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Dexamethasone reverses monocrotaline-induced pulmonary arterial hypertension in rats

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ABSTRACT: **Pulmonary arterial hypertension (PAH) is associated with dysregulated bone morphogenetic protein receptor (BMPR)-II signaling and pulmonary vascular inflammation. We evaluated the effects of dexamethasone on monocrotaline (MCT)-induced PAH in rats for potential reversal of PAH at late time-points.**

Saline-treated control, MCT-exposed, MCT-exposed and dexamethasone-treated rats (5 mg·kg^{-1}·day^{-1}, 1.25 mg·kg^{-1} and 2.5 mg·kg^{-1}·48 h^{-1}) were evaluated at day 28 and day 35 following MCT for haemodynamic parameters, right ventricular hypertrophy, morphometry, immunohistochemistry, and *IL6* and *BMPR2* expression.

Dexamethasone improved haemodynamics and pulmonary vascular remodelling, preventing PAH development at early (day 1–14 and 1–28) and reversing PAH at late (day 14–28 and 21–35) time-points following MCT, as well as improving survival in MCT-exposed rats compared with controls. Both MCT-induced pulmonary *IL6* overexpression and interleukin (IL)-6-expressing adventitial inflammatory cell infiltration were reduced with dexamethasone. This was associated with pulmonary *BMPR2* downregulation following MCT, which was increased with dexamethasone, in whole lung and control pulmonary artery smooth muscle cells. Dexamethasone also reduced proliferation of rat pulmonary artery smooth muscle cells *in vitro*.

Experimental PAH can be prevented and reversed by dexamethasone, and survival is improved. In this model, mechanisms may involve reduction of IL-6-expressing inflammatory cells, restoration of pulmonary *BMPR2* expression and reduced proliferation of vascular smooth muscle cells.

KEYWORDS: **Bone morphogenetic protein receptor, corticosteroids, inflammation, monocrotaline, pulmonary arterial hypertension, type II**

Pulmonary arterial hypertension (PAH) is characterised by a progressive increase in pulmonary vascular resistance, ultimately leading to right ventricular failure and death [1]. The principal pathological finding is remodelling of small pulmonary arteries with marked proliferation of pulmonary artery smooth muscle cells (PASMC), resulting in obstruction of these resistance pulmonary arteries [2]. Inflammatory mechanisms are believed to play a key role in both human and experimental PAH [3]. In idiopathic (I)PAH, infiltrates of macrophages and lymphocytes are found in the range of plexiform lesions with local expression of chemokines CC motif ligand (CCL)2 (monocyte chemotactic protein-1), CCL5 (RANTES (regulated on activation, normal T-cell expressed and secreted)) and CX_3C motif ligand 1 (fractalkine) [4–7]. Histopathological specimens from patients displaying severe PAH in the context of connective tissue diseases suggest that inflammation and remodelling are key contributors to pulmonary vascular disease complicating inflammatory diseases [4]. Proinflammatory cytokines, including interleukin (IL)-1 and IL-6, are elevated in both human IPAH [8] and MCT-induced PAH [9, 10]. Autoimmunity is also demonstrated to contribute to PAH in patients characterised by circulating autoantibodies [11]. Pathogenic autoantibodies target endothelial cells and may induce vascular endothelial apoptosis, promoting PAH development [12].

The suggestion that treatment with corticosteroids and/or immunosuppressants may dramatically improve PAH stems from the improvement seen

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in associated PAH following treatment for coexisting systemic inflammatory conditions, including POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal immunoglobulin and skin changes) syndrome [13], Castleman's disease [14], systemic lupus erythematosus (SLE) [15, 16] and mixed connective tissue disease [15, 16]. Immunosuppressive therapies including rapamycin [17] and cyclosporin [18] have been shown to attenuate the development of PAH in rats exposed to MCT, including established PAH [17, 18]. Earlier studies have shown that steroids prevent the development of MCT-induced PAH [9, 19–21], although no studies have yet shown that steroids reverse established MCT-induced PAH.

Mutations in the bone morphogenetic protein receptor (BMPR)-II gene *(BMPR2)* have been identified in >50% of familial (F)PAH patients and 10–25% of IPAH patients [22, 23]. Reduced levels of *BMPR1a* [24] and *BMPR2* [25, 26] mRNA expression are seen in the lungs of patients with heritable PAH and IPAH, and in other subtypes of PAH. This reduction in pulmonary *BMPR2* is mirrored in MCT-induced PAH [27, 28]. These mutations disrupt bone morphogenetic protein (BMP)/Smad-mediated signalling [29], potentiate BMP/mitogen-activated protein kinase signalling [30] and could underlie the abnormal vascular cell proliferation observed in FPAH [31]. Interestingly, several studies suggest that dysregulation of the BMP pathway leads to vulnerability to an inflammatory second hit [32–34].

The aim of this study was to test the effects of dexamethasone on pulmonary haemodynamics, and *IL6* and *BMPR2* expression in the asymptomatic and symptomatic phases of development of MCT-induced PAH in rats. We hypothesised that dexamethasone treatment could reverse haemodynamics in established MCT-induced PAH, and that haemodynamic improvements would correlate with normalisation of *IL6* and *BMPR2* mRNA levels.

METHODS

Study design

Male Wistar rats (100 g body weight) were maintained in a temperature-controlled room with a 12/12-h light/dark cycle and randomly divided into: 1) a saline-treated control group (n=20); 2) an MCT-exposed group (n=20); 3) an MCT-exposed and 5 $mg \cdot kg^{-1} \cdot day^{-1}$ (day 1–14) dexamethasone-treated group (MCT+Dex D1–14; n=20); 4) an MCT-exposed and 5 $mg \cdot kg^{-1} \cdot day^{-1}$ (*i.p.*; day 1–28) dexamethasone-treated group (MCT+Dex D1–28; n=10); 5) an MCT-exposed and dexamethasone-treated (three dose ranges of 5 $mg \cdot kg^{-1} \cdot day^{-1}$ (Dex5), 2.5 $mg \cdot kg^{-1} \cdot 48\ h^{-1}$ (Dex2.5) and 1.25 $mg \cdot kg^{-1} \cdot 48\ h^{-1}$ (Dex1.25); day 14–28; MCT+Dex D14–28) (n=10 per group); and 6) an MCT-exposed and 5 $mg \cdot kg^{-1} \cdot day^{-1}$ (day 21–35) dexamethasone-treated group (MCT+Dex D21–35) (n=10 per group). All rats had access to standard rat chow and water *ad libitum*. For MCT administration, rats received a single subcutaneous injection of 60 $mg \cdot kg^{-1}$ MCT (Sigma–Aldrich, Lyon, France), which was dissolved in 1 N HCl, and the pH was adjusted to 7.4 with 1 N NaOH. Ten rats from groups 1–3 were sacrificed to perform experiments 14 days after the MCT exposure and the remaining 10 rats from each group were then sacrificed at 28 or 35 days after MCT exposure.

Haemodynamics

As described by STINGER *et al.* [35], a 3.5 French umbilical vessel catheter (Tyco, Plaisir, France), angled to 90° over the distal 1 cm and curved slightly at the tip, was introduced into the right external jugular vein of rats anaesthetised with 35 $mg \cdot kg^{-1}$ ketamine, 4 $mg \cdot kg^{-1}$ xylasine and 0.5 $mg \cdot kg^{-1}$ acepromazine. Following deaths during anaesthesia in preliminary experiments, presumed relative adrenal suppression was managed using a 5 $mg \cdot kg^{-1}$ dose of *i.p.* dexamethasone prior to induction of anaesthesia. With the angle directed anteriorly, the catheter was inserted 2.5 cm proximally, which placed the catheter in the right atrium. The catheter was rotated 90° anticlockwise and inserted 1 cm further, which placed the catheter in the right ventricle, and when advanced an additional 1.5 cm, in the pulmonary artery. Placement at each stage was confirmed by respective pressure contours. Haemodynamic values were automatically calculated by the physiological data acquisition system Cardiomax III (Phymep, Paris, France). Following exsanguination, the lungs were distended by infusion of Optimal Cutting Temperature compound (Miles, Epernon, France) diluted in PBS (1:1) into the trachea, quick-frozen in isopentane on dry ice and stored at -80°C. For Fulton's index of right ventricular hypertrophy, the ratio of the right ventricular weight to left ventricular plus septal weight (RV/LV+S) was calculated.

Gene quantification by quantitative real-time RT-PCR

RNA was extracted from rat lungs using the Total RNA Isolation Mini Kit (Agilent Technologies, Massy, France) and then eluted from silicate columns and reverse-transcribed using the Omniscript Reverse Transcription Kit (Qiagen, Courtaboeuf, France). Constitutively expressed β-actin was selected as an internal housekeeping gene control for the comparative C_T method for the relative quantification of *BMPR2* and *IL6* mRNA expression. *BMPR2*, *IL6* and β-actin expression was quantified by RT-PCR using TaqMan Gene Expression Assays (β-actin Rn00667869_m1, *BMPR2* Rn01437210_m1 and *IL6* Rn00561420_m1), TaqMan Universal PCR Master Mix and an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Courtaboeuf, France).

Immunohistochemistry

Immunohistochemistry was performed on 8-μm sections of frozen tissue (-80°C). After routine preparation, slides were processed with the primary antibody anti-IL-6 (1:600; Abcam rabbit polyclonal ab6672; Abcam, Cambridge, UK), then with the secondary antibody (anti-rabbit; kit En Vision+/HRP; Dako, Trappes, France). Controls used for these antibodies included omission of the primary antibody and incubation with irrelevant immunoglobulins of the same isotype.

Pulmonary artery morphometry

Sections of paraffin-embedded lungs were prepared and stained with haematoxylin and eosin. The slides were evaluated by light microscopy, and the extent of vascular remodelling was assessed by a researcher blinded to the treatment groups. Three whole left lung sections from each rat were evaluated. The percentage medial wall (media/external diameter (ED) × 100) and adventitial thickness (adventitia/ED × 100) of fully muscularised, pre-acinar pulmonary arteries was measured, using 10 randomly chosen vessels from each of the three sections for each rat. For arterioles (<80 μm ED), the degree of muscularisation was score of a scale of 1–3, where: 1=no muscularisation, not occluded; 2=muscularisation, not occluded; and 3=muscularisation, fully occluded).

Primary smooth muscle cell isolation and culture

At baseline (control) and 21 days following exposure to MCT, rats were sacrificed using an overdose of pentobarbital. The lungs were immediately removed and proximal pulmonary arteries were isolated. PASMCs were isolated by enzymatic digestion [36], with purity and verification of PASMCs using immunostaining for smooth muscle α-actin as previously described [37].

Measurement of PASMC proliferation

PASMCs were cultured to 80% confluence in passage 3–6. On day 0 of the proliferation assay, cells were detached with 0.05% trypsin and 0.02% EDTA and seeded in 48-well plates at a density of 5×10^4 cells·well^{-1} (in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS)). After 48 h of incubation (day 2), the cells were serum-starved (in DMEM with 0.1% FCS) for 24 h. On day 3, cells were washed twice with PBS and recultured in DMEM (10% FCS) with dexamethasone (Sigma–Aldrich). On the basis of preliminary experiments examining the antiproliferative effects of dexamethasone on 10% FCS-stimulated rat PASMCs, the final concentrations used were 10^{-7}–10^{-8} M. Controls were cultured in DMEM with 10% FCS. On day 4, 24 h after the addition of dexamethasone, the cells were labelled with ^{3}H-thymidine at 1 μCi·mL^{-1} for 24 h and frozen at -80°C. After labelling was completed in all samples, the cells were washed *in situ* with 500 μL ice-cold uptake buffer, lysed with 500 μL of 0.1 N NaOH and the radioactivity counted using liquid scintillation spectroscopy [37].

Statistical analysis

Data are presented as mean±SEM, unless otherwise stated. Data were analysed using the nonparametric Kruskal–Wallis test followed by Dunn's test for multiple comparisons, the Mann–Whitney U-test and Spearman's rank correlation. Differences were considered significant at p-values <0.05. Analyses were performed using Statview 5.0.

RESULTS

Dexamethasone treatment improves survival in established MCT-induced PAH

Treatment with 5 mg·kg^{-1}·day^{-1} dexamethasone from day 14–28 significantly improved survival assessed at day 28 compared with MCT alone (log rank test p<0.0001; fig. 1). Survival was also significantly improved at day 35 following day 21–35 dexamethasone (data not shown).

Dexamethasone treatment normalises haemodynamics and right ventricular hypertrophy in established MCT-induced PAH

28 days after MCT administration, haemodynamic measurements showed a significant increase in mean pulmonary arterial pressure ($\bar{P}$pa; 40.8±6.4 *versus* 16.4±1.5 mmHg in control animals; p<0.0001; fig. 2a), right ventricular systolic pressure (RVSP; 94.3±7.8 *versus* 35.2±2.4 mmHg in control rats; p<0.0001; fig. 2b) and RV/LV+S (0.6±0.1 *versus* 0.26±0.06 in controls; p<0.0001; fig. 2c). MCT+Dex D14–28 administration normalised the $\bar{P}$pa (p<0.05 for Dex5 and Dex2.5; p=0.066 for Dex1.25), RVSP (p<0.0001 for Dex5 and Dex2.5, and p<0.05 for Dex1.25) and RV/LV+S (p<0.0001 for all doses) in a dose-dependent manner. Dex5 was not statistically different to control rats (p=0.11). MCT+Dex D21–35 also normalised $\bar{P}$pa, RVSP and RV/LV+S (p<0.05 for all compared to MCT alone; fig. 3). Haemodynamic indices were also normalised in all earlier, preventative-phase dexamethasone-treated groups (MCT+Dex D1–14 and D1–28; p<0.001 for both; data not shown).

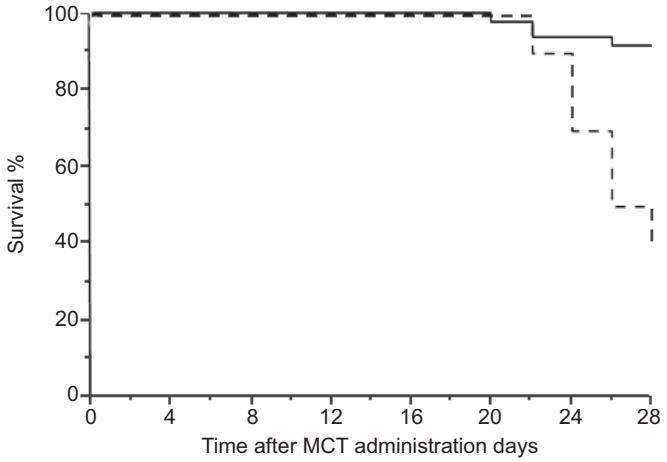

FIGURE 1. Dexamethasone treatment improves survival in monocrotaline (MCT)-induced pulmonary arterial hypertension. Treatment with 5 mg·kg^{-1}·day^{-1} dexamethasone from day 14 to 28 (—) had a significant positive impact on survival at day 28 (log rank test, p=0.001) compared to MCT alone (---).

Dexamethasone treatment reverses pulmonary vascular remodelling in established MCT-induced PAH

Dexamethasone treatment (day 14–28) reduced the degree of muscularisation of peripheral pulmonary arteries and arterioles, as assessed by morphometric analysis. MCT significantly increased the percentage medial thickness of pre-acinar pulmonary arteries (expressed as media/ED×100) at day 28 compared to controls (31.4±10.1 *versus* 10.8±4.93%; p<0.0001). This was reduced in a dose-dependent fashion in all the MCT+Dex D14–28 group at day 28 compared to MCT alone (20.7±9.47%, 18.8±7.7% and 14.7±7.46% for Dex1.25, Dex2.5 and Dex5, respectively, *versus* 31.4±10.1%; p<0.0001 for all). Pulmonary arterial adventitial thickness was also increased at day 28 following MCT compared with control rats (36.5±34.3 *versus* 8.83±4.95%; p<0.0001), which was reduced with all D14–28 dexamethasone doses (24.0±12.6%, 16.3±10.4% and 9.47±5.39 for Dex1.25, Dex2.5 and Dex5, respectively, *versus* 36.5±34.3%; p<0.0001 for Dex5 and Dex2.5, and p<0.05 for Dex1.25; fig. 4a and b). At day 28 following MCT, there was a significant increase in pulmonary arteriolar (*i.e.* vessels <80 μm ED) muscularisation, with an increase seen in the percentage of both nonoccluded and occluded arterioles compared with control rats (p<0.0001). Following MCT+Dex D14–28, the arteriolar muscularisation score was reduced in a dose–dependent manner (p<0.05 for all groups; fig. 4c).

Dexamethasone reduces MCT-induced adventitial infiltration of IL-6-expressing inflammatory cells

In control lungs, immunohistochemistry showed only a weak staining of IL-6 in control lungs (fig. 5a), whereas 28 days after MCT administration, adventitial infiltrating inflammatory cells displayed a strong IL-6 expression in MCT-exposed rats (fig. 5b).

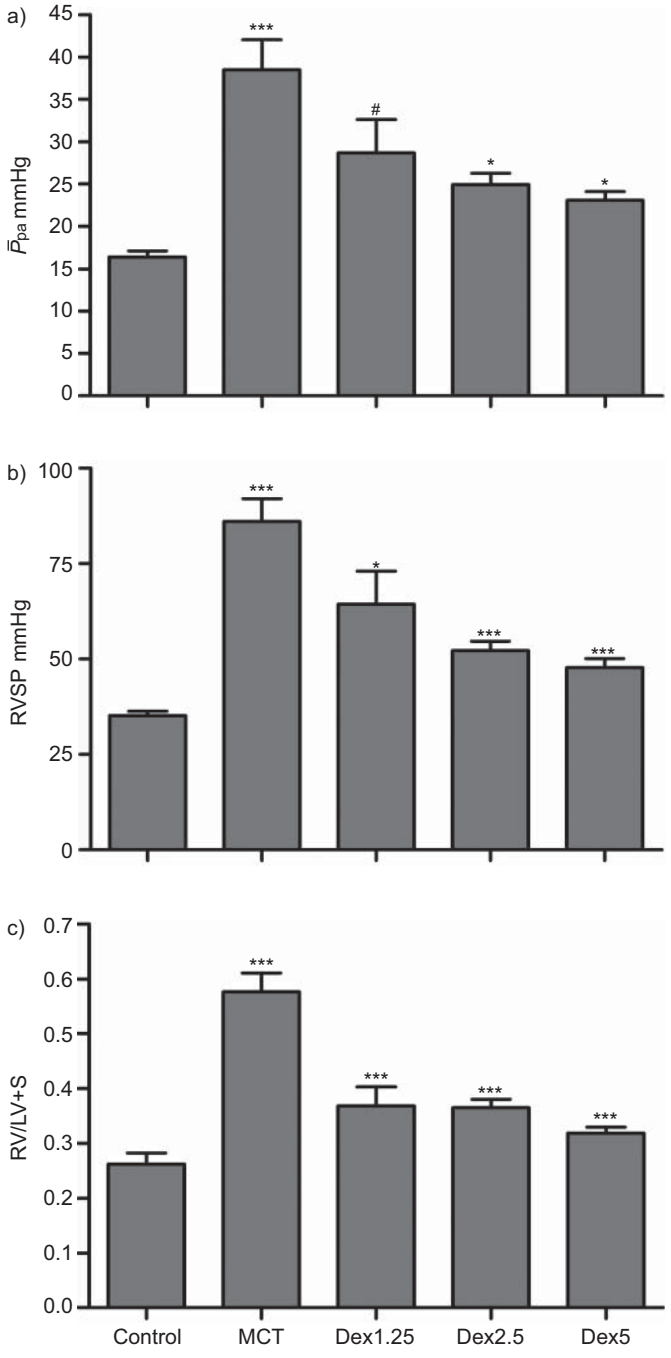

FIGURE 2. Dexamethasone reversed haemodynamics and right ventricular hypertrophy in established monocrotaline (MCT)-induced pulmonary arterial hypertension at day 28. 28 days after MCT administration, a) mean pulmonary arterial pressure ($\bar{P}_{pa}$), b) right ventricular systolic pressure (RVSP) and c) right ventricular weight to left ventricular plus septal weight ratio (RV/LV+S) were significantly increased in MCT-exposed rats. Dexamethasone given at three doses (5 mg·kg^{-1}·day^{-1} (Dex5), 2.5 mg·kg^{-1}·48 h^{-1} (Dex2.5) and 1.25 mg·kg^{-1}·48 h^{-1} (Dex1.25)) from day 14 to 28 after MCT administration normalised the $\bar{P}_{pa}$, RVSP and RV/LV+S. At the highest dose, rats treated with dexamethasone on days 14–28 were no different to control rats (not significant). Data are presented as mean±SEM. #: p=0.066 compared with control; *: p<0.05 compared with control; ***: p<0.001 compared with control.

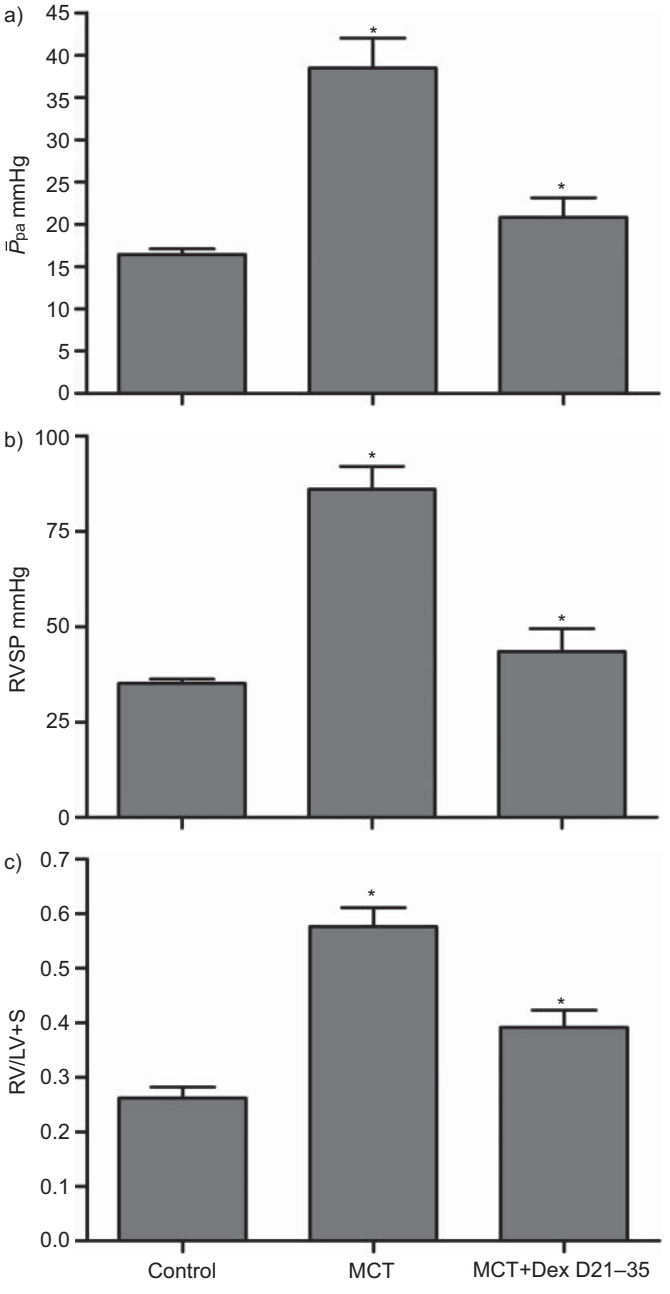

FIGURE 3. Dexamethasone at 5 mg·kg^{-1}·day^{-1} normalised a) mean pulmonary arterial pressure ($\bar{P}_{pa}$), b) right ventricular systolic pressure (RVSP) and c) right ventricular weight to left ventricular plus septal weight ratio (RV/LV+S) in established monocrotaline (MCT)-induced pulmonary arterial hypertension at day 35. Data are presented as mean±SEM. MCT+Dex D21–35: MCT plus 5 mg·kg^{-1}·day^{-1} dexamethasone from day 21 to 35. *: p<0.05 compared with MCT alone.

Lungs from the MCT+Dex D1–28 group exhibited the same faint IL-6 staining as seen in control lungs (fig. 5c).

Dexamethasone inhibits MCT-induced pulmonary IL-6 overexpression

28 days after MCT administration, pulmonary *IL6* mRNA expression measured by quantiative RT-PCR was strongly increased (p<0.01). MCT+Dex D1–28 normalised whole-lung *IL6* mRNA expression (p<0.05 *versus* MCT-exposed lungs; no

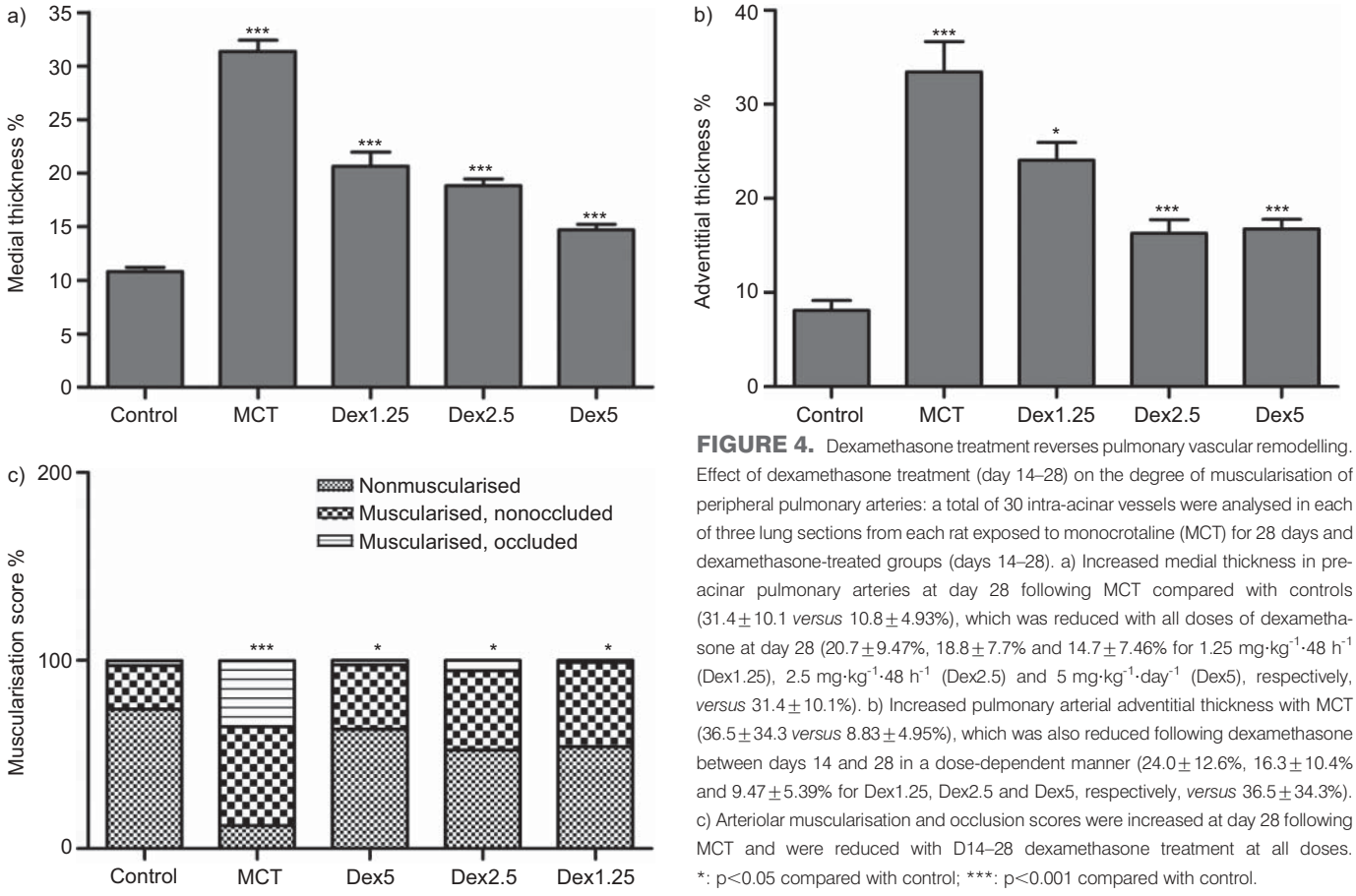

FIGURE 4. Dexamethasone treatment reverses pulmonary vascular remodelling. Effect of dexamethasone treatment (day 14–28) on the degree of muscularisation of peripheral pulmonary arteries: a total of 30 intra-acinar vessels were analysed in each of three lung sections from each rat exposed to monocrotaline (MCT) for 28 days and dexamethasone-treated groups (days 14–28). a) Increased medial thickness in pre-acinar pulmonary arteries at day 28 following MCT compared with controls (31.4±10.1 *versus* 10.8±4.93%), which was reduced with all doses of dexamethasone at day 28 (20.7±9.47%, 18.8±7.7% and 14.7±7.46% for 1.25 mg·kg^{-1}·48 h^{-1} (Dex1.25), 2.5 mg·kg^{-1}·48 h^{-1} (Dex2.5) and 5 mg·kg^{-1}·day^{-1} (Dex5), respectively, *versus* 31.4±10.1%). b) Increased pulmonary arterial adventitial thickness with MCT (36.5±34.3 *versus* 8.83±4.95%), which was also reduced following dexamethasone between days 14 and 28 in a dose-dependent manner (24.0±12.6%, 16.3±10.4% and 9.47±5.39% for Dex1.25, Dex2.5 and Dex5, respectively, *versus* 36.5±34.3%). c) Arteriolar muscularisation and occlusion scores were increased at day 28 following MCT and were reduced with D14–28 dexamethasone treatment at all doses. *: $p<0.05$ compared with control; ***: $p<0.001$ compared with control.

significant difference seen with control lungs). Furthermore, *IL6* was reduced by late (MCT+Dex D14–28) dexamethasone treatment, ($p<0.05$ *versus* MCT-exposed lungs at day28) (fig. 6).

Dexamethasone increases pulmonary BMPR2 *downregulation in MCT-induced PAH*

28 days after MCT administration, pulmonary *BMPR2* mRNA expression was strongly downregulated ($p<0.01$). MCT+Dex D1–28 (fig. 7a) and D14–28 (fig. 7b) increased whole-lung *BMPR2* expression ($p<0.01$ and $p<0.05$ for MCT+Dex D1–28 and D14–28, respectively, *versus* MCT-exposed lungs; not significantly different to control lungs).

Dexamethasone inhibits proliferation of cultured rat PASMC

Proliferation of PASMC isolated from pulmonary hypertensive rats (at day 21 following MCT), as assessed by ^{3}H-thymidine uptake, was inhibited following dexamethasone treatment in a dose-dependent manner, compared with controls in complete medium without dexamethasone (38% reduction at 10^{-8} M dexamethasone and 88% reduction at 10^{-7} M dexamethasone; $p<0.05$ for both doses compared to controls). Similar results were obtained using manual cell counting techniques (data not shown). At 10^{-8} M dexamethasone, cells isolated from control rats were growth-inhibited to a greater extent by dexamethasone compared with PASMCs from MCT-exposed rats ($p<0.05$) (fig. 8).

Dexamethasone increases BMPR2 and reduces IL-6 expression in rat PASMCs

Treatment of rat PASMCs with 10^{-8} M dexamethasone led to an increase in cellular *BMPR2*, as measured by quantitative RT-PCR in control cells ($p<0.05$ compared with untreated cells), whereas there was no significant difference in *BMPR2* following dexamethasone treatment in cells isolated from pulmonary hypertensive rats (fig. 9a). *IL6* mRNA was reduced following dexamethasone treatment ($p<0.05$ for both control and MCT-exposed cells; fig. 9b).

DISCUSSION

The major findings of this study were as follows. 1) Dexamethasone improved survival in rats with established MCT-induced PAH, with normalisation of haemodynamics, right ventricular hypertrophy and pulmonary vascular remodelling at late time points after MCT. 2) Consistent with previous findings, MCT downregulated *BMPR2* expression and increased IL-6 activity: we showed that haemodynamic improvements with dexamethasone treatment were associated with a normalization of *BMPR2*. 3) Reduced pulmonary *IL6* overexpression and a reduction in the adventitial infiltration of IL-6-expressing inflammatory cells. 4) Dexamethasone inhibited rat PASMC proliferation, which was associated with a reduction in *IL6* expression and an increase of *BMPR2* in control PASMCs. 5) PASMCs isolated from pulmonary hypertensive rats appeared relatively resistant to the *BMPR2* mRNA increase and the anti-proliferative effects of dexamethasone.

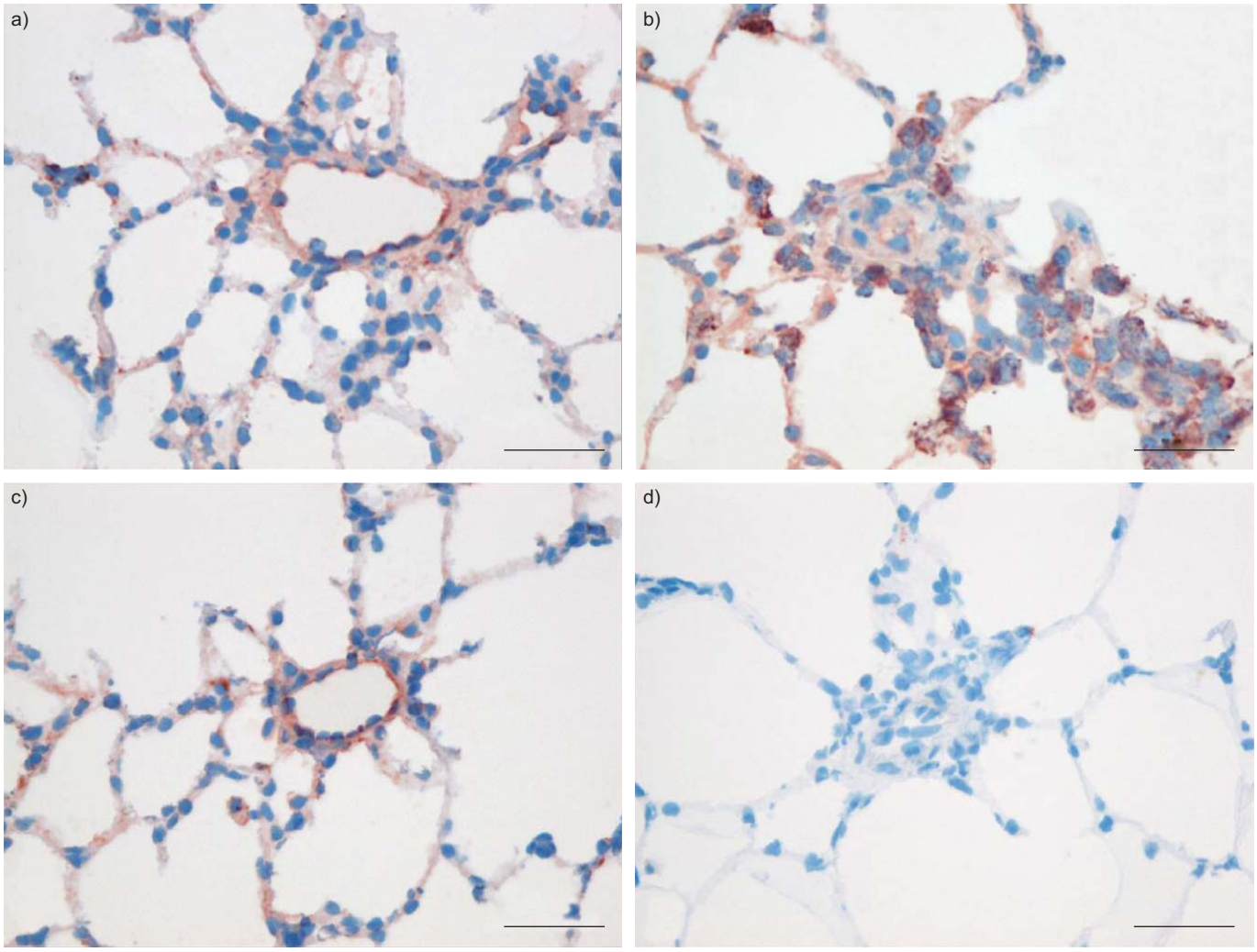

FIGURE 5. Effects of dexamethasone on infiltration of interleukin (IL)-6-expressing inflammatory cells. Immunohistochemical staining for IL-6 (in red) of frozen rat lung tissue sections, counterstained with haematoxylin (nuclei in blue). a) Control lung showing pulmonary arteriole with single layer arteriolar wall and weak staining of IL-6. b) Extensive vascular narrowing of small muscular pulmonary arteries and adventitial infiltrating inflammatory cells displaying a strong IL-6 expression in monocrotaline (MCT) exposed rats. c) Lungs from dexamethasone-treated rats (days 1–28) showing a normal medial wall thickness of small pulmonary arteries and exhibiting a similar weak IL-6 staining as seen in control rats. d) Negative control (omission of primary antibody). Scale bars=50 μm.

Monocrotaline is an ''inflammatory'' model of PAH, comprising an initial asymptomatic inflammatory phase, followed by a less inflammatory symptomatic phase from day 14, with increased medial volume in both major and intra-acinar pulmonary arteries by 21 days exposure [38, 39]. Previous studies have similarly shown that preventive immunosuppressive therapy is effective in MCT-induced PAH when given before the onset of pulmonary vascular remodelling (*i.e.* prior to day 14) [9, 17, 18, 40–43]. However, although there have been some studies showing reversal of PAH at later time-points with various anti-inflammatory therapies, none has yet used glucocorticoids. The reversal of MCT-induced PAH beyond the onset of vascular remodelling is of clinical relevance. Although patients with IPAH are not believed to have steroid-responsive disease, clinical improvement of PAH is seen in associated inflammatory conditions where immunosuppression (including glucocorticoids) was otherwise indicated, including PAH associated with connective tissue diseases [44] and SLE [45]. Interestingly, this improvement in PAH occurs particularly in those with earlier, less severe PAH [16], perhaps suggesting that immunosuppressive therapies may be more effective in proliferating, active lesions in early disease than in those with established, fixed pulmonary vascular lesions.

Among the wide spectrum of biological actions of glucocorticoids, dexamethasone has been shown to inhibit vascular cell proliferation [46]. The antiproliferative findings in this study are consistent with a study of prednisolone on platelet-derived growth factor-stimulated PASMCs from PAH patients [47], although that study used much higher doses (equating to 3×10^{-5} and 3×10^{-4} M dexamethasone).

Mutations in *BMPR2* have been shown to be important in familial IPAH [22]. BMPs are members of the transforming growth factor (TGF)-β superfamily, which, through type 1 and 2 receptors, contribute to regulation of cell proliferation,

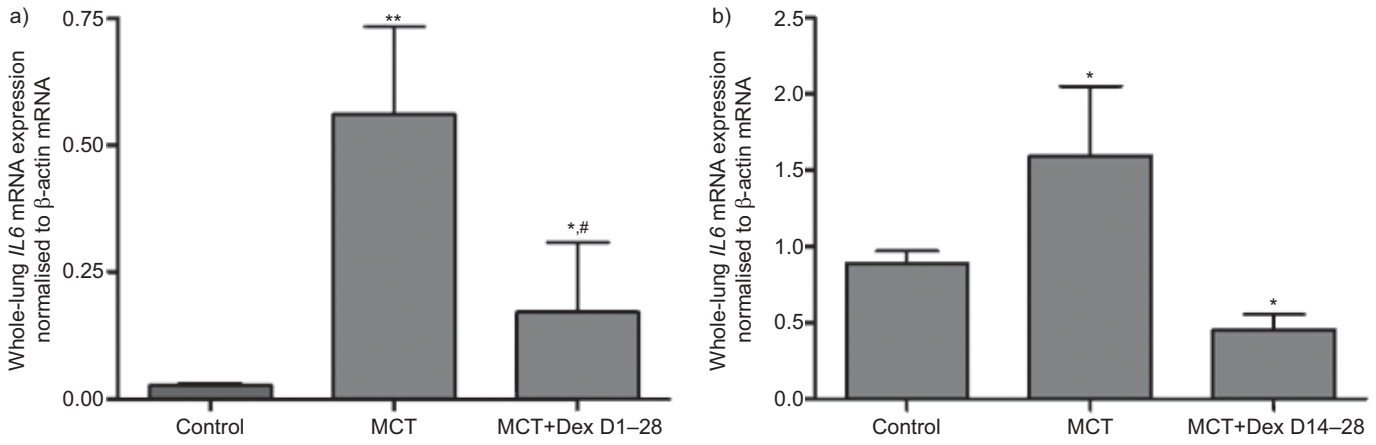

FIGURE 6. Impact of dexamethasone on pulmonary *IL6* mRNA expression in monocrotaline (MCT)-induced pulmonary arterial hypertension. 28 days after MCT administration, *IL6* mRNA expression was upregulated in lungs of MCT exposed rats. 5 mg·kg^{-1}·day^{-1} dexamethasone for days a) 1–28 (MCT+Dex D1–28) and b) 14–28 (MCT+Dex D14–28) normalised whole-lung *IL6* mRNA expression. *IL6* mRNA was normalised to the housekeeping gene β-actin. $^{\#}$: not significant compared with control; *: $p<0.05$; **: $p<0.01$.

differentiation and apoptosis. In humans, a variety of cell types, including PASMCs and endothelial [48] cells, synthesise and secrete BMPs. BMPR-II-positive cells have also been shown to be closely associated with the inflammatory cell infiltrate in IPAH lesions [49]. One of the normal roles of the BMP signalling pathway is believed to be the prevention of runaway positive feedback loops in inflammatory cytokines. Reduced expression of BMPR-II has been reported in most types of human PAH and an attractive theory is that the dysregulated BMPR-II signalling is followed by an inflammatory second hit early in the pathogenesis of PAH. Our data are consistent with previous studies [27, 28] showing a reduction in BMPR-II in MCT-induced PAH in rats and, as far as we are aware, this is the first study to show a glucocorticoid-induced increase in the low *BMPR2* levels in MCT-induced PAH. The importance of IL-6 has been shown in several studies of PAH. Patients with PAH have increased circulating IL-6 levels [8] and IL-6 is capable, on its own, of causing growth of vascular smooth muscle cells [50] and PAH [51]. In transgenic mice, IL-6 overexpression induces PAH associated with downregulation of TGF-β signalling [52]. Consistent with previous studies [9, 51], we found that MCT-induced PAH is also associated with increased IL-6 production. Kaposi's sarcoma-associated herpes virus, which may cause PAH in HIV-negative Castleman's disease, encodes a viral, constitutively active form of IL-6 [53]. In addition, recent studies have suggested the involvement of viral infection or autoimmunity [12] in the development of PAH. Thus, there is substantial evidence that the unknown second hit is likely to be inflammatory in character. HAGEN *et al.* [34] have identified a negative feedback loop between IL-6 and the BMP pathway, in which increased IL-6 induces BMP pathway activity and increased BMP pathway activity suppresses IL-6. Furthermore, IL-6 enhances proliferation *via* activation of signal transducer and activator of transcription 3 [54], persistent activation of which has been shown to reduce BMPR-II expression, which may contribute to

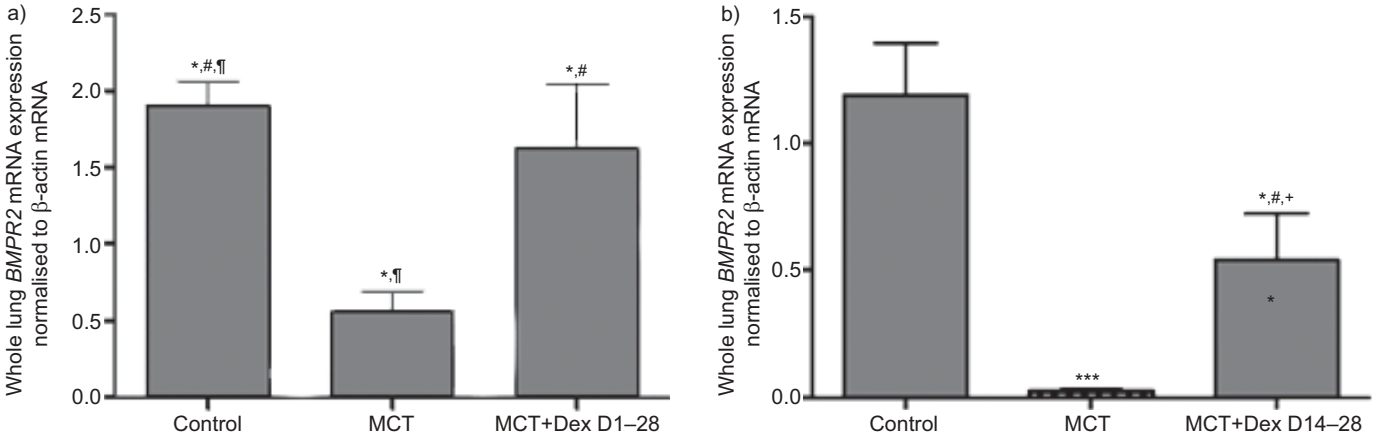

FIGURE 7. Impact of dexamethasone on pulmonary *BMPR2* mRNA expression in monocrotaline (MCT)-induced pulmonary arterial hypertension. 28 days after MCT administration, *BMPR2* mRNA expression was strongly downregulated in lungs of MCT-exposed rats. a) Dexamethasone increased whole lung *BMPR2* mRNA expression in rats treated with dexamethasone between days 1 and 28 (MCT+Dex D1–28) compared with MCT exposed rats and b) following day 14–28 dexamethasone treatment (MCT+Dex D14–28). $^{\#}$: not significant between groups; ¶: $p<0.0001$; $^{+}$: not significant compared with control; *: $p<0.05$; ***: $p<0.001$.

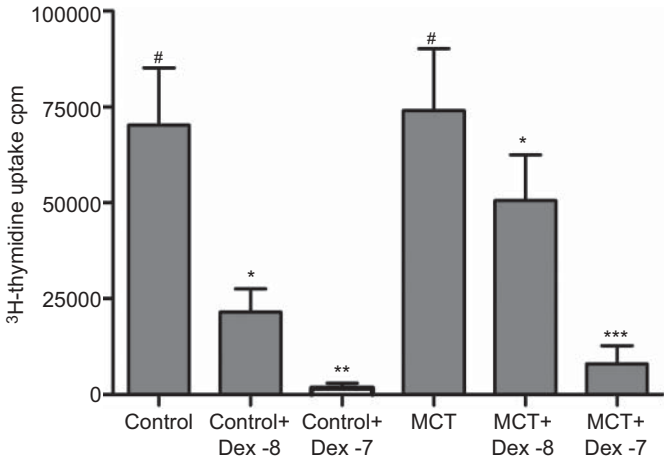

FIGURE 8. Dexamethasone reduces proliferation of cultured rat pulmonary artery smooth muscle cells (PASMCs). Proliferation of PASMCs was assessed by ^{3}H-thymidine uptake and confirmed using manual counting (data not shown). Proliferation of PASMCs was inhibited following dexamethasone treatment in a dose-dependent manner compared to untreated controls, for both PASMC exposed to monocrotaline (MCT) and non-MCT-exposed cells (control). The concentrations of dexamethasone used were 10^{-7} (Dex -7) and 10^{-8} M (Dex -8). PASMCs isolated from Control+Dex -8 were growth-inhibited to a greater extent than MCT+Dex -8. #: not significant between groups; *: $p<0.05$ between groups; ***: $p< 0.001$ compared to control.

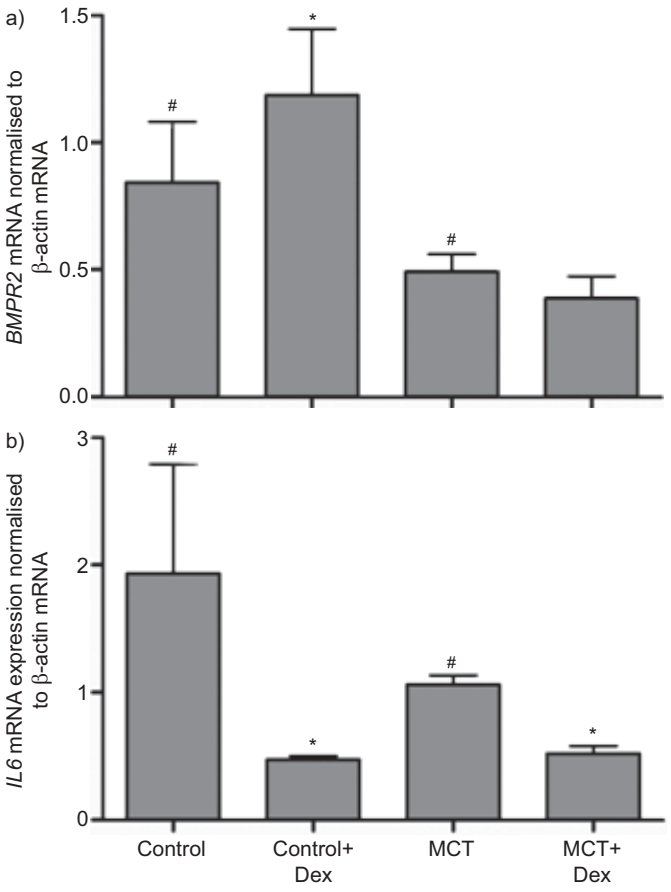

FIGURE 9. Dexamethasone a) increases *BMPR2* and b) reduces *IL6* expression in cultured rat pulmonary artery smooth muscle cells (PASMCs). PASMCs were isolated from control and pulmonary hypertensive rats (day 21 following monocrotaline (MCT)), treated with 10^{-8} M dexamethasone (Dex) and RT-PCR was performed for cellular *BMPR2* and *IL6* mRNA. Dexamethasone treatment increased *BMPR2* in control+Dex but not MCT+Dex, compared with MCT alone. *IL6* was reduced in PASMC from both control+Dex and MCT+Dex compared with controls. #: not significant between groups; *: $p<0.05$ compared with controls.

the loss of BMPR-II during PAH development [55]. Further work showing that, although asymptomatic *BMPR2*$^{+/-}$ mice do not develop pulmonary hypertension spontaneously, under inflammatory stress, they are more susceptible than wild-type mice [32] and mice expressing a dominant-negative *BMPR2* allele in smooth muscle develop elevated right ventricular pressures, with an increase in cytokines and markers of immune response, when the transgene is activated [33]. BMPR-II dysfunction and resulting loss of activity may, therefore, result in unopposed IL-6 production in the context of an as yet unknown inflammatory stimulus.

Our data suggest that dexamethasone interrupts the IL-6–BMPR-II negative feedback loop [34], probably mainly through a dexamethasone-induced reduction in IL-6-expressing inflammatory cells and, possibly, also a direct PASMC effect. The resulting increase in pulmonary *BMPR2* mRNA may thus restore the required dampening effects of BMPR-II signalling on IL-6 function. The mechanisms through which glucocorticoids interact with BMPR-II are unclear, but a gene expression profiling study of asthmatics receiving glucocorticoids suggests an important interaction between the sensitivity of the glucocorticoid receptor and BMPR-II [56].

Limitations of our study include the absence of BMPR-II protein expression analysis to confirm the findings in gene transcription assays, the lack of immunohistochemistry for BMPR-II and the lack of IL-6 immunohistochemistry at later treatment time-points, although we replaced this with quantitative data using RT-PCR for *IL6* and *BMPR2* at these time-points.

In conclusion, we have shown that treatment with dexamethasone improves haemodynamics, reduces remodelling and improves survival in established MCT-induced PAH, with normalisation of *BMPR2* expression and inflammatory responses. These findings provide new insight into the potential role of immunosuppressants in the treatment of human PAH *via* the regulation of the BMPR-II and IL-6 pathways.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

Statements of interest for D. Montani, R. Souza, G. Simonneau and M. Humbert can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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