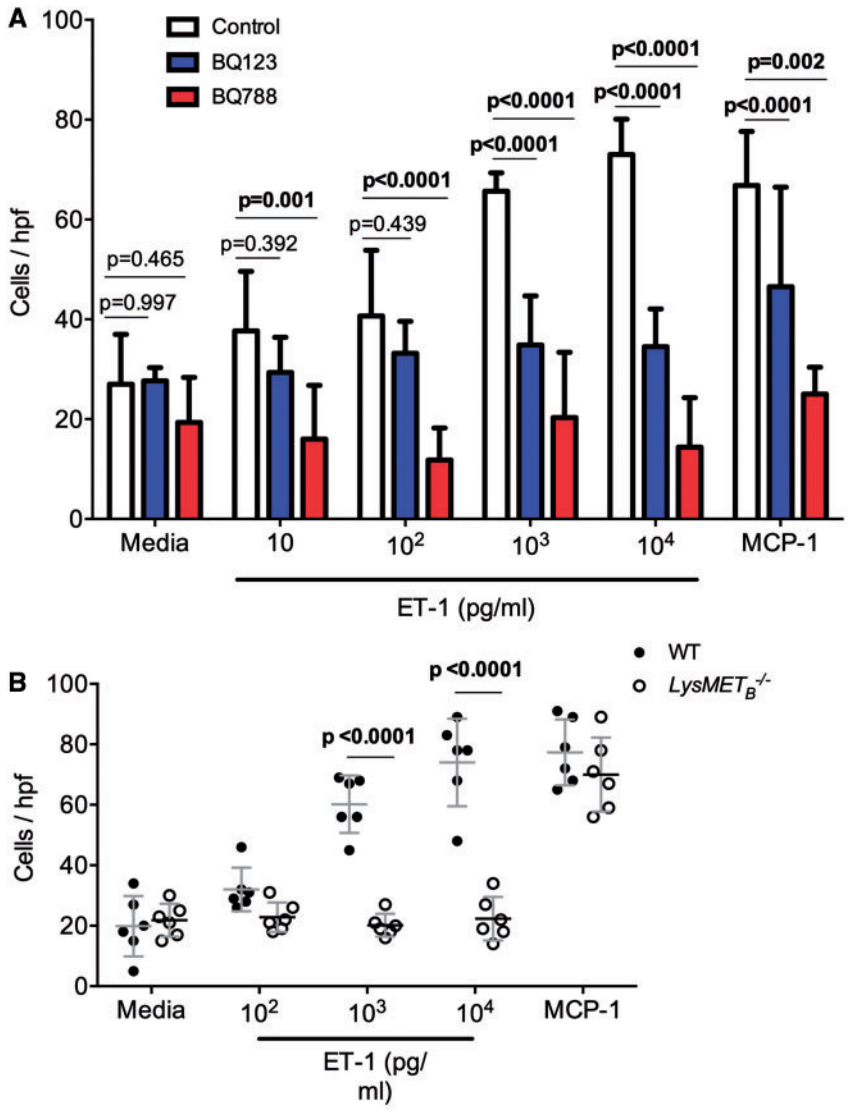

Figure 3 Macrophage demonstrate chemokinesis towards endothelin-1. (*A*) Bone marrow-derived Mϕ chemokinesis in response to increasing doses of endothelin-1 in the presence or absence of selective endothelin-A (BQ123) or endothelin-B (BQ788) antagonism; MCP-1 was used as a positive control. The number of Mϕ per high powered field (mean ± standard deviation; $n = 6$ mice per group) was compared by two-way analysis of variance, with main effects of endothelin-1 or MCP-1 treatment ($P < 0.0001$) and receptor antagonism ($P < 0.0001$) and the interaction ($P < 0.0001$). Effects within rows were compared, using one family per row and a family *P*-value of 0.01. Adjusted *P*-values are shown. (*B*) Chemokinesis of bone marrow-derived Mϕ in response to endothelin-1 or MCP-1. Bone marrow-derived Mϕ were isolated from $LysMET_B^{-/-}$ (open circles; $n = 6$) and control (closed circles; $n = 6$) mice. Data were compared by two-way analysis of variance, with main effects of endothelin-1 or MCP-1 treatment ($P < 0.0001$) and genotype ($P < 0.0001$) and the interaction ($P < 0.0001$). Between genotype comparisons were made and adjusted *P*-values are shown.

chemokinesis to ET-1 and (see Supplementary material online, *Figure S11C* and *D*).

Macrophage depletion augments the pressor response to endothelin-1 but not angiotensin II

To understand the role of Mϕ in the pressor response to ET-1, we administered incremental doses of intravenous (i.v.) ET-1 to CD11b-DTR mice given diphtheria toxin (DT) and to controls. Mϕ depletion *per se* was not associated with a difference in baseline mean arterial pressure (MAP) (*Figure 6A*) or a shift in vasoconstrictor-vasodilator capacity of conduit or resistance vessels (see Supplementary material online, *Figure S12*). Administration of ET-1 following Mϕ depletion resulted in an exaggerated hypertensive response compared with controls (*Figure 6B*) with a greater maximal change in mean arterial pressure (MAP) in Mϕ-deficient mice (*Figure 6C*). At a dose of ET-1 1 nmol/kg the maximal change in MAP

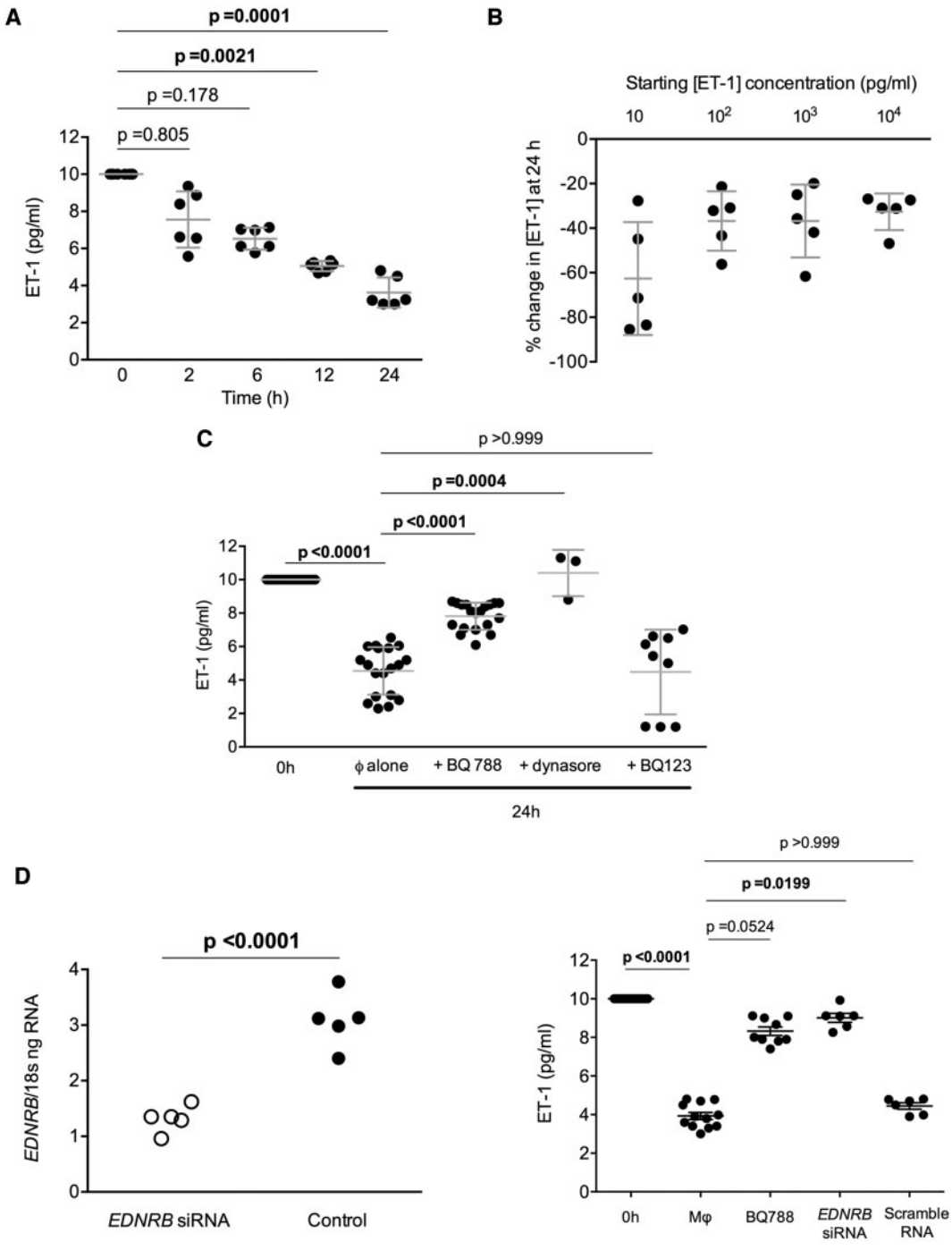

Figure 4 Macrophages clear endothelin-1 through endothelin-B receptor-mediated endocytosis. Endothelin-1 concentration in cell culture supernatant from bone marrow-derived Mϕ (*A*) over 24 h after incubation with a starting endothelin-1 concentration of 10 pg/mL, (*B*) at 24 h following incubation with endothelin-1 (10–10^4 pg/mL), (*C*) after incubation with an endothelin-A (BQ123) or endothelin-B (BQ788) antagonist or dynasore, an inhibitor of dynamin-dependent endocytosis receptor antagonism, and (*D*) following bone marrow-derived Mϕ endothelin-B receptor knockdown with siRNA (efficiency of knockdown left panel). Kruskal–Wallis tests were used with Dunn's test for multiple planned comparisons. Adjusted *P*-values are shown. For (*D*) left panel an unpaired *t*-test was used; the two-tailed *P*-value is shown.

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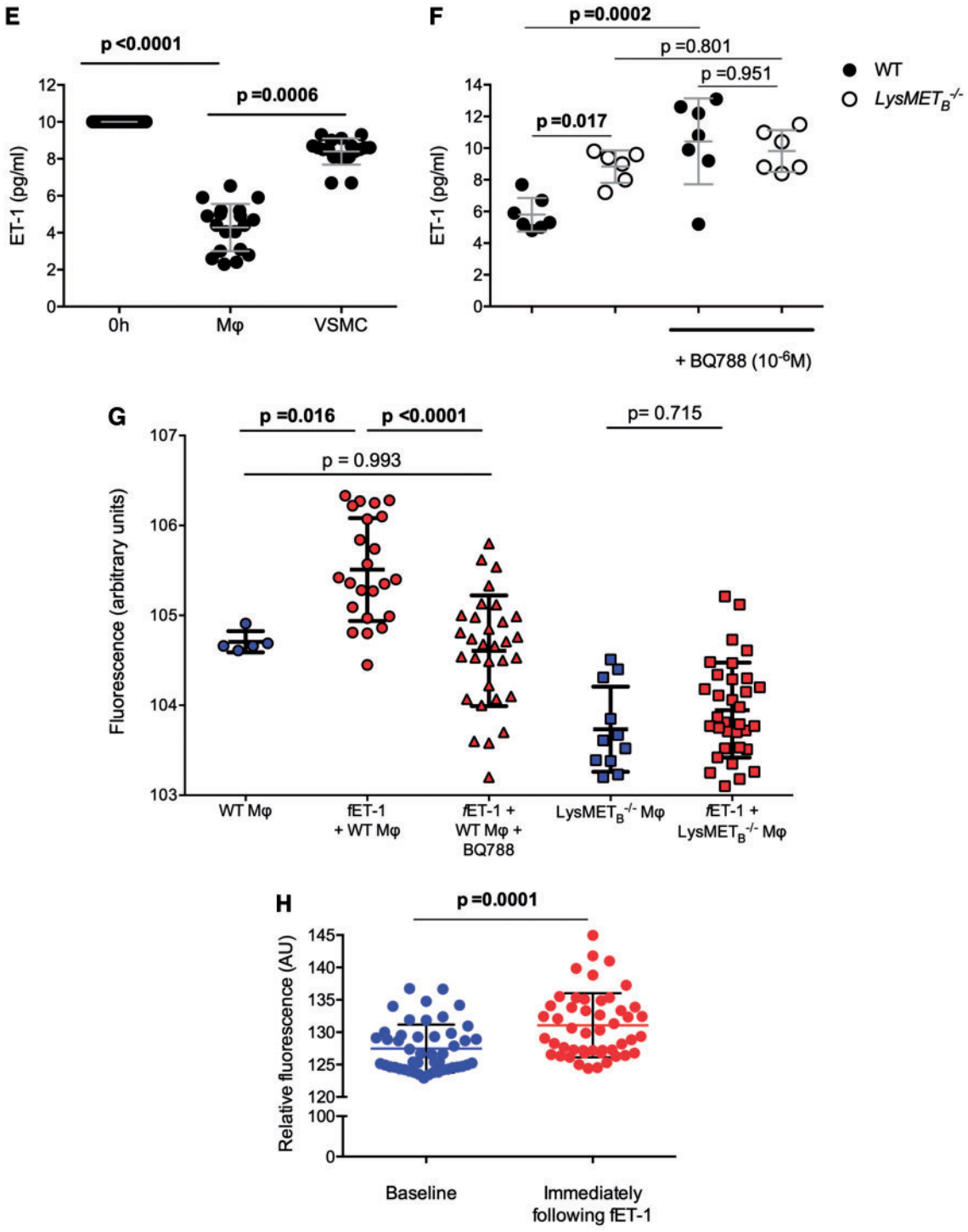


Figure 4 (*E*) Vascular smooth muscle cells do not remove endothelin-1 from their media *in vitro*. Data from individual experiments are shown and were compared by Kruskal–Wallis; with Dunn's test for multiple planned comparisons as shown. (*F*) Wild type (white circles) and *LysMET*$_B^{-/-}$ (black circles) bone marrow-derived Mϕ were exposed to endothelin-1 10 pg/mL *in vitro* in the presence or absence of BQ788, an endothelin-B receptor antagonist. Endothelin-1 was measured in the supernatant at 24 h. One-way analysis of variance ($P = 0.0003$) was used and adjusted *P*-values for multiple comparisons are shown. (*G*) Uptake of fluorescent endothelin-1 by wild type or *LysMET*$_B^{-/-}$ bone marrow-derived Mϕ in the presence or absence of BQ788. One-way analysis of variance ($P < 0.0001$) was used and adjusted *P*-values for planned comparisons, as shown. (*H*) Rapid uptake of endothelin-1 by bone marrow-derived Mϕ.

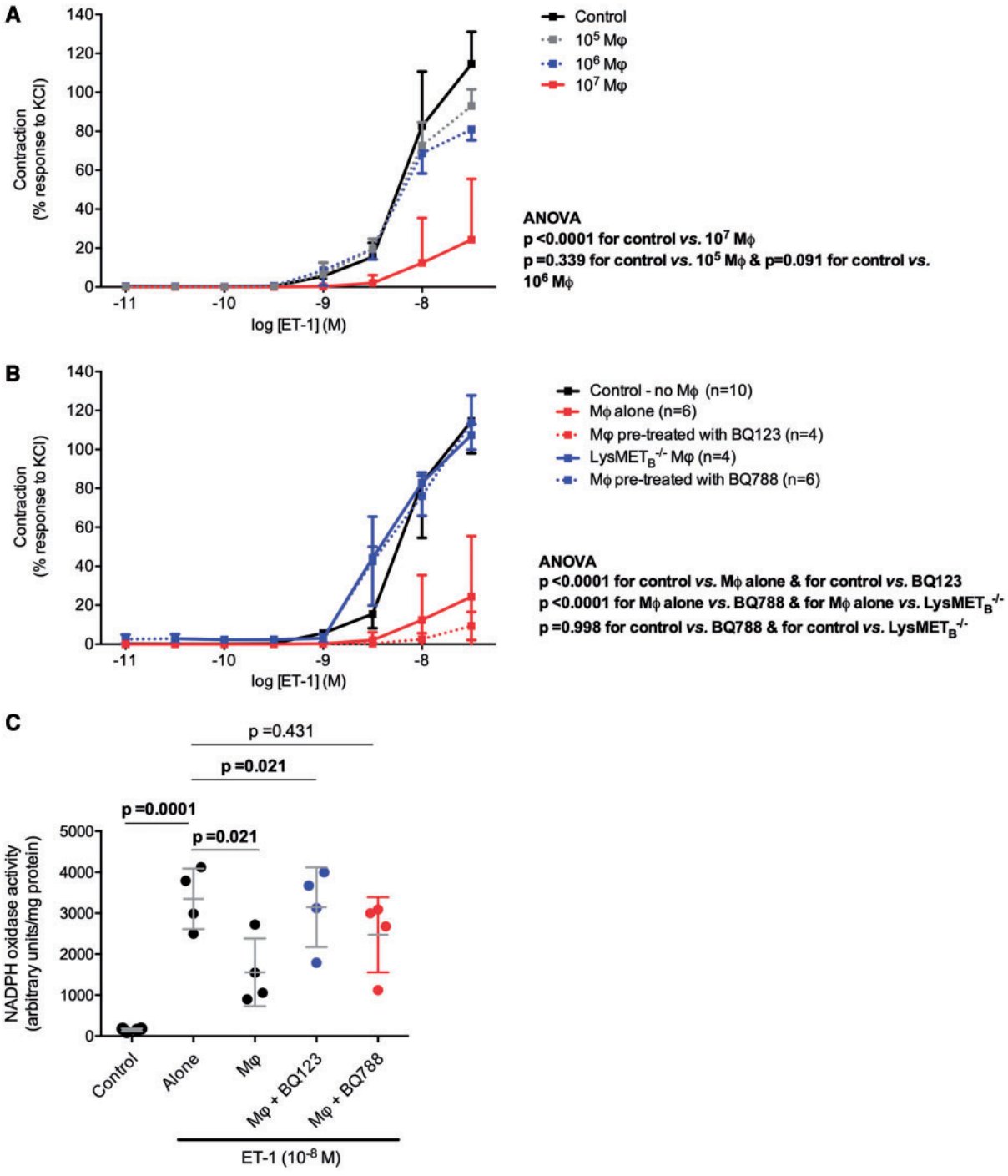


Figure 5 Macrophages modify vascular contraction and oxidative stress in response to endothelin-1. (*A*) Contraction of mesenteric artery segments to increasing (endothelin-1) (Control, black line, $n = 10$) and following pre-incubation with varying number of Mϕ ($n = 6$ per group). (*B*) Vascular responses to 10^7 Mϕ (red line, $n = 6$), Mϕ + BQ123 (dotted red line, $n = 4$), Mϕ from *LysMET*$_B^{-/-}$ mice (blue line, $n = 4$), and Mϕ + BQ788 (dotted blue line, $n = 6$). Data (mean ± standard deviation) were compared by two-way analysis of variance, with main effects of endothelin-1, pre-treatment and the interaction (all $P < 0.0001$). Adjusted *P*-values for planned comparisons are shown and only the comparisons where $P < 0.05$ at 10^7 endothelin-1 are shown. (*C*) The effects of Mϕ on oxidative stress. Mean ± standard deviation were compared by one-way analysis of variance ($P = 0.0002$), using Sidak correction for multiple comparisons, with adjusted *P*-values are shown.

was approximately two-fold greater in Mϕ deficient mice compared with control groups. Notably, Mϕ depletion did not affect the acute pressor response to ANG II (*Figure 6D*).

To specifically explore the role of the Mϕ ET_B receptor in the pressor response to ET-1, we administered i.v. ET-1 to *LysMET*$_B^{-/-}$ and control mice. Baseline MAP did not differ between the two groups (*Figure 6E*). Similar to the response seen following systemic Mϕ depletion, *LysMET*$_B^{-/-}$ mice demonstrated an exaggerated pressor response to ET-1 (*Figure 6F*). At a dose of ET-1 0.1 nmol/kg the maximal change in MAP was approximately two-fold that seen in control animals.

Macrophage depletion augments the chronic hypertensive response to endothelin-1 and adoptive transfer of wild type monocytes prevents this

A 3-week infusion of ET-1 led to sustained increases in systolic and diastolic BP in CD11b-DTR mice (*Figure 7A* and *B*). Both systolic and diastolic BP increased by approximately 10–12 mmHg above baseline. DT was used to deplete Mϕ over a period of 7–10 days resulting in gradual increases in both systolic and diastolic BP (*Figure 7C* and *D*). As the effects of DT weaned, and Mϕ repopulated, BP gradually returned to pre-depletion levels. The maximal increase in systolic BP was approximately 20 mmHg and for diastolic BP this was approximately 15 mmHg. In the negative control arm, CD11b-DTR mice were injected with phosphate buffered saline, and here, BP remained stable throughout the experiment.

In keeping with an important role for circulating monocytes in these effects, adoptive transfer of wild type monocytes prevented the rise in BP seen with DT (*Figure 7E* and *F*). As might be expected, Mϕ depletion was associated with a rise in circulating ET-1 with a fall to pre-depletion levels with Mϕ repopulation (*Figure 7G*).

Macrophage depletion augments the chronic hypertensive response to a high salt diet

Next, we explored the role of Mϕ in a second model of hypertension that associated with a high salt diet. High salt led to rises in both systolic and diastolic BP of approximately 10 mmHg above baseline (see Supplementary material online, *Figure S13A*). Mϕ depletion led to further rises in both and, as previously seen, these effects diminished with Mϕ repopulation (see Supplementary material online, *Figure S13B* and *C*).

Myeloid ET_B receptor deficiency augments the chronic hypertensive response to endothelin-1 and angiotensin II

In line with an important role for the myeloid ET_B receptor in protecting from the deleterious effects of ET-1, ET-1 administration into *LysMET*$_B^{-/-}$ mice led to a two- to three-fold exaggerated hypertensive response to ET-1 compared with littermate control mice (*Figure 8A*). To confirm our findings, we exposed *LysMET*$_B^{-/-}$ and littermate control mice to a 2-week infusion of ANG II, a model of hypertension that is ET-1 dependent.[20,21] Baseline systolic and diastolic BP were similar between the two groups of animals during both the active (night) and inactive (day) phases. *LysMET*$_B^{-/-}$ mice had a significantly exaggerated hypertensive response to ANG II compared with their controls. For example, at night, systolic BP rose on average by approximately 25 mmHg in control mice but by approximately 40 mmHg in knockouts. For diastolic BP, these corresponding figures were approximately 25 mmHg and approximately 35 mmHg (*Figure 8B*).

In patients with anti-neutrophil cytoplasmic antibody vasculitis different immunotherapies variably affect blood pressure and the endothelin system

Cyclophosphamide (CYC) and mycophenolate mofetil (MMF) are standard therapies for patients with small vessel vasculitis associated with autoantibodies to neutrophil cytoplasmic antigens (ANCA). Cyclophosphamide not only depletes B and T cells but also effectively depletes circulating and tissue Mϕ[28]; MMF suppresses T- and B-cell function but does not deplete Mϕ. Thus, we investigated changes in BP and the ET system following treatment in 20 patients with ANCA vasculitis: 10 received CYC as their immunosuppressive therapy whereas the other 10 received MMF. Demographics and other treatments, including corticosteroid dose, antihypertensive treatment, and the use of plasmapharesis, were similar between groups (see Supplementary material online, *Table S2*).

Baseline systolic and diastolic BP, plasma and urine ET-1 did not differ between the two groups (see Supplementary material online, *Table S2*). Interestingly, plasma ET-1 was higher in patients with ANCA vasculitis than in healthy volunteers (3.74 ± 0.38 pg/mL vs. 1.21 ± 1.2 pg/mL, $P = 0.02$). Whereas CYC reduced mean circulating monocyte count MMF did not (baseline vs. week 6: CYC: 0.87 ± 0.15 vs. $0.26 \pm 0.17 \times 10^9$, $P < 0.05$; MMF: 0.77 ± 0.25 vs. $0.69 \pm 0.29 \times 10^9$, $P = 0.756$). Cyclophosphamide treatment was associated with a greater increase in BP compared with treatment with MMF (*Figure 9A and B*, $P < 0.01$ for CYC vs. MMF). There was a positive correlation between the change in peripheral monocyte count and the extent to which BP rose in those patients receiving CYC (*Figure 9C* and *D*). Plasma ET-1 fell following treatment with MMF (*Figure 9E*, $P < 0.001$ for both weeks 0 vs. 6 and 6 vs. 12), whereas CYC treatment only led to an initial fall in plasma ET-1 ($P < 0.05$ for weeks 0 vs. 6). Plasma ET-1 was lower at week 12 in those treated with MMF (*Figure 9E*). Urine ET-1 fell with both CYC and MMF (*Figure 9F*, $P < 0.01$ for CYC and $P < 0.0001$ for MMF for weeks 0 vs. 12) but to a greater degree with MMF ($P < 0.01$ for MMF vs. CYC). Flow-mediated dilation (FMD) of the brachial artery was similar at baseline between the two groups (6.7 ± 0.2 for MMF vs. 6.6 ± 0.1% for CYC, $P = 0.589$). At 12 weeks, FMD had not changed in those patients receiving MMF treatment) whereas those patients receiving CYC had lower FMD in keeping with worse endothelial function (-1.2%, $P < 0.05$ vs. baseline).

In these same patients, we characterized Mϕ expression of ET_A and ET_B receptors and compared this to health (see Supplementary material online, *Table S2* and *Figure S9G* and *H*). There were no differences in expression of the ET_A receptor between the groups and in keeping with our previous data its expression was lower than that of the ET_B receptor. Interestingly, patients presenting with ANCA vasculitis demonstrated reduced expression of the ET_B receptor on

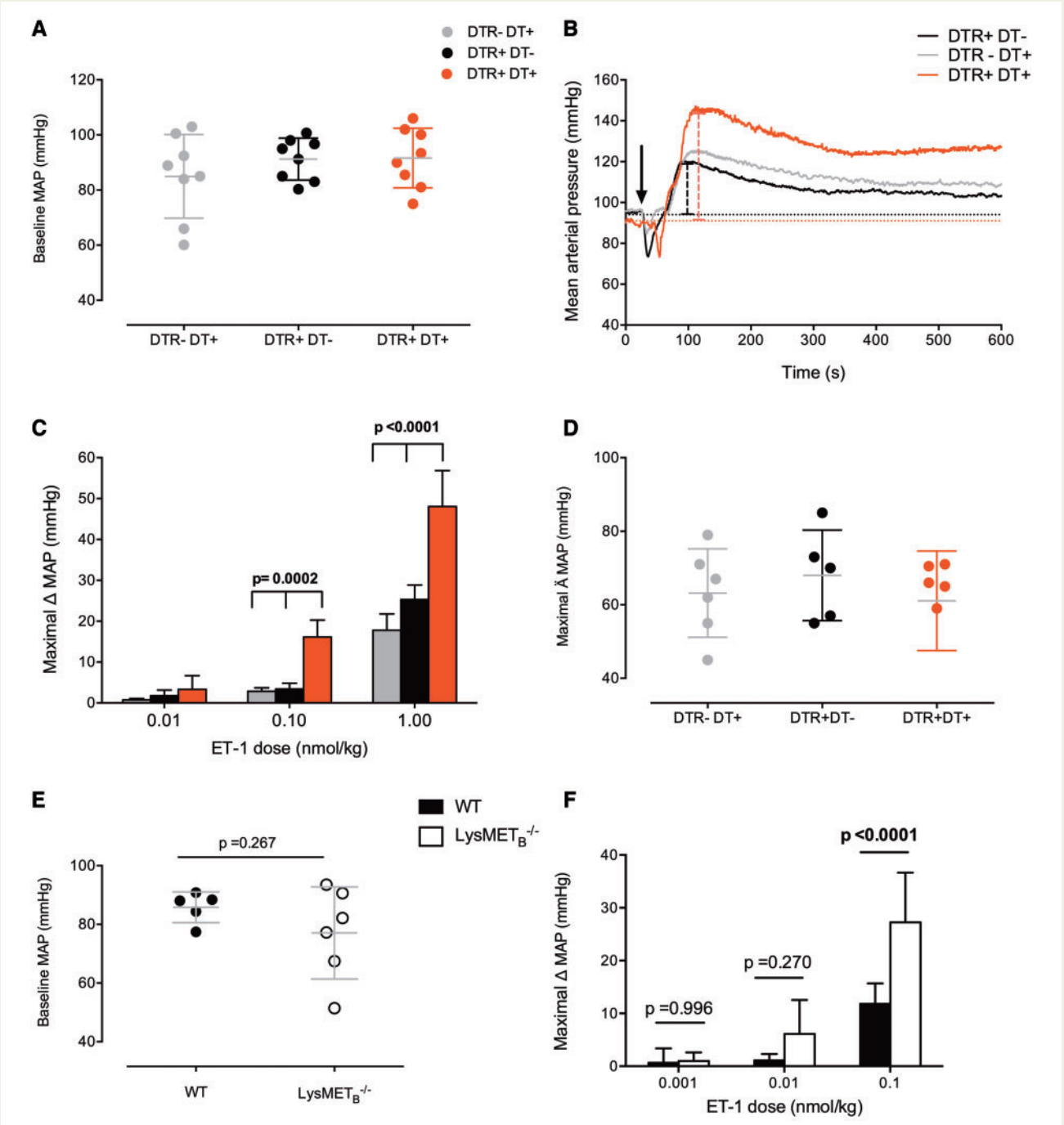


Figure 6 Role of Mϕ in the pressor response to endothelin-1. Acute blood pressure response to incremental doses of endothelin-1 following acute depletion of circulating monocytes and resident Mϕ ($DTR^{+}DT^{+}$), and in controls [those with the diphtheria toxin receptor (DTR) construct but given saline, $DTR^{+}DT^{-}$, and mice given diphtheria toxin (DT), $DTR^{-}DT^{+}$; $n = 10$ mice/group]. (*A*) Baseline blood pressure. (*B*) Example of the differences seen among the three groups in the acute pressor response to endothelin-1. Intravenous endothelin-1 administration is defined by the black arrow. The dotted and dashed lines represent baseline and maximal mean arterial pressure, respectively. (*C*) Maximal change in mean arterial pressure, compared by two-way analysis of variance (main effect of genotype, of endothelin-1 dose and the interaction all $P = 0.0001$); Holm–Sidak planned comparisons were made and adjusted *P*-values are shown. (*D*) Acute blood pressure response to ANG II (1 nmol/kg) following depletion of circulating monocytes and resident Mϕ. (*E*) Baseline mean arterial pressure and (*F*) maximal change in mean arterial pressure in response to endothelin-1 in *LysMET*$_B^{-/-}$ and littermate control mice ($n = 6$ mice per group), compared by two-way analysis of variance [main effect of genotype $P = 0.0128$, of endothelin-1 dose ($P = 0.0001$) and the interaction $P = 0.0004$]; Holm–Sidak planned comparisons were made and adjusted *P*-values are shown.

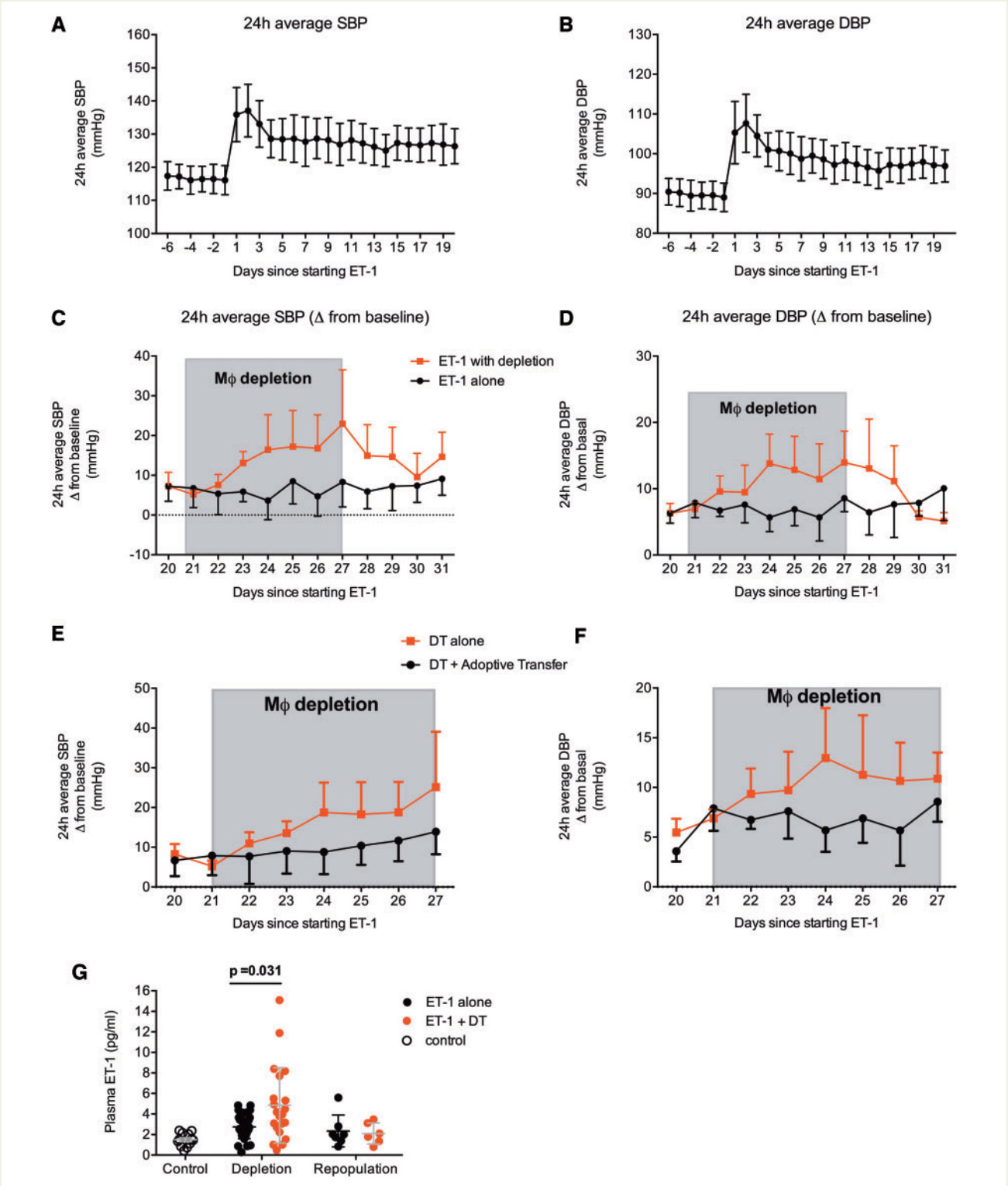


Figure 7 Effects of chronic Mϕ depletion on endothelin-1-mediated hypertension. Effects on systolic and diastolic blood pressure of endothelin-1 infusion (10 pmol/kg/min administered via minipump) for 3 weeks (mean ± standard deviation, $n = 12$ mice, *A* and *B*). From Day 21, half the mice ($n = 6$) underwent Mϕ depletion over the period shown in grey. Groups were compared by two-away analysis of variance, assessing the main effect of Mϕ depletion, duration in days and the interaction. The effect of Mϕ depletion was $P < 0.0001$ for (*C*) systolic blood pressure and (*D*) diastolic blood pressure. Effects of diphtheria toxin alone or diphtheria toxin with adoptive transfer of $CD11b^{+}Gr1^{+}$ monocytes at Day 21 and Day 24 on systolic and diastolic blood pressure are shown in (*E*) and (*F*). Two-way analysis of variance reported a significant ($P = 0.0003$) effect of group. (*G*) The effects of Mϕ depletion and repopulation on plasma endothelin-1 ($n = 5$ mice per group), assessed by one-way analysis of variance ($P < 0.0001$), with the adjusted *P*-values for planned comparisons are shown.

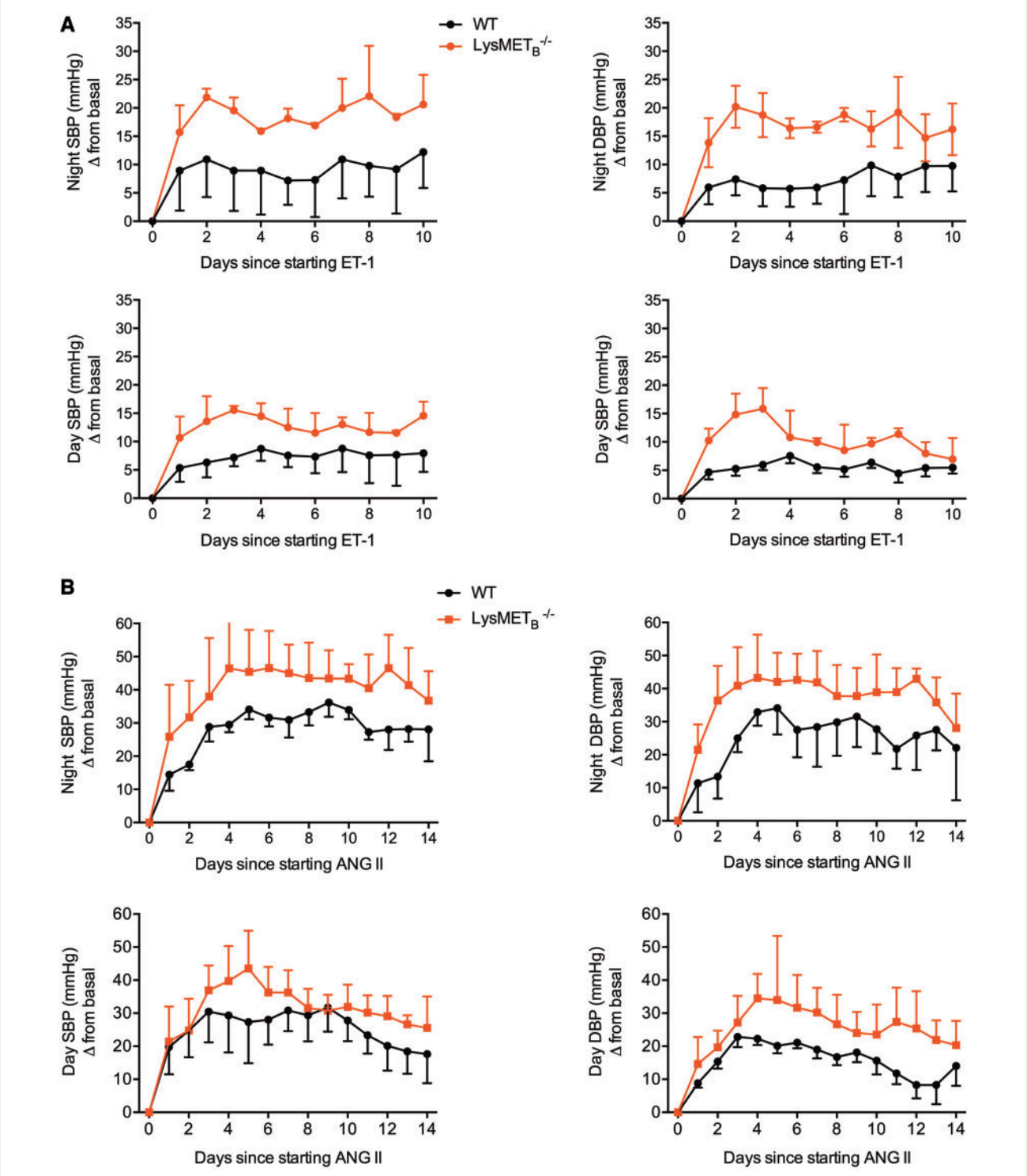


Figure 8 Effects of myeloid endothelin-B receptor deficiency on endothelin-1 and angiotensin II-mediated hypertension. Night-time and daytime telemetry systolic and diastolic blood pressure in *LysMET$_B^{-/-}$* and wild type controls receiving 2 weeks of endothelin-1 (5 pmol/kg/min; $n = 8$ mice per group) (*A*) or angiotensin II (1 μg/kg/min; $n = 12$ mice/group) (*B*). Data are mean ± standard deviation and two-way analysis of variance compared the main effects of genotype, treatment (endothelin-1 or angiotensin II) and the interaction. In all cases, there was a significant ($P<0.0001$) effect of genotype.

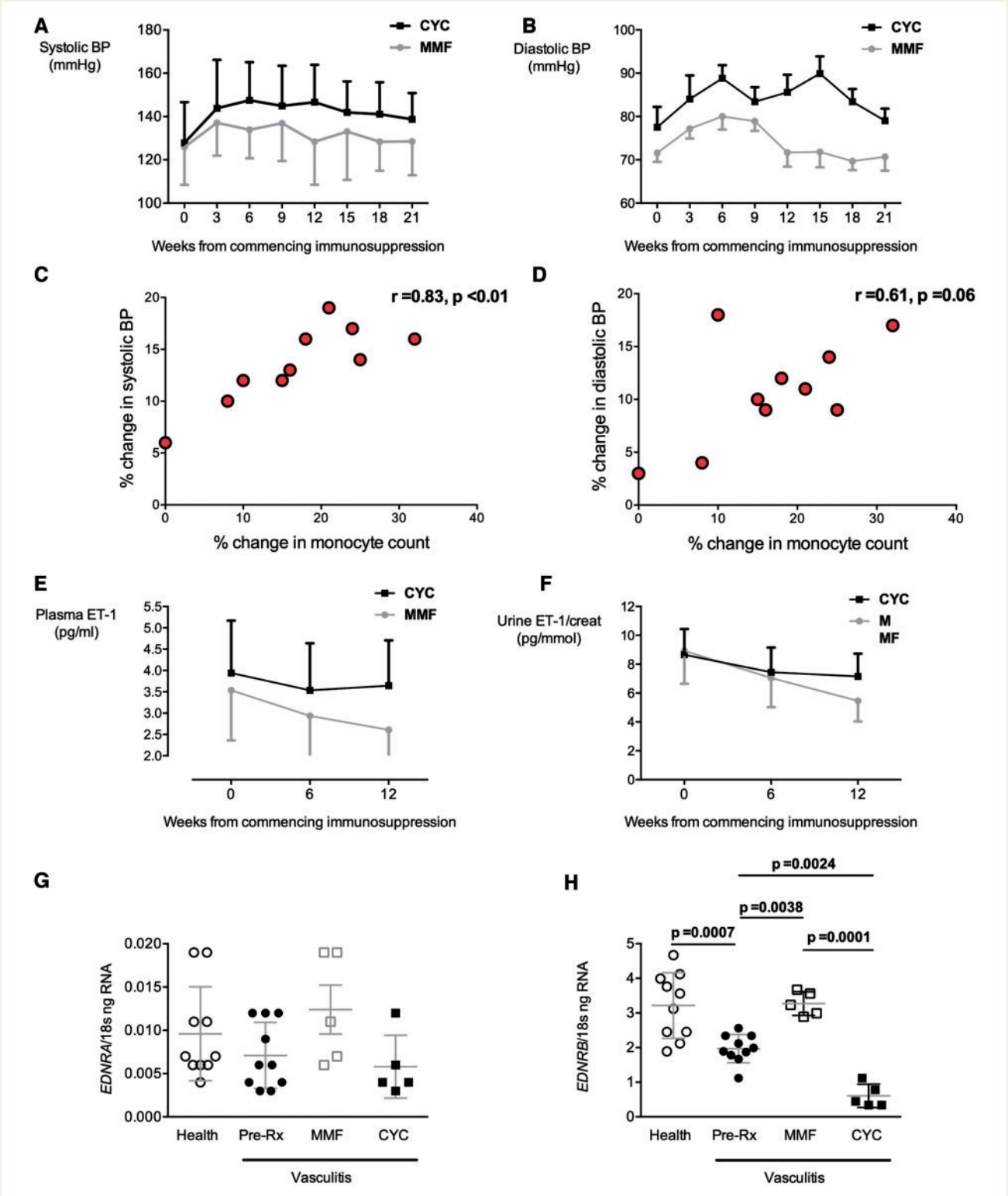


Figure 9 Effects of treatment with cyclophosphamide and mycophenolate mofetil in patients with autoantibodies to neutrophil cytoplasmic antigens vasculitis. (*A* and *B*) Systolic and diastolic blood pressure, with two-way analysis of variance showing a significant effect of treatment ($P = 0.001$). (*C* and *D*) The relationship between change in circulating monocyte count and changes in systolic and diastolic blood pressure is shown. (*E*) Plasma endothelin-1 (effect of treatment $P = 0.0225$) and (*F*) urine endothelin-1 (effect of treatment $P = 0.2053$). (*G* and *H*) Monocyte expression of endothelin-A ($P = 0.1310$) and endothelin-B receptors ($P = 0.0001$) in patients with autoantibodies to neutrophil cytoplasmic antigens vasculitis, compared by one-way analysis of variance with adjusted *P*-values are shown.

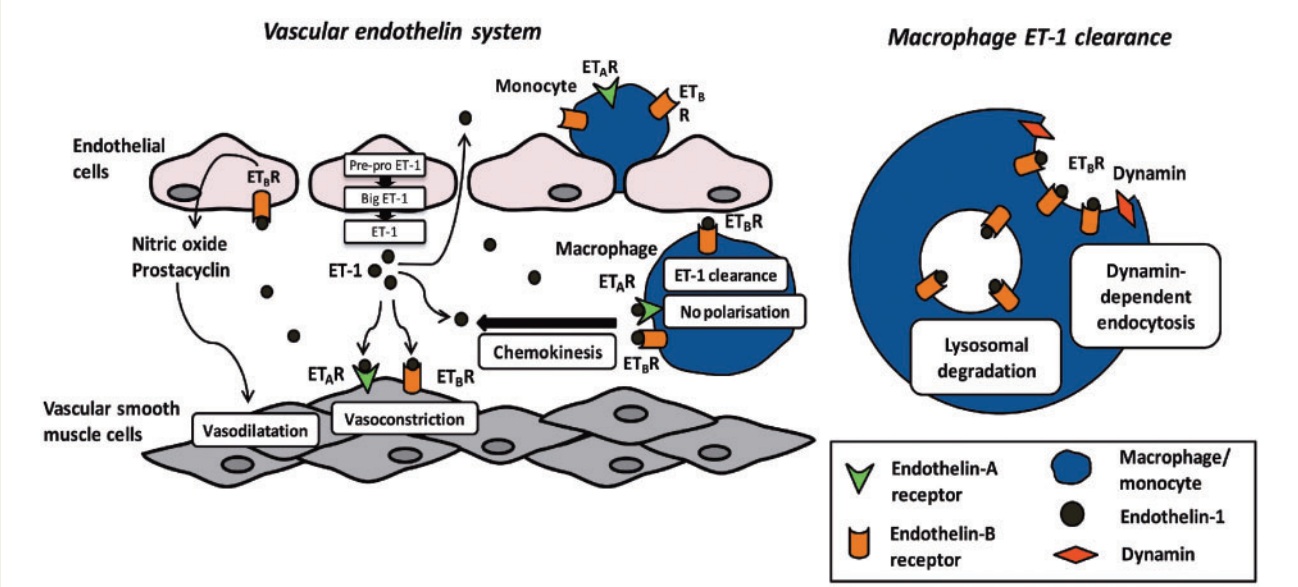


Figure 10 Macrophage-endothelin system interplay. Endothelin-1 is produced predominantly by vascular endothelial cells. *EDN1* gene transcription produces pre-pro endothelin-1 which is cleaved to big endothelin-1 and then endothelin-1. Endothelin-1 is largely secreted abluminally where binding to endothelin-A and endothelin-B receptors on vascular smooth muscle cells causes vasoconstriction. Endothelin-B receptor activation on endothelial cells results in the release of prostacyclin and NO and consequently vasodilatation. Macrophages express both endothelin-A and endothelin-B receptors and display chemokinesis towards endothelin-1. However, endothelin-1 does not polarize Mϕ to a pro-inflammatory or anti-inflammatory phenotype. Our data suggest that Mϕ clear endothelin-1 through endothelin-B receptor-mediated uptake. Binding of endothelin-1 to endothelin-B receptor on Mϕ results in dynamin-dependent endocytosis. This endothelin-B receptor bound endothelin-1 is then transported to the lysosomes for degradation.

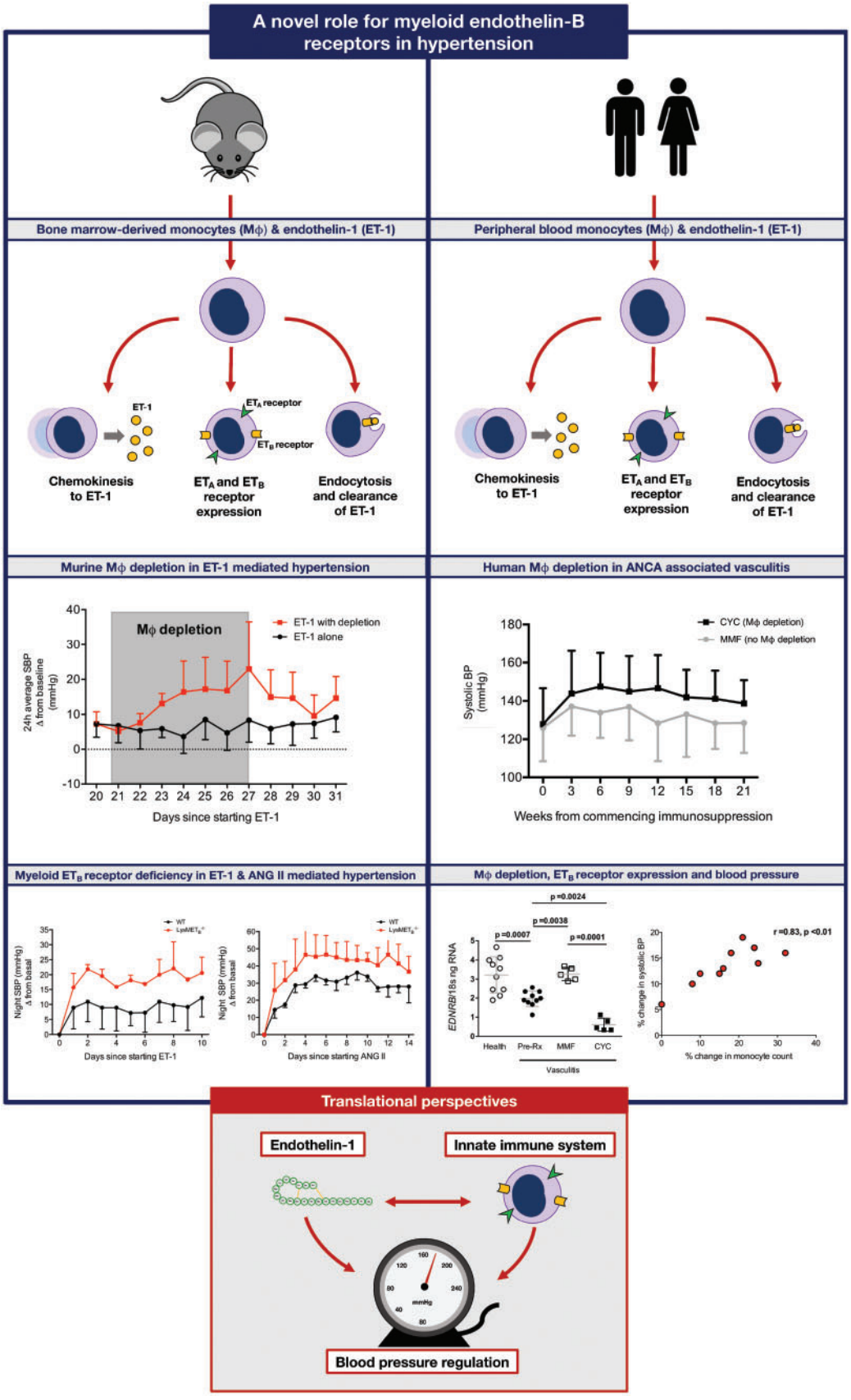
their Mϕ compared with levels seen in health. This was normalized by treatment with MMF but further reduced following treatment with CYC.

Discussion

For the first time, we have demonstrated that the Mϕ ET_B receptor in both mouse and humans provides a novel clearance mechanism for ET-1. Its functional importance *in vivo* is demonstrated by the exaggerated pro-hypertensive effect of ET-1 and ANG II in mice with a deletion of the Mϕ ET_B receptor or following systemic Mϕ depletion. Interestingly, and unexpectedly, we found no evidence that ET-1 was able to polarize mouse or human Mϕ towards a classical pro-inflammatory or alternative anti-inflammatory phenotype but both displayed chemokinesis towards ET-1. Overall, these data provide us with new knowledge, and a clarification of mechanisms underlying the pathological basis of hypertension in relation to the immune and ET systems (*Figure 10*).

To date, many of the studies investigating the role of the innate immune system in the pathogenesis of, and response to, hypertension have focused on the role of T cells in relation to ANG II-mediated hypertension.[29–31] Few have examined the role of Mϕ and only one study relates to ET-1-mediated vascular injury.[32] Recent studies have explored the effects of altering the phenotype of bone marrow-derived cells[9] or Mϕ[10] on hypertension and its complications, whereas others have elegantly depleted neutrophils and Mϕ[8] to this end. The results of these studies are often contradictory but, nevertheless, suggest that Mϕ may contribute to, and protect from, hypertension. The study by Machnik *et al.*[7] showed that salt loading increases Mϕ accumulation in the subcutaneous space. These Mϕ are stimulated by the hypertonic environment to produce vascular endothelial growth factor C. This leads to proliferation of lymphatics. This is protective, because clodronate-mediated Mϕ depletion prevents the lymphatic proliferation and leads to hypertension in response to salt loading. These landmark findings support a role for Mϕ acting as a buffering mechanism, protecting against the development of hypertension. Our study extends beyond these, providing a mechanistic understanding of the interaction between Mϕ and local vascular contractility and the impact on BP.

The Mϕ system comprises a spectrum of cell types, and depletion strategies vary in their specificity and efficacy.[33] Hence, we used two depletion models here, the CD11b-DTR (reduction in Mϕ number) and lysozyme M (LysM; alternation in Mϕ phenotype)-Cre systems. DT administration on CD11b-DTR mice achieved 90% ablation of circulating monocytes. In terms of resident cells, there was a significant reduction in renal Mϕ but less of an effect in the liver and spleen. Analysis of the main monocyte subsets ($Gr1^+$ $CCR2^+$ $CX3CR1^-$ and $Gr1^-$ $CCR2^-$ $CX3CR1^+$) demonstrated that both are equally depleted over the time course of our studies. DT administration did not deplete neutrophils. These findings are in keeping with earlier studies.[34,35] Using the LysM-Cre system, there is functional depletion (80–100%) of mature Mϕ but also neutrophils.



Take home figure A novel role for myeloid endothelin-B receptors in hypertension.

Additionally, this system may partially (16–20%) deplete dendritic cells (DCs),[36] an effect that is more pronounced in the CD11b-DTR mice.[35]

Both CD11b-DTR mice administered DT and *LysMET$_B^{-/-}$* mice were more sensitive to the pressor effects of ET-1. Taken together it is likely these effects were due to an absence of circulating monocytes and/or vascular Mϕ. Given the acute nature of the response, we hypothesize that in control mice circulating monocytes remove the intravenously administered ET-1 to some extent thus attenuating the amount reaching the target VSMC. In contrast, the absence of this ET-1 clearance in Mϕ-deplete mice allows more to be available to act on these cells promoting an exaggerated vasoconstrictor and so hypertensive response. There may be a contribution from DCs and neutrophils to these effects. Although there is currently no evidence that murine or human DCs are able to regulate ET-1, by its clearance or degradation, one study has shown that DCs are able to synthesize ET-1 in response to inflammatory stimuli[37] although another has suggested the opposite.[38] Interestingly, the few data on neutrophils and ET-1 suggest that these cells can both produce and degrade ET-1.[39]

Our data also demonstrate that Mϕ are important in hypertension. We used three models of hypertension—chronic ET-1 and ANG II infusion, which are both dependent on the ET system,[20,21,40,41] and a high salt diet. Mϕ depletion with repeated doses of DT resulted in gradual increases in both systolic and diastolic BP in chronic ET-1 and salt-dependent hypertension. Interestingly, once the DT administration was stopped (and Mϕ were allowed to repopulate) as well as monocyte adoptive transfer resulted in both systolic and diastolic BP returning to pre-depletion levels. This suggests that the importance of both circulating and/or organ-based Mϕ and future work should focus on discriminating which of these is more important here. The importance of the ET_B receptor in chronic hypertension is provided by data from our mice genetically deficient for ET_B on Mϕ alone. Here, two separate models of hypertension, chronic ET-1 and chronic ANG II infusion, elicited exaggerated rises in both systolic and diastolic BP in knockout animals compared with littermate controls.

ET-1 is considered to be pro-inflammatory, so it was surprising that it was unable to polarize Mϕ phenotype, at least *in vitro*. This was true at a range of ET-1 concentrations (10–10 000 pg/mL; mean plasma ET-1 in mice 2–3 pg/mL[42]), whether the ET-1 was administered prior to, following or concomitantly with classical (LPS/INFγ) or alternative (IL-4/IL-13) stimulation. The few data supporting a pro-inflammatory effect of ET-1 on Mϕ used immortalized Mϕ tumour cell lines and lacked robust methodology.[43,44] Interestingly, and in keeping with earlier studies,[37,45] LPS/INFγ stimulation of mouse Mϕ did increase ET-1 concentrations in the supernatant at 24 h and this was completely blocked by an inhibitor of ECE suggesting that this increase in immunoreactive ET-1 is a result of *de novo* production by Mϕ.

Mouse BMDM demonstrated chemokinesis towards ET-1 and this was reduced by selective ET_A antagonism and completely abrogated by selective ET_B blockade. Two recent studies support our findings[46,47] but both found that the ability of Mϕ to move towards ET-1 was more dependent on the ET_A receptor than the ET_B. Of note, both studies investigated that the role of Mϕ and the ET system in the setting of cancer (bladder and breast) where there may well be several different Mϕ phenotypes with a different balance of ET_A:ET_B receptors. In support of ET_B-mediated chemokinesis, BMDM from *LysMET$_B^{-/-}$* displayed no migration towards ET-1. This was not due to an inability to move as they retained their chemokinetic response to MCP-1. Our data showing that Mϕ produce ET-1 in response to an inflammatory stimulus allows us to postulate that this may in turn lead to recruitment of further Mϕ to the area of inflammation as a mechanism to propagate or regulate the response of the innate immune system.

Thus, although Mϕ are not activated by ET-1 they migrate towards it and clear the peptide through ET_B receptor-mediated uptake providing a novel clearance mechanism for the peptide. In EC, the ET_B receptor resides within caveolae. Binding of ET-1 to endothelial ET_B stimulates rapid budding and internalization of the caveolae containing the ET_B receptor bound ET-1.[48] This mechanism is dynamin-dependent. In our *in vitro* studies, both mouse and human Mϕ removed ET-1 from their surrounding media, an effect that was significantly reduced by selective antagonism (or knockdown) of the ET_B receptor but unaffected by ET_A blockade. In keeping with ET-1 clearance by caveolar ET_B receptors, inhibiting dynamin GTPase activity with dynasore completely prevented ET-1 removal by Mϕ. Mϕ are multi-functional cells and are able to degrade peptides through the secretion of proteases as well as through the activity of the cell surface metalloprotease, NEP.[49] However, broad protease and NEP inhibition did not affect Mϕ ET-1 uptake.

To demonstrate the clinical relevance of our findings, we studied patients with ANCA-associated vasculitis, a potentially life-threatening autoimmune condition. Circulating ET-1 was higher in those with vasculitis than in health, probably contributed to by systemic inflammation and endothelial dysfunction. Both MMF and CYC are standard therapies for this condition but they differ in their mechanisms of action. Whereas, MMF inhibits T- and B-cell proliferation and function,[50] CYC is directly cytotoxic and depletes not only these cells but also circulating and tissue Mϕ.[28] In keeping with this action, we demonstrate that CYC has a tendency to reduce the circulating monocyte count by approximately 50% whereas MMF does not. Although this is an accepted measure of circulating monocytes[51] it does not account for tissue-based Mϕ. Nevertheless, we show here that those patients receiving CYC have a greater increase in BP and deterioration in endothelial function than those receiving MMF. In part, this may be due to loss of Mϕ-ET regulation. This is supported by the greater fall in plasma ET-1 in the MMF, but not CYC group, and the down-regulation of the Mϕ ET_B receptor with CYC but not MMF. Thus, hypertension in CYC-treated patients may respond well to ET antagonists. This hypothesis should be explored in future clinical studies as there are few data that relate to the impact of vasculitis and its treatment on BP or vascular function—which is important because ET antagonism may have broader cardiovascular benefits.[52–55]

In summary, our study has identified a new interaction between Mϕ and the endothelin system, whereby Mϕ are drawn to ET-1, without any evident effect on polarization of phenotype, and clear ET-1 from the surrounding milieu. *In vivo*, this cellular action has a significant impact on acute vascular function and BP; importantly this pathway exerts a restraining effect on BP in chronic hypertension. Our final study strongly suggests that this system is operational in humans and therefore represents an intriguing opportunity to modulate BP and reduce cardiovascular risk in multi-system inflammatory conditions. In future, studies such as ours might lead to newer and

currently available BP lowering treatments being used in a more rational way for specific groups of patients, such as those with cancer, as outlined in recent guidelines.[56]

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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