

# TNF $\alpha$ -induced GM-CSF release from human airway smooth muscle cells depends on activation of an ET-1 autoregulatory positive feedback mechanism

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► Additional details of the methods and results, and an additional figure are published online only at <http://thorax.bmj.com/content/vol64/issue12>

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## ABSTRACT

**Background:** There is an urgent need to inhibit endothelin-1 (ET-1) induced chronic inflammatory processes in early stages of lung diseases in order to prevent untreatable irreversible stages often accompanied by lung fibrosis and pulmonary hypertension. Nothing is known about the airway inflammation-inducing and/or maintaining role of ET-1 in human airway smooth muscle cells (HASMCS).

**Objective:** ET-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) expression in response to tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and ET-1 stimulation was investigated, and the impact of mitogen-activated protein kinase (MAPK) pathways in this context was studied. To elucidate the anti-inflammatory properties of the dual endothelin receptor antagonist bosentan that targets both endothelin receptor subtypes A (ET<sub>A</sub>R) and B (ET<sub>B</sub>R), its effect on the TNF $\alpha$ /ET-1/GM-CSF network was investigated.

**Methods:** ET-1 and GM-CSF expression and activation of MAPKs were investigated via quantitative reverse transcription-PCR (RT-PCR), western blotting and ELISA.

**Main results:** Both TNF $\alpha$  and ET-1 activated p38<sup>MAPK</sup> and extracellular signal-regulated kinase (ERK)-1/-2 signalling. ET-1 expression was induced by TNF $\alpha$  and by ET-1 itself. Both effects were inhibited by bosentan and by specific ET<sub>A</sub>R or p38<sup>MAPK</sup> blockade. ET-1- and TNF $\alpha$ -induced GM-CSF expression were both reduced by bosentan as well as by specific inhibition of either ET<sub>A</sub>R, ET<sub>B</sub>R, p38<sup>MAPK</sup> or ERK-1/-2.

**Conclusion:** TNF $\alpha$  activates an ET<sub>A</sub>R- and p38<sup>MAPK</sup>-dependent ET-1 autoregulatory positive feedback loop to maintain GM-CSF release from HASMCS. Since bosentan impairs ET-1 autoregulation and TNF $\alpha$ -induced ET-1 release, as well as TNF $\alpha$ - and ET-1-induced GM-CSF release, the present data suggest therapeutic utility for bosentan in treating particularly the early stages of chronic inflammatory airway diseases.

Airway smooth muscle cells (ASMCs) are believed to play a critical role in the establishment of inflammation in chronic inflammatory airway diseases and also in pulmonary fibrosis for which current literature suggests airway inflammation as an important factor for disease genesis and progression.<sup>1,2</sup> An inflammatory environment can induce ASMCs to secrete chemokines, cytokines and growth factors. Hence, ASMCs are suggested to perpetuate inflammation within the airway wall by promoting recruitment, activation and trafficking of inflammatory cells.<sup>1</sup> This can lead to airway wall and airway vessel remodelling, which is understood as the main cause of irreversible airway

obstruction or pulmonary hypertension of patients with chronic inflammatory airway diseases.<sup>3</sup> Thus, the therapeutic counter-regulation of inflammatory mechanisms of ASMCs might be an auspicious strategy to combat airway inflammation-associated diseases in early reversible stages.

Chronic inflammatory airway diseases and pulmonary fibrosis are characterised by elevated tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) levels in sputum or bronchoalveolar lavage fluid (BALF), indicating a central role for TNF $\alpha$  in the establishment and/or maintenance of pathogenesis.<sup>4-6</sup> As TNF $\alpha$  is believed to act as an initiating cytokine in airway inflammation regulating infiltration/recruitment of immune effector cells into the lung,<sup>7</sup> TNF $\alpha$ -mediated processes are of interest in terms of therapeutically intervening airway inflammation at early disease stages. TNF $\alpha$  signals through TNF receptors 1 and 2 (TNFR1 and 2) which both are expressed on human ASMCs (HASMCS).<sup>8</sup>

In ASMCs, TNF $\alpha$  induces the release of granulocyte-macrophage colony-stimulating factor (GM-CSF).<sup>9</sup> GM-CSF is a proinflammatory and profibrotic cytokine with key functions in the establishment of airway inflammation,<sup>10,11</sup> and recent studies predict therapeutic utility for GM-CSF neutralisation in chronic inflammatory airway diseases and pulmonary fibrosis. GM-CSF deficiency/neutralisation impairs airway inflammation in murine models of allergic asthma and chronic obstructive pulmonary disease (COPD).<sup>12-14</sup> GM-CSF is increased in the BALF of patients with pulmonary fibrosis, stimulates macrophages to release profibrotic cytokines and might induce fibrosis by direct stimulation of ASMCs.<sup>10</sup>

Endothelin-1 (ET-1), a secreted peptide, signals through ET<sub>A</sub> and ET<sub>B</sub> transmembrane receptors (ET<sub>A</sub>R and ET<sub>B</sub>R), both belonging to the G-protein superfamily, and both are expressed on HASMCS.<sup>15</sup> Activation of the ET-1 gene leads to the release of big-ET-1, an inactive ET-1 precursor extracellularly processed into active ET-1 (hereafter referred to as ET-1).<sup>16</sup> Accumulating evidence is indicating the role of ET-1 as a proinflammatory cytokine. ET-1 is known to prime neutrophils, activate mast cells and stimulate monocytes to produce a variety of proinflammatory cytokines.<sup>16</sup> The hypothesis that ET-1 might contribute to airway inflammation in chronic inflammatory airway diseases finds support from studies demonstrating increased ET-1 levels in exhaled breath condensates of patients with asthma (in correlation with disease severity) and in sputum of patients with COPD.<sup>17,18</sup> ET-1 also has profibrotic properties and its levels are

elevated in sputum, BALF and fibrotic tissue of patients with cystic or idiopathic pulmonary fibrosis (IPF).<sup>17–19</sup> ET-1 is abundantly expressed in the vascular endothelium of the lung and, to a lesser extent, by other cell types, including pulmonary artery smooth muscle cells and lung fibroblasts.<sup>20</sup> Whereas many studies have focused on the effects of ET-1 on vascular smooth muscle cells,<sup>20</sup> there is no information about ET-1 expression in HASMCs and the ASMC inflammatory response to ET-1 stimulation.

Bosentan, approved as a treatment for pulmonary arterial hypertension combating vasoconstriction, is an endothelin receptor antagonist with almost equal affinity for ET<sub>A</sub>R and ET<sub>B</sub>R, and thus commonly referred to as a dual blocker.<sup>20</sup> Concerning the inflammatory and profibrotic properties of ET-1, bosentan might also be useful as a treatment for chronic inflammatory airway diseases and pulmonary fibrosis in early disease stages. In rat models of emphysema and eosinophilic airway inflammation, application of bosentan results in a decrease of inflammation due to a substantial reduction in leucocyte number and/or a decrease in the level of proinflammatory cytokines in the BALF and in lung tissue.<sup>21–22</sup>

The aim of this study was to elucidate for the first time the expression and inflammatory properties of ET-1 in HASMCs. To this end, we investigated ET-1 expression in response to TNF $\alpha$  stimulation. Since secreted inflammatory factors have been known temporarily to induce their own expression to potentiate inflammation rapidly (autoregulatory positive feedback mechanism), we investigated the autoregulation of ET-1 expression in cultured HASMCs. We studied the release of GM-CSF in response to TNF $\alpha$  and ET-1. To elucidate the anti-inflammatory properties of bosentan in HASMCs, we studied its effect on the TNF $\alpha$ /ET-1/GM-CSF network. Finally, we investigated the impact of endothelin receptor subtypes and mitogen-activated protein kinase (MAPK) pathways in these processes.

## METHODS

### Isolation and cultivation of HASMCs

HASMCs were dissected from lobar or main bronchus tissue obtained from patients undergoing lung resection as previously described.<sup>23</sup> Cultivation and characterisation of HASMCs was performed as described elsewhere.<sup>24</sup> For details see the Supplementary online material.

### Stimulation of HASMCs

Before stimulation, subconfluent cell monolayers were deprived of serum for 24 h as previously described.<sup>24</sup> Details of the media used for serum withdrawal and stimulation are given in the Supplementary online material. HASMCs were stimulated with ET-1 (Sigma, Hamburg, Germany; catalogue no. E7764) at 100 nM or with human TNF $\alpha$  (R&D systems, Minneapolis, Minnesota, USA; catalogue no. 210-TA) at 20 ng/ml for the indicated times. Bosentan (Actelion Pharmaceuticals, Freiburg, Germany), BQ123 (Sigma; catalogue no. B150) or BQ788 (Sigma; cat catalogue no. B157) were added at 10<sup>-6</sup> M or at the indicated concentrations 60 or 120 min before ET-1 or TNF $\alpha$  stimulation, respectively. Pretreatment with monoclonal blocking antibodies to TNFR1 (clone H398; Alexis, Lausen, Switzerland; catalogue no. ALX-804-200) and TNFR2 (clone 80M2; Alexis; catalogue no. ALX-804-450) each at 10  $\mu$ g/ml or with PD098059 (10  $\mu$ M; Calbiochem/VWR, Darmstadt, Germany; catalogue no. 513000) and SB203580 (10  $\mu$ M, Calbiochem/VWR; catalogue no. 559389) was carried out 60 or 30 min before stimulation, respectively.

### Semi-quantitative reverse transcription-PCR (RT-PCR)

RNA was isolated with the RNeasy spin column chromatography (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Semi-quantitative RT-PCR for ET-1 and GM-CSF (with PCR cycle conditions to analyse the output in the linear range) was performed as described before.<sup>25</sup> Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. PCR conditions and primer sequences are given in the Supplementary online material.

### Enzyme-linked immunosorbent assays

Quantitative ELISAs for GM-CSF, ET-1 or big-ET-1 were done with supernatants of subconfluent HASMCs on a 6-well plate. Intracellular ELISA for phosphorylated (active) p38<sup>MAPK</sup> with total p38<sup>MAPK</sup> as a reference was carried out on subconfluent HASMCs on a 96-well plate. GM-CSF ELISA was performed as described before.<sup>26</sup> ET-1, big-ET-1 (Biomedica, Vienna, Austria; catalogue nos BI-20052 and BI-20082) and phosphorylated p38<sup>MAPK</sup> ELISAs (R&D Systems; catalogue no. KBC869) were performed according to the instructions of the manufacturer. ELISA protocols are given in the Supplementary online material.

### Western blotting

Protein extraction from HASMCs and immunodetection of phosphorylated (active) extracellular signal-regulated kinase (ERK)-1/2 and of GAPDH were performed as previously described.<sup>24</sup> Details are given in the Supplementary online material.

### Statistical analysis

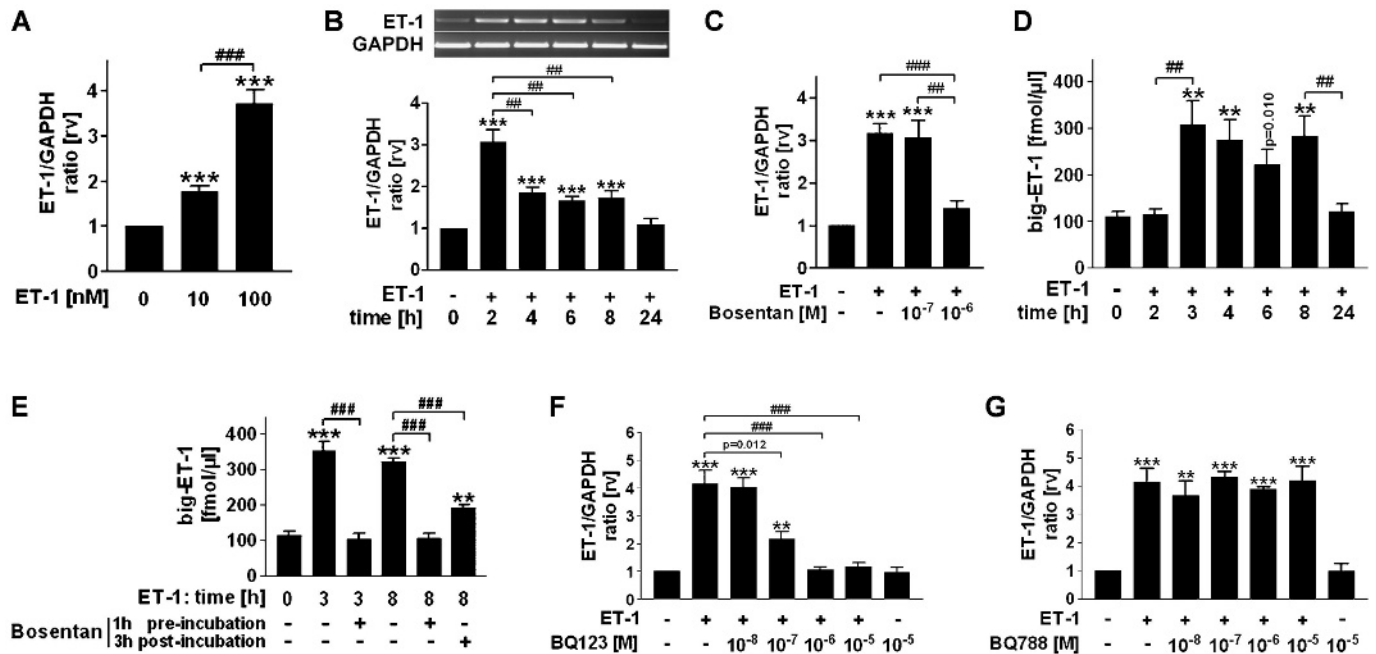
Statistical analyses were performed to examine the effects of TNF $\alpha$  and ET-1 alone or in combination with enzyme inhibitors on gene transcription, enzyme activation and cytokine release by HASMCs. The Gaussian distribution for each data set was confirmed by histogram analyses and Kolmogorov–Smirnov test. Results are expressed as mean (SEM). Comparisons over time or across different stimulations on matched HASMC samples of one subject for *n* independent subjects were analysed by one-way repeated measures analysis of variance (ANOVA) with 95% CIs. For separate comparisons of each stimulation, post hoc Bonferroni–Holm tests were performed. A *p* value <0.05 was considered as statistically significant.

## RESULTS

### ET-1 induces its own expression in HASMCs via ET<sub>A</sub>R and p38<sup>MAPK</sup>

In dose–response and time–response experiments ET-1 induced its own transcription in HASMCs at the highest level (~3-fold above the unstimulated control) at 100 nM after 2 h of stimulation (fig 1A,B). These conditions were used in the subsequent experiments. Notably, the ET-1-inducing effect on ET-1 transcription was maintained for 8 h (fig 1B). A minimum concentration of 10<sup>-6</sup> M bosentan was required to block ET-1-induced ET-1 transcription almost completely (fig 1C). ET-1 induced the release of big-ET-1 (the inactive ET-1 precursor) after 3–8 h of stimulation, and this effect was completely blocked by pretreatment with bosentan (fig 1D,E). ET-1-induced ET-1 transcription was completely blocked by BQ123, a highly selective ET<sub>A</sub>R inhibitor,<sup>15–27</sup> but not by BQ788 (fig 1F,G), a highly selective ET<sub>B</sub>R inhibitor,<sup>15–27</sup> demonstrating a predominant role for ET<sub>A</sub>R in this process.

ET-1 at 1, 10 (data not shown) and 100 nM (fig 2A) rapidly activated ERK-1/2 after 5–30 min of stimulation in a concentration-independent manner. ET-1 transiently activated p38<sup>MAPK</sup>



**Figure 1** Endothelin-1 (ET-1) induces its own expression in human airway smooth muscle cells (HASMCs) via the endothelin receptor  $ET_A$ R. HASMCs were stimulated with ET-1 at the indicated concentrations (A) or at 100 nM (B–G) for 2 h (A, C, F, G) or for the times indicated (B, D, E). In (C), (F) and (G) bosentan or selective inhibitors of  $ET_A$ R (BQ123) or  $ET_B$ R (BQ788) were added to the medium 1 h before ET-1 stimulation at the concentrations indicated; in (E) bosentan at  $10^{-6}$  M was added at the times indicated before (pre-incubation) or after (post-incubation) ET-1 addition. (A–C, F, G) After incubation, the RNA was extracted and subjected to semi-quantitative reverse transcription-PCR (RT-PCR) with ET-1-specific primers. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. In (B, top) one representative example of a time-course set of RT-PCRs is shown. Semi-quantitative RT-PCR signals were evaluated by densitometry. Values for ET-1 were normalised to GAPDH and related to solvent controls (0 nM, –). (D, E) After incubation, concentrations of big-ET-1 in supernatants were measured by ELISA. Each graph represents the mean (SEM) of  $n = 4$  (E–G) or  $n = 5$  (A–D) individual experiments. One-way repeated measures analysis of variance (ANOVA):  $p < 0.0001$  (A–G). Post hoc Bonferroni–Holm tests: exact  $p$  values down to 0.01 are indicated in the graph (related to the indicated values or to solvent controls if placed on top of a bar); \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  related to solvent controls; ## $p < 0.01$ ; ### $p < 0.001$  related to values as indicated; rv, relative values.

with four peak levels: after 5–30 min, and 2, 4 and 6.5 h of stimulation (fig 2B). ET-1-induced ERK-1/2 and  $p38^{MAPK}$  activation was almost completely impaired by pretreatment with bosentan (fig 2C,D). In contrast to PD098059, a specific inhibitor of the ERK-1/2 pathway, the  $p38^{MAPK}$ -specific inhibitor SB203580 almost completely inhibited ET-1-induced ET-1 transcription (fig 2E). These data provide evidence for an  $ET_A$ R- and  $p38^{MAPK}$ -dependent ET-1 autoregulatory positive feedback loop in HASMCs.

This hypothesis was supported by the following data: the exogenously added ET-1 was no longer detectable in the supernatant after 2 h of stimulation; however, we detected ET-1 4 h after stimulation (Supplementary fig 1), indicating ET-1 de novo synthesis. Postincubation with bosentan 3 h after ET-1 stimulation reduced both the release of big-ET-1 after 8 h of ET-1 stimulation and the 4 h  $p38^{MAPK}$  activation peak (figs 1E, 2D). We conclude that the 4 h peak level results from the re-activation of  $p38^{MAPK}$  through de novo synthesised ET-1 and contributes to the big-ET-1 expression observed after 8 h of stimulation and thus to the maintenance of big-ET-1 release (for a detailed discussion see Supplementary online material).

### ET-1 induces GM-CSF expression via $ET_A$ R, $ET_B$ R, ERK-1/2 and $p38^{MAPK}$ signalling

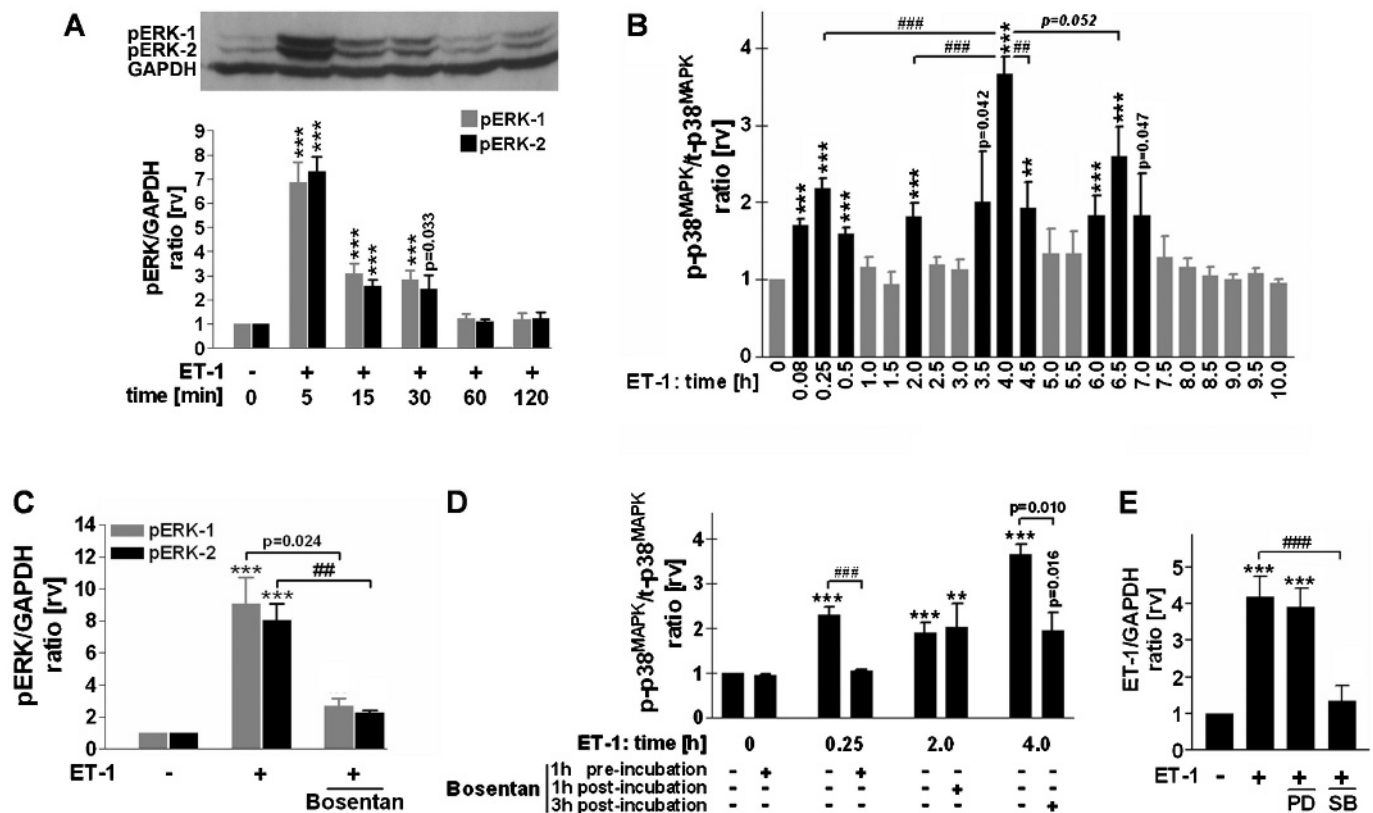
In dose-response and time-response experiments ET-1 induced GM-CSF transcription in HASMCs at the highest level (five- to eightfold above the unstimulated control) at 100 nM after 4 h

of stimulation (fig 3A,B). Notably, the inducing effect on GM-CSF transcription was detected at 2–8 h of stimulation (fig 3B). As individual treatments with bosentan, BQ123, BQ788, PD098059 and SB203580 all abolished ET-1-induced GM-CSF transcription (fig 3C–F) we conclude that ET-1 induces GM-CSF expression via  $ET_A$ R,  $ET_B$ R, ERK-1/2 and  $p38^{MAPK}$  signalling.

### TNF $\alpha$ induces ET-1 expression via $ET_A$ R and $p38^{MAPK}$

TNF $\alpha$  did not influence ET-1 mRNA levels at 1 ng/ml (data not shown). However, at 10 ng/ml (data not shown) and 20 ng/ml, TNF $\alpha$  (fig 4A) strongly induced ET-1 transcription after 2–8 h of stimulation without statistically significant differences between these concentrations and times. This effect was reduced by specific blocking of TNFR1 or TNFR2 (fig 4B). TNF $\alpha$  induced ET-1 release from HASMCs after 4–72 h of stimulation (fig 4C). Surprisingly, TNF $\alpha$ -induced ET-1 transcription after 4 h and ET-1 release after 6 h were both partially blocked by bosentan (fig 4D,E). TNF $\alpha$ -induced ET-1 transcription was also reduced by BQ123 but not by BQ788 (fig 4F,G).

TNF $\alpha$  activated ERK-1/2 at 1, 20 and 50 ng/ml (fig 5A, data not shown) after 5–30 min of stimulation in a concentration-independent manner. TNF $\alpha$  activated  $p38^{MAPK}$  at three time peaks: after 5–45 min, and after 5 and 9 h of stimulation (fig 5B). Bosentan did not significantly reduce ERK-1/2- or  $p38^{MAPK}$  activation in response to 15 min TNF $\alpha$  stimulation (data not shown). TNF $\alpha$ -induced ET-1 transcription was almost



**Figure 2** The endothelin-1 (ET-1) autoregulatory positive feedback loop depends on p38 mitogen-activated protein kinase (p38<sup>MAPK</sup>). Human airway smooth muscle cells (HASMCS) were stimulated with ET-1 at 100 nM for the indicated times (A, B, D), for 5 min (C) or for 2 h (E). Bosentan at 10<sup>-6</sup> M was added to the medium 1 h before ET-1 stimulation (C) or at the times indicated before (pre-incubation) or after (post-incubation) ET-1 addition (D). In (E) PD098059 (10 μM) or SB203580 (10 μM) was added 30 min before ET-1 stimulation. (A, C) After incubation, proteins were extracted and subjected to quantitative immunodetection of activated phosphorylated extracellular signal-regulated kinase-1/-2 (pERK-1/-2) with a pERK-1/-2-specific antibody. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. (B, D) After incubation, the activity of p38<sup>MAPK</sup> was measured by an intracellular ELISA for phosphorylated p38<sup>MAPK</sup> (p-p38<sup>MAPK</sup>) with total p38<sup>MAPK</sup> (t-p38<sup>MAPK</sup>) as a reference. (E) After incubation, the RNA was extracted and subjected to semi-quantitative reverse transcription-PCR (RT-PCR) with ET-1-specific primers. GAPDH was used as a standard. Semi-quantitative RT-PCR signals were evaluated by densitometry. Values for pERK-1/-2 and ET-1 or for p-p38<sup>MAPK</sup> were normalised to GAPDH or to t-p38<sup>MAPK</sup>, respectively, and related to solvent controls (0 h, -). Each graph represents the mean (SEM) of n = 4 (B, D), n = 5 (E), n = 6 (C) or n = 7 (A) individual experiments. One-way repeated measures analysis of variance (ANOVA): p < 0.0001 (A, B, D, E); p = 0.0014 (C, ERK-1); p = 0.0001 (C, ERK-2). Post hoc Bonferroni-Holm tests: exact p values down to 0.01 are indicated in the graph (related to the indicated values or to solvent controls if placed on top of a bar); \*\*p < 0.01; \*\*\*p < 0.001 related to solvent controls; in (B) statistically significant upregulations compared with the solvent control are indicated by black bars; ##p < 0.01; ###p < 0.001 related to values as indicated; rv, relative values.

completely blocked by SB203580 (fig 5C) but not by PD098059 (data not shown). Thus, our data demonstrate that TNF $\alpha$ -induced ET-1 transcription depends on ET<sub>A</sub>R and p38<sup>MAPK</sup> signalling.

### TNF $\alpha$ induces GM-CSF release via activation of endothelin signalling

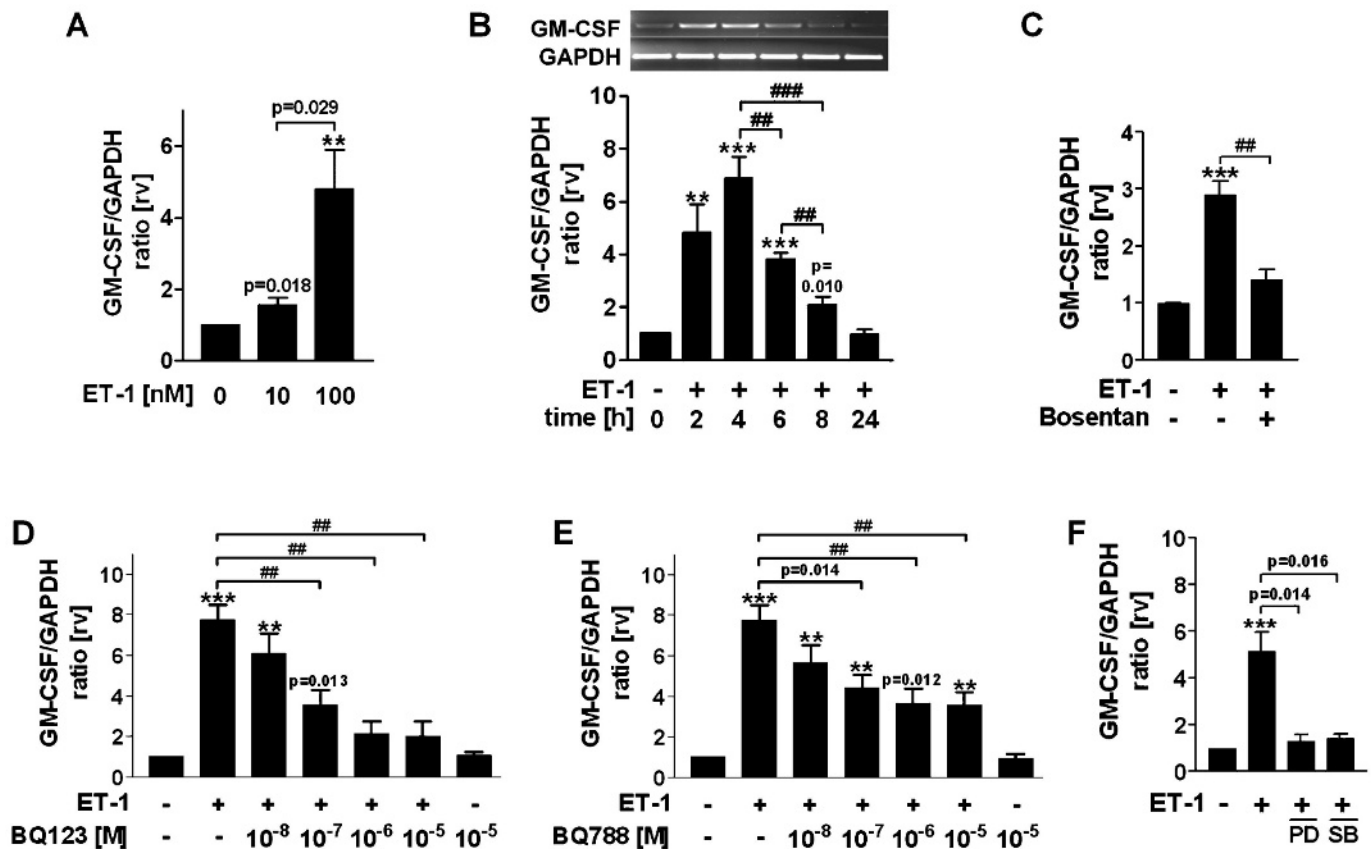
TNF $\alpha$  induced GM-CSF transcription at two peak levels: after 2 and 8–12 h of stimulation (fig 6A). Whereas the 2 h peak was completely reduced by specific blocking of TNFR1 but not of TNFR2, the 8 h peak was partially reduced by individual blocking of both receptor subtypes (fig 6B,C). Bosentan did not modulate GM-CSF transcription after 2 h of TNF $\alpha$  stimulation; however, the drug clearly impaired the induction of GM-CSF transcription after 8 h of TNF $\alpha$  stimulation (fig 6D). Both BQ123 and BQ788 individually blocked TNF $\alpha$ -induced GM-CSF transcription after 8 h with an efficiency similar to bosentan (fig 6E,F). This indicates that unlike the short-term effect, the

long-term effect of TNF $\alpha$  on GM-CSF expression depends on the activation of each endothelin receptor subtype.

TNF $\alpha$  induced a strong GM-CSF release after 72 h, and this effect was reduced by ~65% by bosentan (fig 6G). TNF $\alpha$ -induced GM-CSF release was also partially reduced by PD098059 and completely impaired by SB203580 (fig 6G). The combined treatment of TNF $\alpha$ -stimulated HASMCs with bosentan and PD098059 did not result in an additional reduction of GM-CSF expression compared with the individual treatments (fig 6G). These data demonstrate that TNF $\alpha$ -induced GM-CSF release is mediated by ERK-1/-2 and p38<sup>MAPK</sup> signalling, and depends, at least in part, on endothelin receptor activity.

### DISCUSSION

We found that ET-1 rapidly induces its own expression in HASMCs, suggesting an ET-1 autoregulatory positive feedback loop (fig 7). In contrast to blocking ET<sub>B</sub>R and ERK-1/-2, inhibiting ET<sub>A</sub>R and p38<sup>MAPK</sup> activity almost completely



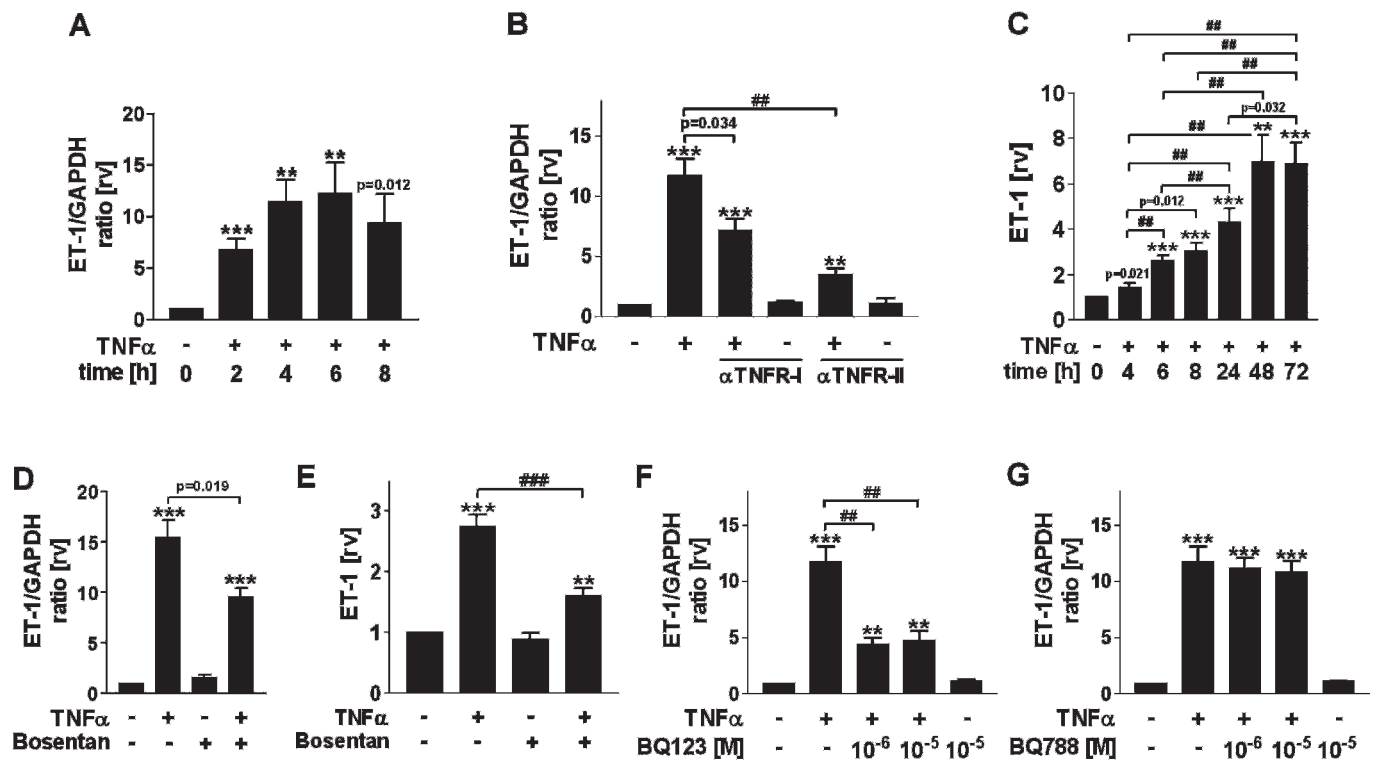
**Figure 3** Endothelin-1 (ET-1) induces granulocyte–macrophage colony-stimulating factor (GM-CSF) expression in human airway smooth muscle cells (HASMCs) via the endothelin receptors  $ET_{A}R$  and  $ET_{B}R$ , extracellular signal-regulated kinase (ERK)-1/-2 and p38 mitogen-activated protein kinase ( $p38^{MAPK}$ ). HASMCs were stimulated with ET-1 at the indicated concentrations (A) or at 100 nM (B–F) for 4 h (A, C–F) or for the indicated times (B). In (C) bosentan at  $10^{-6}$  M and in (D) and (E) selective inhibitors of  $ET_{A}R$  (BQ123) or  $ET_{B}R$  (BQ788) at the indicated concentrations were added to the medium 1 h before ET-1 stimulation. In (F) PD098059 (10  $\mu$ M) or SB203580 (10  $\mu$ M) was added 30 min before ET-1 stimulation. After incubation, the RNA was extracted and subjected to semi-quantitative reverse transcription-PCR (RT-PCR) with GM-CSF-specific primers. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. In (B, top) one representative example of a time-course set of RT-PCRs is shown. Semi-quantitative RT-PCR signals were evaluated by densitometry. Values for GM-CSF were normalised to GAPDH and related to solvent controls (0 nM, –). Each graph represents the mean (SEM) of  $n = 4$  (D–F) or  $n = 5$  (A–C) individual experiments. One-way repeated measures analysis of variance (ANOVA):  $p = 0.0029$  (A);  $p < 0.0001$  (B–F). Post hoc Bonferroni–Holm tests: exact  $p$  values down to 0.01 are indicated in the graph (related to the indicated values or to solvent controls if placed on top of a bar); \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  related to solvent controls; ## $p < 0.01$ ; ### $p < 0.001$  related to values as indicated; rv, relative values.

impaired ET-1-induced ET-1 transcription, demonstrating that activation of the  $ET_{A}R/p38^{MAPK}$  pathway is necessary to initiate and/or maintain this ET-1 feedback mechanism (for further discussion, see the Supplementary online material). The induction of GM-CSF expression by ET-1 has been described for other cell types—for example, lung fibroblasts,<sup>16</sup> but we were first able to demonstrate this ET-1 effect in HASMCs, thereby underlining ET-1 proinflammatory functions. In contrast to ET-1 autoregulation, its effect on GM-CSF transcription depends on the activation of both endothelin receptor subtypes and on  $p38^{MAPK}$  and ERK-1/-2 pathways. Individual  $ET_{A}R$  or  $ET_{B}R$  blockade is sufficient to impair ET-1-induced GM-CSF transcription by ~75% or 50%, respectively, indicating that the combined activation of both receptor subtypes is required for maximum effects.

Here, we demonstrate for the first time that TNF $\alpha$  induces rapid ET-1 transcription and release by HASMCs. In a rat model of airway inflammation, increased ET-1 release in the lungs was observed after 3 h of provocation prior to the main inflammatory response.<sup>28</sup> Since both TNF $\alpha$  and ET-1 are suggested as

initial cytokines in airway inflammation,<sup>7, 28</sup> ET-1 release by HASMCs in response to TNF $\alpha$  might be an initial event in the amplification of inflammation in the airways. Mechanistic insights have been described for cerebrovascular endothelial cells, where the effect of TNF $\alpha$  on ET-1 mRNA upregulation depends on the generation of reactive oxygen species and on the activation of ERK-1/-2,  $p38^{MAPK}$  and their common downstream target mitogen- and stress-activated protein kinase (MSK).<sup>29</sup> Consistently, we have shown here the complete reduction of TNF $\alpha$ -induced ET-1 transcription by a  $p38^{MAPK}$ -specific inhibitor, but, in contrast, that experimental blocking of ERK-1/-2 has no effect. This indicates that TNF $\alpha$  activates ET-1 transcription in HASMCs via the  $p38^{MAPK}/MSK$  rather than the ERK/MSK pathway. However, our data cannot rule out an MSK-independent signalling mechanism.

It has previously been described that TNF $\alpha$ -induced GM-CSF release from HASMCs depends on the activation of the c-Jun N-terminal kinase pathway.<sup>23</sup> We have extended these studies by demonstrating that ERK-1/-2 or  $p38^{MAPK}$  blockade also impairs TNF $\alpha$ -induced GM-CSF release, suggesting that all three



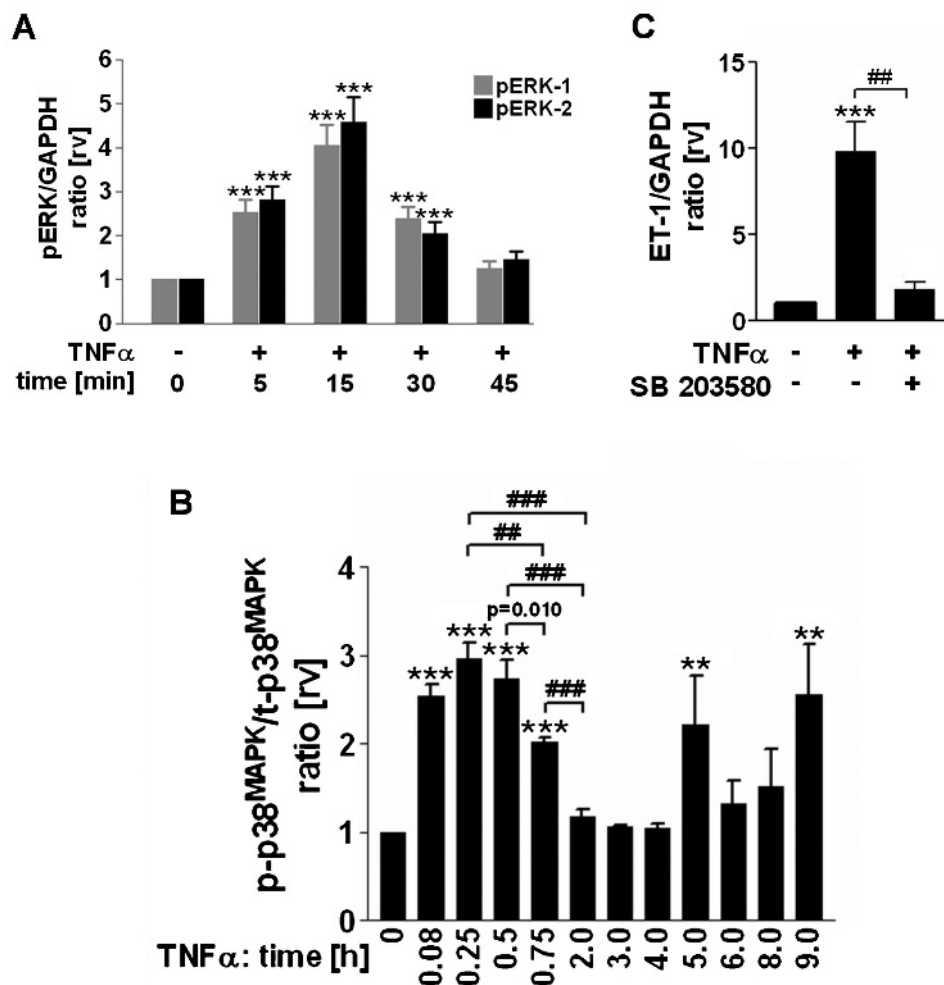
**Figure 4** Tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) induces endothelin-1 (ET-1) expression in human airway smooth muscle cells (HASMCS) via the endothelin receptor ET<sub>A</sub>R. HASMCs were stimulated with human TNF $\alpha$  at 20 ng/ml for the indicated times (A, C) for 4 h (B, D, F, G) or for 6 h (E). (B) Monoclonal blocking antibodies specific for TNF receptor 1 (TNFR1;  $\alpha$ TNFR-I, 10  $\mu$ g/ml) or TNFR2 ( $\alpha$ TNFR-II, 10  $\mu$ g/ml) were individually added to the medium 60 min prior to TNF $\alpha$  stimulation. Bosentan (10<sup>-6</sup> M) (D, E) or selective inhibitors for ET<sub>A</sub>R (BQ123) or ET<sub>B</sub>R (BQ788) (at the indicated concentrations) (F, G) were individually added to the medium 2 h prior to TNF $\alpha$  stimulation. (A, B, D, F, G) After incubation, the RNA was extracted and subjected to semi-quantitative reverse transcription-PCR (RT-PCR) with ET-1-specific primers. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. Semi-quantitative RT-PCR signals were evaluated by densitometry. Values for ET-1 were normalised to GAPDH and related to solvent controls (-). (C, E) After incubation, ET-1 concentrations in supernatants were measured by ELISA, and ET-1 values were normalised to solvent controls. A value of 1 corresponds on average to an ET-1 concentration of 0.45 fmol/ml. Each graph represents the mean (SEM) of n = 4 (B, F, G) or n = 5 (A, C–E) individual experiments. One-way repeated measures analysis of variance (ANOVA): p = 0.0069 (A); p < 0.0001 (B–G). Post hoc Bonferroni–Holm tests: exact p values down to 0.01 are indicated in the graph (related to the indicated values or to solvent controls if placed on top of a bar); \*\*p < 0.01; \*\*\*p < 0.001 related to solvent controls; ##p < 0.01; ###p < 0.001 related to values as indicated; rv, relative values.

prominent proinflammatory MAPK pathways are substantially involved in this process. We showed that TNF $\alpha$  induces GM-CSF transcription at two peak levels after 2 and 8–12 h of stimulation. While the short-term effect depends exclusively on TNFR1, both TNF $\alpha$  receptor subtypes mediate the long-term effect and—notably—also TNF $\alpha$ -induced ET-1 expression, indicating a link between these two signalling events. In addition, bosentan partially inhibited the long-term (but not the short-term) TNF $\alpha$  effect, demonstrating that endothelin receptor activity is necessary to obtain complete induction and/or to maintain the complete level of GM-CSF expression in response to TNF $\alpha$ . Consistently, bosentan reduced TNF $\alpha$ -induced GM-CSF release from HASMCs.

These observations raise the question of a mechanistic link between TNF $\alpha$  and endothelin receptor activation. Since TNF $\alpha$  induces ET-1 release, and ET-1 induces its own expression and that of GM-CSF, we suggest the following model for the long-term effect of TNF $\alpha$  on GM-CSF expression: TNF $\alpha$  activates the ET-1 autoregulatory positive feedback loop by initial activation of the ET-1 gene via the p38<sup>MAPK</sup> pathway. As a consequence, ET-1 autocrinally maintains the increased level of ET-1 expression via ET<sub>A</sub>R and p38<sup>MAPK</sup>, and concurrently induces the expression and release of GM-CSF via both endothelin

receptor subtypes and p38<sup>MAPK</sup> and ERK-1/-2 signalling (fig 7). Our data demonstrating that blocking ET<sub>A</sub>R (but not ET<sub>B</sub>R) activity attenuates TNF $\alpha$ -induced ET-1 expression strongly support the hypothesis of an activation of the ET-1 feedback loop in response to TNF $\alpha$  (for a detailed discussion see the Supplementary online material). In contrast, the short-term TNF $\alpha$  effect on GM-CSF expression is bosentan insensitive and thus independent from endothelin signalling, and might include the canonical and rapid activation of MAPKs by TNFR1. This hypothesis is supported by our data showing a rapid increase of p38<sup>MAPK</sup> and ERK-1/-2 activity as early as 5 min after TNF $\alpha$  stimulation.

HASMCS are believed to contribute to the establishment of inflammation in chronic inflammatory airway diseases and lung fibrosis,<sup>1</sup> and our data suggest that this could be mediated by ET-1 and GM-CSF upregulation in response to pathogenically elevated TNF $\alpha$  levels. Accordingly, the inhibiting effects of bosentan on the TNF $\alpha$ /ET-1/GM-CSF network in HASMCs suggest therapeutic utility for bosentan in the treatment of inflammation in chronic inflammatory airway diseases and lung fibrosis, particularly in early stages. Maximal TNF $\alpha$ -induced GM-CSF expression requires the activity of both ET<sub>A</sub>R and ET<sub>B</sub>R, suggesting that a dual endothelin receptor blocker might



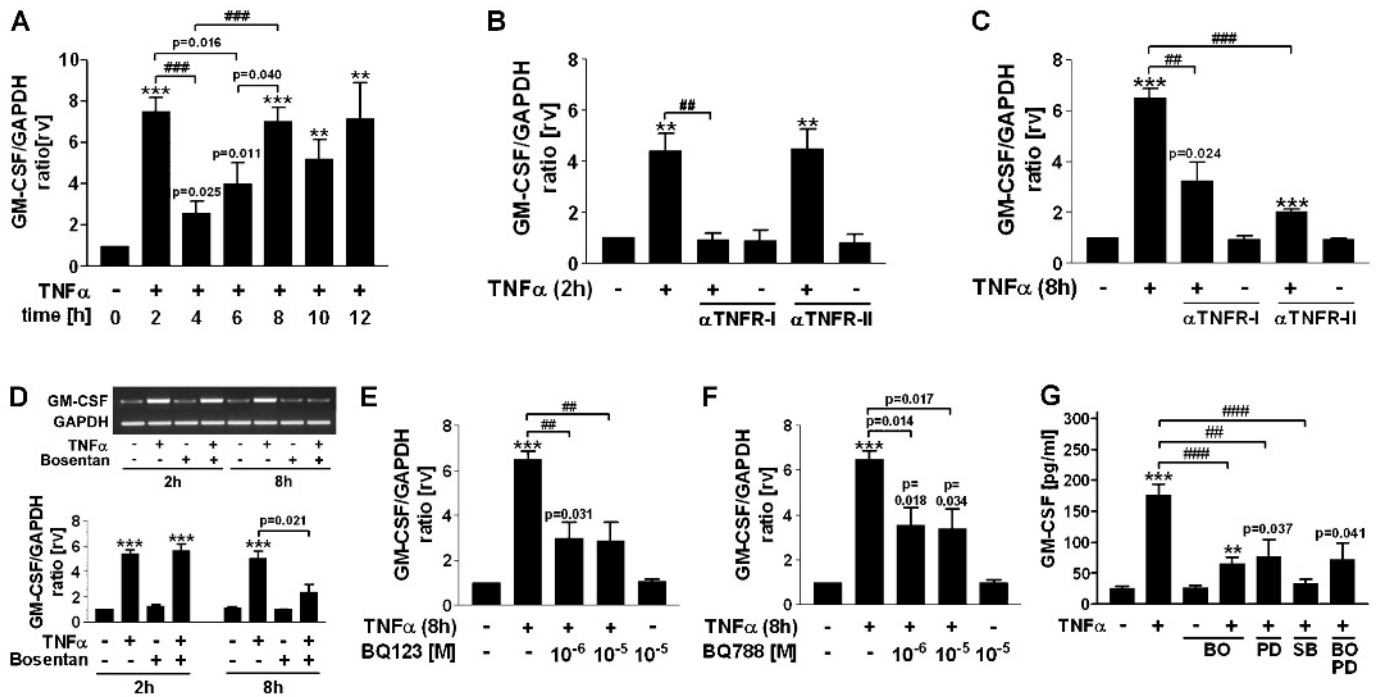
**Figure 5** Tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) induces ET-1 expression in human airway smooth muscle cells (HASMCs) via p38 mitogen-activated protein kinase (p38<sup>MAPK</sup>). HASMCs were stimulated with human TNF $\alpha$  at 20 ng/ml for the indicated times (A, B) or for 4 h (C). In (C) SB203580 (10  $\mu$ M) was added 30 min before TNF $\alpha$  stimulation. (A) After incubation, proteins were extracted and subjected to quantitative immunodetection of phosphorylated extracellular signal-regulated kinase-1/-2 (p-ERK-1/-2). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. (B) After incubation, the amount of phosphorylated (p-) p38<sup>MAPK</sup> was determined in relation to total (t-) p38<sup>MAPK</sup> by intracellular ELISA. (C) After incubation, the RNA was extracted and subjected to semi-quantitative reverse transcription-PCR (RT-PCR) with ET-1-specific primers. GAPDH was used as a standard. Semi-quantitative RT-PCR signals were evaluated by densitometry. Values for pERK-1/-2, p-p38<sup>MAPK</sup> or ET-1 were normalised to GAPDH (A, C) or to t-p38<sup>MAPK</sup> (B), respectively, and related to solvent controls (0 h, -). Each graph represents the mean (SEM) of n = 5 (B, C) or n = 7 (A) individual experiments. One-way repeated measures analysis of variance (ANOVA): p < 0.0001 (A–C, in A values for ERK-1 and ERK-2 were analysed separately). Post hoc Bonferroni–Holm tests: exact p values down to 0.01 are indicated in the graph; \*\*p < 0.01; \*\*\*p < 0.001 related to solvent controls; ##p < 0.01; ###p < 0.001 related to values as indicated; rv, relative values.

be advantageous in therapy over selective blockers. The current study was designed to elucidate a novel signalling pathway with relevance for airway inflammation in order to provide a basis for developing new therapeutic strategies. The efficiency of dual versus selective endothelin receptor antagonists available for therapy in inhibiting the TNF $\alpha$ /ET-1/GM-CSF network or other inflammatory/profibrotic cytokines whose expression is mediated by TNF $\alpha$ /ET-1 has to be deciphered in further studies.

The BUILD-1 study evaluated the efficacy of bosentan in IPF. Bosentan was associated with a trend toward delayed time to disease progression or death, and improvement in quality of life, both of which were more pronounced (and reached statistical significance in a post hoc analysis) in a subgroup of patients with a biopsy-confirmed IPF diagnosis.<sup>19</sup> These observations are currently being investigated in the BUILD-3 trial. Our data suggest that this beneficial effect of bosentan might be a result

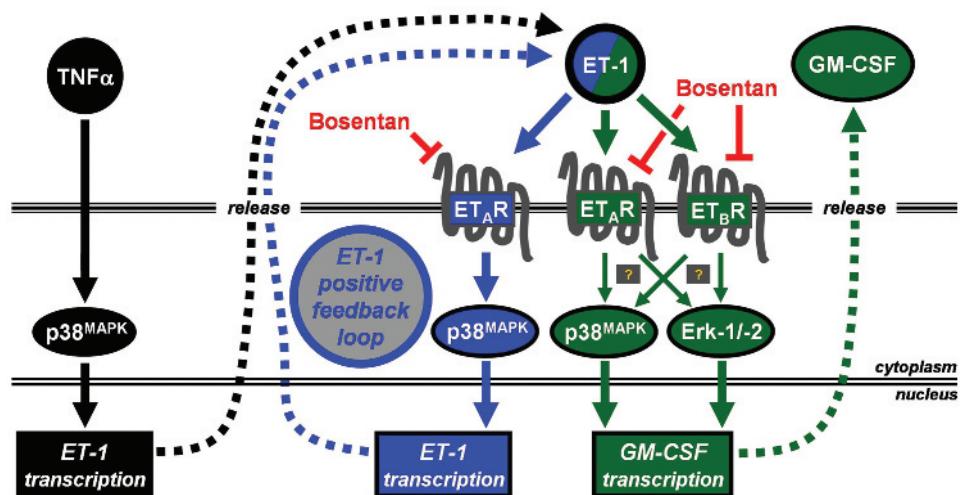
of antagonising airway inflammation associated with the TNF $\alpha$ /ET-1/GM-CSF network.

In summary, we demonstrated that TNF $\alpha$  induces GM-CSF release in HASMCs via the activation of an ET-1 autoregulatory positive feedback loop. Moreover, we provided evidence for the anti-inflammatory properties of the dual receptor antagonist bosentan in HASMCs, and our data predict therapeutic utility for bosentan in the treatment of chronic inflammatory airway diseases and lung fibrosis in the early stages. In this context, treatment with bosentan might be an auspicious therapy helping to reduce the application of high doses of corticosteroids and other immunomodulatory drugs. Furthermore, treatment with bosentan in early disease stages might be useful to avoid the induction of pulmonary hypertension as a late and serious complication of chronic inflammatory airway diseases and lung fibrosis.



**Figure 6** The maintenance of tumour necrosis factor  $\alpha$  (TNF $\alpha$ )-induced granulocyte–macrophage colony-stimulating factor (GM-CSF) expression depends on signalling through TNF receptor 1 (TNFR1) and 2, endothelin receptors ET<sub>A</sub>R and ET<sub>B</sub>R, and p38 mitogen-activated protein kinase (p38<sup>MAPK</sup>) and extracellular signal-regulated kinase (ERK)-1/-2. Human airway smooth muscle cells (HASMCS) were stimulated with human TNF $\alpha$  at 20 ng/ml for the indicated times (A–F) or for 72 h (G). In (B) and (C) monoclonal blocking antibodies specific for TNFR1 ( $\alpha$ TNFR-I, 10  $\mu$ g/ml) or TNFR2 ( $\alpha$ TNFR-II, 10  $\mu$ g/ml) were individually added to the medium 60 min prior to TNF $\alpha$  stimulation. Bosentan (BO; 10<sup>-6</sup> M) (D, G) or selective inhibitors for ET<sub>A</sub>R (BQ123) or ET<sub>B</sub>R (BQ788) (at the indicated concentrations) (E, F) were individually added to the medium 2 h prior to TNF $\alpha$  stimulation. In (G) PD098059 (10  $\mu$ M) or SB203580 (10  $\mu$ M) were added 30 min before TNF $\alpha$  stimulation. (A–F) After incubation the RNA was extracted and subjected to semi-quantitative reverse transcription PCR (RT-PCR) with GM-CSF-specific primers. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. One representative example of a set of RT-PCRs is shown in (D, top). Semi-quantitative RT-PCR signals were evaluated by densitometry. Values for GM-CSF were normalised to GAPDH and related to solvent controls (–). (G) After incubation the absolute amounts of GM-CSF protein in supernatants were determined by ELISA. Each graph represents the mean (SEM) of n = 4 (B–F), n = 5 (A) or n = 9 (G) individual experiments. One-way repeated measures analysis of variance (ANOVA): p < 0.0001 (A, C, D (values for 2 h and 8 h were analysed separately), E, G); p = 0.0001 (B, F). Post hoc Bonferroni–Holm tests: exact p values down to 0.01 are indicated in the graph (related to the indicated values or to solvent controls if placed on top of a bar); \*\*p < 0.01; \*\*\*p < 0.001 related to solvent controls; ##p < 0.01; ###p < 0.001; related to values as indicated; rv, relative values.

**Figure 7** Model to explain the involvement of endothelin signalling in tumour necrosis factor  $\alpha$  (TNF $\alpha$ )-induced granulocyte–macrophage colony-stimulating factor (GM-CSF) expression and release. TNF $\alpha$  is purported initially to activate the endothelin-1 (ET-1) gene via the p38 mitogen-activated protein kinase (p38<sup>MAPK</sup>) pathway (indicated in black). As a consequence, the ET-1 autoregulatory positive feedback loop (indicated in blue) is activated, which depends on the endothelin receptor ET<sub>A</sub>R and p38<sup>MAPK</sup>. In turn, elevated ET-1 levels induce and maintain GM-CSF expression and release via ET<sub>A</sub>R, ET<sub>B</sub>R, p38<sup>MAPK</sup> and extracellular signal-regulated protein kinase (ERK)-1/-2 (indicated in green). The individual impact of the endothelin receptor subtypes on the activation of p38<sup>MAPK</sup> and ERK-1/-2 signalling is unclear (indicated by question marks). Inhibition of endothelin receptor activity by bosentan (indicated in red) impairs the long-term effect of TNF $\alpha$  on GM-CSF expression.





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**Competing interests:** None.

**Ethics approval:** This study has been approved by the ethics committee of the University of Cologne, Cologne, Germany.

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## SUPPLEMENTARY ONLINE MATERIAL

### METHODS

#### Cultivation of HASMCs

HASMCs were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Karlsruhe, Germany; cat#-31885-023) with 10% FCS (Sigma; cat#-N-4637), sodium pyruvate (1 mM; Invitrogen; cat#-11360-039), L-glutamine (2 mM; Sigma cat#-G-7513), non-essential amino acids (1%; Invitrogen; cat#-11140-035), penicillin (100 U/ml), streptomycin (100 µg/ml; Sigma; cat#-P-4333), and amphotericin B (1.5 µg/ml; Sigma cat#-A-2942) in a humidified atmosphere at 37° C, 5% CO<sub>2</sub>. Subconfluent cells were passaged with trypsin (0.25%; Sigma; cat#-T-4674) and 1 mM EDTA. HASMCs were used for experiments at passages 2-7. Cells were plated at equal density into 6-well plates. HASMCs were characterized by positive immunostaining for calponin, smooth muscle  $\alpha$ -actin, and myosin heavy chain.

#### Stimulation of HASMCs: Media

*Serum withdrawal:* serum-free and low glucose (1g/l) DMEM (Invitrogen; cat#-41966-029) supplemented with 1mM sodium pyruvate, 2mM L-glutamine, non-essential amino acids, 100U/ml penicillin, 100µg/ml streptomycin, 1.5µg/ml amphotericin B, 1mM insulin, 5mg/ml apo-transferrin and 100µM ascorbic acid. *Stimulation:* serum-free and low glucose (1g/l) DMEM supplemented with 1mM sodium pyruvate, 2mM L-glutamine, non-essential amino acids, 100U/ml penicillin, 100µg/ml streptomycin, and 1.5µg/ml amphotericin B.

#### Semiquantitative RT-PCR (PCR conditions and primer sequences)

PCR conditions for ET-1: denaturation, 94°C, 30 sec; annealing, 61°C, 30 sec; extension, 72°C, 30 sec; 39 cycles; ET-1 forward primer 5'-GAG AGG TCC ATT GTC ATC CCC-3', ET-1 reverse primer 5'-TGT GGC GAC TCT GCA CTC-3'. PCR conditions for GM-CSF: denaturation, 94°C, 30 sec; annealing, 55°C, 30 sec; extension, 72°C, 30 sec; 35 cycles; GM-CSF-primer pairs were purchased from R&D systems (cat#-RDP-34). PCR conditions for GAPDH: denaturation, 94°C, 45 sec; annealing, 60°C, 45 sec; extension, 72°C, 45 sec; 27 cycles; GAPDH forward primer 5'-TCT AGA CGG CAG GCT AGG TCC ACC-3', GAPDH reverse primer 5'-ACG GTA CCT TAA ACG GTA CCC ACC-3'.

#### ELISA protocols

ET-1 ELISA was performed according to the instructions of the manufacturer (Biomedica, Vienna, Austria cat#-BI 20052). Briefly, 50 µl of supernatants together with monoclonal mouse anti-human-endothelin antibody were incubated in 96-wells (coated with polyclonal anti-human ET-1 antibody) for 16 hours at room temperature. Following washing, HRP-coupled anti-mouse IgG was added for one hour at room temperature. After washing, detection was performed with TMB as a substrate and measuring extinction at 450 nm with 620 nm as reference. Quantification was done by interpolation from a standard curve with the lower limit of detection of 0.3 fmol/ml.

Big-ET-1 ELISA (that does not crossreact with ET-1) was performed according to the instructions of the manufacturer (Biomedica; cat#-BI 20082). Briefly, 50 µl of supernatants of HASMC cultures together with HRP-coupled monoclonal mouse anti-human big-ET-1 antibody were incubated in 96-wells (coated with polyclonal sheep anti-human big-ET-1 antibody directed against amino acids 22-38) for 4 hours at room temperature. After washing, detection was performed with TMB as a substrate and measuring extinction at 450 nm with

620 nm as reference. Quantification was done by interpolation from a standard curve with the lower limit of detection of 0.02 fmol/ml.

Intracellular ELISA for active p38<sup>MAPK</sup> (R&D Systems; cat#-KBC869) on subconfluent HASMCs on a 96-well plate was performed according to the instructions of the manufacturer. This assay is based on the simultaneous detection of phosphorylated (active) p38<sup>MAPK</sup> and total p38<sup>MAPK</sup> as a reference. Briefly, cells were fixed and lysed with 4% formaldehyde for 20 minutes at room temperature. After washing, fixed cells were treated with quenching buffer (20 minutes at room temperature), and, after additional washing, with 10% fetal bovine serum for blocking (one hour, room temperature). After washing, a mixture of rabbit anti-human-phospho-p38<sup>MAPK</sup> (T180/Y182) antibody and mouse anti-human-total-p38<sup>MAPK</sup>-antibody was added for 16 hours at 4° C. In order to distinguish between phosphorylated p38<sup>MAPK</sup> and the total cellular p38<sup>MAPK</sup> content the respective specific antibodies added have to be derived from different species. After washing, a secondary antibody mixture (HRP-conjugated anti-rabbit IgG and AP-conjugated anti-mouse IgG) was added for two hours at room temperature. After washing, HRP-fluorogenic substrate was added for 60 minutes and, afterwards, AP-fluorogenic substrate was added for 5 minutes. Phospho-p38<sup>MAPK</sup> was measured using a fluorescence plate reader with excitation at 540 nm and emission at 600 nm, total p38<sup>MAPK</sup> was measured with excitation at 360 nm and emission at 450 nm. Values for phospho-p38<sup>MAPK</sup> were normalized to values for total p38<sup>MAPK</sup>.

### **Western Blotting**

The activation status of ERK-1 and ERK-2 was assessed by Western immunoblot analysis using antibodies that recognize the dual phosphorylated (activated) form of the enzymes. After treatment, HASMCs were lysed in lysis buffer (20 mM Tris base, pH 7.4; 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM NaCl, 5 mM EDTA), supplemented with the proteinase inhibitors PMSF (500 µM), Na-orthovanadate (2 mM), leupeptin (10 µg/ml), aprotinin (25 µg/ml), pepstatin (10 µg/ml), NaF (1.25 mM), and Na-pyrophosphate (1 mM). Insoluble protein was removed by centrifugation at 12,000 x g for 5 min and aliquots of the resulting supernatant were diluted 1:4 in Laemelli buffer (62.5 mM Tris-HCl, 10% v/v glycerol, 1% w/v SDS, 1% β-mercaptoethanol, 0.01% w/v bromophenol blue, pH 6.8) and boiled for 5 min. Denatured proteins (40 µg) were subsequently separated by SDS/PAGE upon 10% vertical slab gels and transferred to Hybond ECL membranes (Amersham, Buckinghamshire, UK) in blotting buffer (Tris base 50 mM, pH 8.3; glycine 192 mM) supplemented with 20% vol/vol methanol. The filter was incubated for 1 h in TBS-Tween-20 (25 mM Tris-base, 150 mM NaCl, pH 7.6 and 0.1% Tween 20, 10% [w/v] nonfat milk and incubated 2 h in TBS-Tween 20 containing 3% BSA and either primary antibodies raised against pERK-1/-2 (Cell Signaling Technology/New England Biolabs, Frankfurt, Germany; cat#-9101) or GAPDH (each diluted 1:1,000). Following 3 x 10-min washes in TBS-Tween 20, the membranes were incubated for 60 min with a goat anti-rabbit peroxidase-conjugated IgG antibody diluted 1:5,000 in TBS-Tween 20 supplemented with 1% nonfat milk for ERK-1/-2, and for 60 min with a rabbit, anti-mouse peroxidase-conjugated IgG antibody diluted 1:4,000 in TBS-Tween 20 supplemented with 5% nonfat milk for GAPDH and then washed again (3 x 10 min). Antibody-labeled proteins were subsequently visualized by enhanced chemiluminescence (Amersham).

## DISCUSSION

### **Hypothesis: TNF $\alpha$ -induced GM-CSF release depends on activation of an p38<sup>MAPK</sup>-dependent ET-1 autoregulatory positive feedback mechanism – additional support.**

**ET-1 autoregulatory positive feedback mechanism.** It has been reported for other cell types that large amounts of ET-1 rapidly (within a few minutes) and pseudo-irreversibly bind to its receptors.[1,2] Due to the rapid internalization of the ET-1/endothelin-receptor complexes (again within a few minutes) followed by their intracellular dissociation and an externalization of unoccupied and functional endothelin receptors to the plasma membrane, free (unbound) ET-1 has an extremely short half-life time in the extracellular milieu.[1,3] This might also apply to HASMC culture since we were not able to detect significant ET-1 amounts in the supernatant anymore two hours after stimulation with 100nM ET-1 (Supplementary Fig. 1). Three experiments strongly support the hypothesis that stimulation with ET-1 induces *de novo* synthesis and release of big-ET-1 followed by its maturation to active ET-1 (autoregulatory positive feedback): (1) the rapid increase of ET-1 mRNA levels in response to ET-1 stimulation (Fig. 1B), (2) the release of big-ET-1 after ET-1 stimulation (Fig. 1D), and (3) the presence of ET-1 in the culture medium four hours after exogenous ET-1 addition although two hours after stimulation the exogenously added ET-1 was not detectable in the supernatant anymore (Supplementary Fig. 1).

**The ET-1 autoregulatory positive feedback depends on p38<sup>MAPK</sup> activity.** The induction of big-ET-1 release by ET-1 is maintained for up to eight hours (Fig. 1D), and ET-1 induces four peaks of p38<sup>MAPK</sup>-activation: after five to 30 minutes, two, four and 6.5 hours of stimulation (Fig. 2B). Pre-treatment with Bosentan would be expected to abolish big-ET-1 release and all p38<sup>MAPK</sup>-activation peaks. Indeed, we demonstrated this for short- (3h) and long-term (8h) big-ET-1 release (Fig. 1E) and exemplary for the initial (5-30 min) p38<sup>MAPK</sup>-activation peak (Fig. 2D). We hypothesize that the long-term p38<sup>MAPK</sup>-activation peaks are the consequence of p38<sup>MAPK</sup> re-activation through *de novo* synthesized ET-1. Strong support comes from the experiment showing that the third (4h) p38<sup>MAPK</sup>-activation peak was attenuated through the addition of Bosentan to the culture medium at a time (3 hours after ET-1 addition) when the exogenously added ET-1 is not detectable anymore (see above) and the second (2h) peak of p38<sup>MAPK</sup>-activation has already receded (Fig. 2D). Notably, addition of Bosentan at the same time also partially impaired the long-term (8h) expression of big-ET-1 in response to ET-1 stimulation (Fig. 1E). This indicates that p38<sup>MAPK</sup> re-activation is required for the maintenance of ET-1 *de novo* synthesis and thus for the maintenance of the autoregulatory positive ET-1 feedback mechanism in HASMCs. As Bosentan inhibits endothelin signalling through extracellular binding to endothelin receptors these data also exclude the possibility that continued signalling via internalized ET-1/endothelin receptor complexes (as it has been proposed for other cell types [4,5]) governs the re-activation of p38<sup>MAPK</sup> or the maintenance of big-ET-1 release. As Bosentan added after the first (5-30 min) peak of p38<sup>MAPK</sup>-activation did not abolish the second (2h) peak, this early re-activation of p38<sup>MAPK</sup> might be independent of *de novo* synthesised ET-1. In agreement we did not find ET-1 in the HASMC supernatant after two hours of ET-1 stimulation (Supplementary Figure 1). Instead, this second p38<sup>MAPK</sup>-activation peak could be induced by other, yet unidentified, factors that are rapidly up-regulated by ET-1 stimulation or by signalling from internalized ET-1/endothelin-receptor complexes.

**The ET-1 autoregulatory positive feedback depends on ET<sub>A</sub>R but not on ET<sub>B</sub>R.** As selective ET<sub>A</sub>R- but not ET<sub>B</sub>R-antagonism blocks ET-1-induced ET-1 transcription, our data demonstrate that ET-1-induced ET-1 expression is triggered by ET<sub>A</sub>R-mediated p38<sup>MAPK</sup>

signalling but not by ET<sub>B</sub>R. This raises the question why ET<sub>B</sub>R activation does not contribute to ET-1 expression? ET-1 binding to its receptors results in the activation of complex intracellular signalling networks that include besides MAP-kinases numerous other signalling pathways, e.g. depending on phospholipase C or Rho kinase.[6,7] The ET<sub>A</sub>R-triggered signalling network might contain besides p38<sup>MAPK</sup> at least one other, yet unidentified, pathway with a key role for ET-1 gene induction, which, however, is not activated by ET<sub>B</sub>R. As the differences between the ET<sub>A</sub>R- and ET<sub>B</sub>R-triggered signalling networks and targeted transcription factors are not well understood for HASMCs or any other cell type, the mention of candidate pathways would remain highly speculative. Nevertheless, this theory can just as well explain the failure of activated ET<sub>B</sub>R to induce ET-1 expression as the hypothesis that the p38<sup>MAPK</sup> pathway itself might not be associated with ET<sub>B</sub>R in HASMCs. However, the latter assumption might be rather unlikely as ET<sub>B</sub>R-dependent p38<sup>MAPK</sup> activation has been reported for cell types closely related to HASMCs.[8]

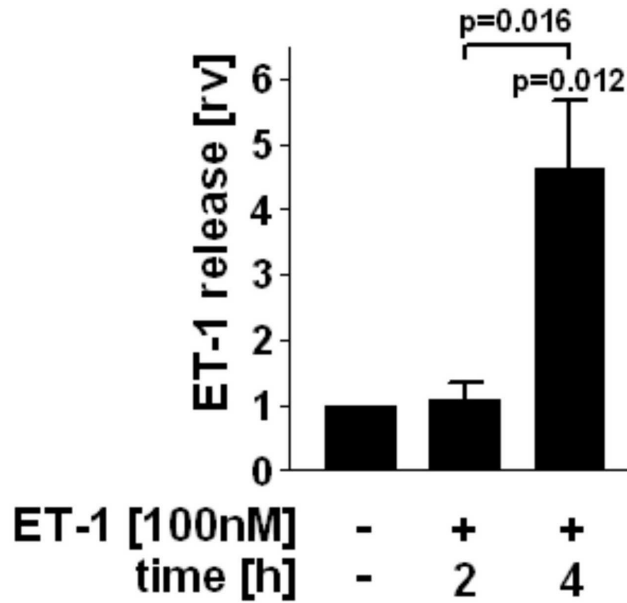
**Induction of the ET-1 autoregulatory feedback mechanism by TNF $\alpha$  in order to maintain GM-CSF expression.** ET-1 release induced by ET-1 itself and by TNF $\alpha$  as well as ET-1-induced GM-CSF transcription all are very rapid processes with measurable outcomes after 2-4 hours of stimulation (Fig. 1D; 3B; 4C). A comparable rapid release of *de novo* synthesised ET-1 in response to TNF $\alpha$  has been demonstrated for cultured rat mesangial cells.[9] The reducing effect of endothelin receptor inhibitors on TNF $\alpha$ -induced GM-CSF transcription in HASMCs after eight hours of stimulation could sufficiently be explained by a cascade of TNF $\alpha$ -induced ET-1 release with subsequent ET-1-induced GM-CSF transcription. However, there are two major arguments for an involvement of the ET-1 feedback mechanism as a substantial part of this cascade maintaining GM-CSF expression. First support is found when matching the ET-1- with the TNF $\alpha$ -induced p38<sup>MAPK</sup> activation patterns (compare Fig. 2B with 5B). Besides the expected short-term activation peak (5-45 min) in response to TNF $\alpha$ , p38<sup>MAPK</sup> re-activation was observed after five and nine hours of stimulation. Given that TNF $\alpha$  induces (low but measurable) ET-1 release after four and six hours (Fig. 4C) and that initial p38<sup>MAPK</sup> activation by ET-1 occurs within five to 30 minutes (Fig. 2B) it is reasonable to suggest that the TNF $\alpha$ -induced five hour p38<sup>MAPK</sup> re-activation peak reflects an “initial” p38<sup>MAPK</sup> activation through ET-1 that is expressed and released in response to TNF $\alpha$ . Moreover, exogenous ET-1 addition leads to p38<sup>MAPK</sup> re-activation via *de novo* synthesized ET-1 about four hours after initial p38<sup>MAPK</sup> activation (Fig. 2B,D). This time lag matches with the time lag between the five and the nine hour p38<sup>MAPK</sup> re-activation peaks in response to TNF $\alpha$  underlining the hypothesis of an induction of the ET-1 autoregulatory positive feedback by TNF $\alpha$ .

Further and strong support for this hypothesis comes from the experiments in Figures 4D-F showing that TNF $\alpha$ -induced ET-1 expression can partially be abolished by ET<sub>A</sub>R inactivation with BQ123 and by Bosentan. As the literature does not provide any evidence for a direct TNF $\alpha$ /ET<sub>A</sub>R interaction the only reasonable explanation for the effects of BQ123 and Bosentan is that ET-1 released in response to TNF $\alpha$ -stimulation activates ET<sub>A</sub>R to further maintain ET-1 expression implying the activation of the ET-1 autoregulatory positive feedback by TNF $\alpha$ . Bosentan already blocks ET-1 release after six hours of TNF $\alpha$ -stimulation (Fig. 4E) and ET-1 can induce GM-CSF transcription within two hours (Fig. 3B). Thus, a cascade of TNF $\alpha$ -induced ET-1 release with subsequent ET-1-induced ET-1 release and ET-1-induced GM-CSF transcription would also coincide with the long-term effect (eight to twelve hours, Fig. 6A) of TNF $\alpha$  on GM-CSF transcription. Moreover, the activation of the ET-1 positive feedback mechanism by TNF $\alpha$  can also explain (1) the increasing TNF $\alpha$ -induced ET-1 release for up to 72 hours, and (2) the maintenance of TNF $\alpha$ -induced GM-CSF release over a 48 hour-period (after 24 to 72 hours of stimulation, Fig. 6G; [10]).

Summarized, our data provide strong evidence for an activation of the ET-1 autoregulatory positive feedback mechanism by TNF $\alpha$  in HASMCs to maintain ET-1 and GM-CSF expression.

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**Supplementary Figure 1. ET-1 is rapidly removed from the extracellular milieu in HASMC culture and induces its own release.** HASMCs were stimulated with ET-1 at 100nM for times as indicated. After incubation concentrations of ET-1 in supernatants were measured by ELISA. ET-1 values were normalized to solvent controls. The graph represents the mean  $\pm$  SEM of n=4 individual experiments. One-way repeated measures ANOVA: p=0.0034. Post hoc Bonferroni-Holm tests: exact p-values are indicated in the graph related to non-stimulated controls if placed on top of a bar or to values as indicated; rv, relative values.