



# Myeloid-cell-specific deletion of inducible nitric oxide synthase protects against smoke-induced pulmonary hypertension in mice

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**Deletion of iNOS in myeloid cells protects mice against smoke-induced PH. Cross-talk of M2 macrophages with PSMCs drives smoke-induced pulmonary vascular remodelling and depends on iNOS expression in macrophages and ERK activity in PSMCs.** <https://bit.ly/3itDtuV>

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## Abstract

**Background** Pulmonary hypertension (PH) is a common complication of COPD, associated with increased mortality and morbidity. Intriguingly, pulmonary vascular alterations have been suggested to drive emphysema development. Previously, we identified inducible nitric oxide synthase (iNOS) as an essential enzyme for development and reversal of smoke-induced PH and emphysema, and showed that iNOS expression in bone-marrow-derived cells drives pulmonary vascular remodelling, but not parenchymal destruction. In this study, we aimed to identify the iNOS-expressing cell type driving smoke-induced PH and to decipher pro-proliferative pathways involved.

**Methods** To address this question we used 1) myeloid-cell-specific iNOS knockout mice in chronic smoke exposure and 2) co-cultures of macrophages and pulmonary artery smooth muscle cells (PASCs) to decipher underlying signalling pathways.

**Results** Myeloid-cell-specific iNOS knockout prevented smoke-induced PH but not emphysema in mice. Moreover, iNOS deletion in myeloid cells ameliorated the increase in expression of CD206, a marker of M2 polarisation, on interstitial macrophages. Importantly, the observed effects on lung macrophages were hypoxia-independent, as these mice developed hypoxia-induced PH. *In vitro*, smoke-induced PASC proliferation in co-cultures with M2-polarised macrophages could be abolished by iNOS deletion in phagocytic cells, as well as by extracellular signal-regulated kinase inhibition in PASCs. Crucially, CD206-positive and iNOS-positive macrophages accumulated in proximity of remodelled vessels in the lungs of COPD patients, as shown by immunohistochemistry.

**Conclusion** In summary, our results demonstrate that iNOS deletion in myeloid cells confers protection against PH in smoke-exposed mice and provide evidence for an iNOS-dependent communication between M2-like macrophages and PASCs in underlying pulmonary vascular remodelling.

## Introduction

COPD represents one of the five leading causes of death worldwide. Pathological changes in COPD are caused by inhalation of noxious agents such as cigarette smoke and comprise chronic airway inflammation and progressive alveolar destruction, resulting in chronic bronchitis and emphysema [1, 2]. Underlying



molecular mechanisms leading to COPD include increased oxidative stress, the imbalance between proteolytic activity and antiproteolytic defence and influx of inflammatory cells [3, 4]. Additionally, recent findings from pre-clinical models [5–8] and COPD patients [9] prompted the hypothesis that pulmonary vascular alterations are an early phenomenon of the COPD pathology and a possible driver of emphysema [10]. In smoke-exposed mice and guinea pigs, pulmonary vascular remodelling precedes parenchymal destruction [5–8] and established therapies for pulmonary hypertension (PH) affect the development of emphysema [11–13]. Moreover, histopathological signs of pulmonary vascular remodelling have been found in most COPD patients and smokers who had not developed COPD [9]. In addition, abnormally high mean pulmonary arterial pressure is present in up to 90% of COPD patients. According to the current World Health Organization classification, COPD-associated pulmonary hypertension (COPD-PH) is included in group 3 of PH (PH due to lung diseases/hypoxia) [14–16]. Although PH is associated with increased risk of exacerbations and decreased survival in COPD patients, effective pharmacological options are not available [14]. Some of the mechanisms implicated in the pathogenesis of COPD-PH are endothelial dysfunction, hypoxia, vascular pruning and loss of capillary bed [17]. Moreover, activation of inflammatory cells might contribute to PH development in COPD, as increased systemic levels of several cytokines such as tumour necrosis factor- $\alpha$ , C-reactive protein [18] and interleukin (IL)-6 [19] were associated with PH in COPD patients and the number of perivascular inflammatory cells correlated with pulmonary vascular alterations [20]. These findings are of even more interest as the prominent role of inflammation in pulmonary vascular remodelling was already recognised in other forms of PH [21–23], although it was previously shown that vascular gene regulation occurring in COPD-PH largely differs from other types of this pulmonary vascular disease [5]. Furthermore, we recently identified inducible nitric oxide synthase (iNOS) as the key player in the pathogenesis of smoke-induced PH as well as emphysema. In smoke-exposed mice, iNOS inhibition prevented and reversed parenchymal destruction, PH and pulmonary vascular remodelling. Moreover, we demonstrated that iNOS expression in bone-marrow-derived cells drives the pulmonary vascular alterations, but not emphysema development [5]. However, it remained unclear which bone-marrow-derived cell type drives the process and what the respective mechanism is. We hypothesised that pathological signalling leading to smoke-induced PH is triggered by elevated iNOS expression in myeloid cells, that macrophages play a critical role and that a similar process as in the mouse may occur in humans. iNOS is an inducible, calcium-independent and high-throughput isoform of nitric oxide synthase, an enzyme generating nitric oxide from L-arginine and molecular oxygen. This enzyme has a prominent role in the immune system, which goes beyond its antimicrobial and antifungal activities and involves effects on the phenotype of the expressing cell, but also on the function and composition of neighbouring immune cells [24].

## Materials and methods

### *Animal model*

Adult male and female iNos LysM-Cre mice (Nos2<sup>tm2904.1A</sup>Arte-Tg(CAG-flpe)2ArteLyz2<sup>tm1(cre)lfo</sup>/), aged 3–4 months, and age- and sex-matched wild-type (WT) controls (C57BL/6N; Charles River Laboratories, Sulzfeld, Germany) were randomly allocated to either tobacco smoke exposed or unexposed groups, and to either hypoxic or normoxic groups. All animal experiments were approved by the regional board for animal welfare (Regierungspräsidium Giessen, Germany).

### *Immunohistochemical staining of human lung sections*

Human lung samples from donors and COPD patients were used. The study complied with the Declaration of Helsinki, and the tissue donation protocol was approved by the ethics committee of the faculty of medicine at Justus-Liebig University of Giessen, Germany. Immunohistochemical staining was performed as reported previously [25] with subtle modifications. Primary antibodies were used as follows: CD68 (Cat#ab955, Abcam, Cambridge, UK; 1:200); CD206 (Cat#ab64693, Abcam, Cambridge, UK; 1:100), anti-iNOS (Cat#NB300–605, Novus Biologicals, Littleton, CO, USA; 1:250), phospho p42/p44 (Cat#4370, Cell Signaling Technology, Danvers, MA, USA; 1:200).

### *Statistical analysis*

Statistical analyses were performed using Prism 6 (GraphPad, LaJolla, CA, USA). All data are expressed as mean  $\pm$  SEM. Comparison between multiple groups was performed by two-way ANOVA and Tukey's *post hoc* test for comparison between different groups of nonmatched samples (animal experiments) and Sidak's *post hoc* test for matched samples (cell-culture experiments). Independent t-test was used for comparing equality of means between two groups. p-values <0.05 were considered statistically significant.

Information about all other methods used in this study can be found in the supplementary material.

## Results

### *iNOS deletion in myeloid cells prevents PH and right ventricular hypertrophy in smoke-exposed mice*

Considering our previous finding that iNOS-expressing bone-marrow-derived cells drive development of smoke-induced PH, we generated myeloid-cell-specific iNOS knockout mice by crossing iNOS<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> mice with lysozyme M promoter-driven Cre-expressing mice (hereafter LysM-Cre), to assess the role of myeloid-cell-specific iNOS expression in development of smoke-induced PH *in vivo*. Successful generation of the knockout mice was confirmed *in vitro* by quantification of iNOS expression in bone-marrow-derived macrophages (supplementary figure S1a, b), and *in situ*, by immunofluorescent staining of mouse lung sections (supplementary figure S1c).

Haemodynamic measurements following chronic smoke exposure revealed that myeloid-cell-specific iNOS knockout mice were protected against PH compared to WT mice when exposed to smoke for 3 and 8 months (figure 1a). In addition, right ventricular (RV) hypertrophy and impaired RV function occurring after smoke exposure in WT animals were absent in iNOS LysM-Cre mice, as shown by Fulton index and echocardiography (figure 1b, c). Moreover, muscularisation of small pulmonary vessels increased in cigarette-smoke-exposed WT mice compared to unexposed (room air) controls after 3 and 8 months (figure 1d–f). In contrast, iNOS LysM-Cre mice were fully protected against smoke-induced pulmonary vascular remodelling.

### *Myeloid-cell-specific iNOS knockout does not prevent emphysema development in mice after chronic smoke exposure*

In parallel, we investigated the development of emphysema in iNOS LysM-Cre mice. We first used fluorescent molecular tomography (FMT) combined with micro-computed tomography ( $\mu$ CT) and an annexin V-based imaging probe to quantify apoptosis *in vivo* and found an increase in apoptosis in lungs of both WT and iNOS LysM-Cre mice after 8 months of smoke exposure, compared to the respective unexposed (room air) controls (figure 2a). Functional measurements with the FlexiVent system (figure 2b), assessment of functional residual capacity by  $\mu$ CT (figure 2c) and morphometric analysis including design-based stereology (figure 2d and supplementary figure S2) confirmed emphysema development in both WT and iNOS LysM-Cre mice after 8 months of chronic smoke exposure. Baseline values and temporal dynamics of lung function changes were different in knockout animals, possibly due to congenital effects.

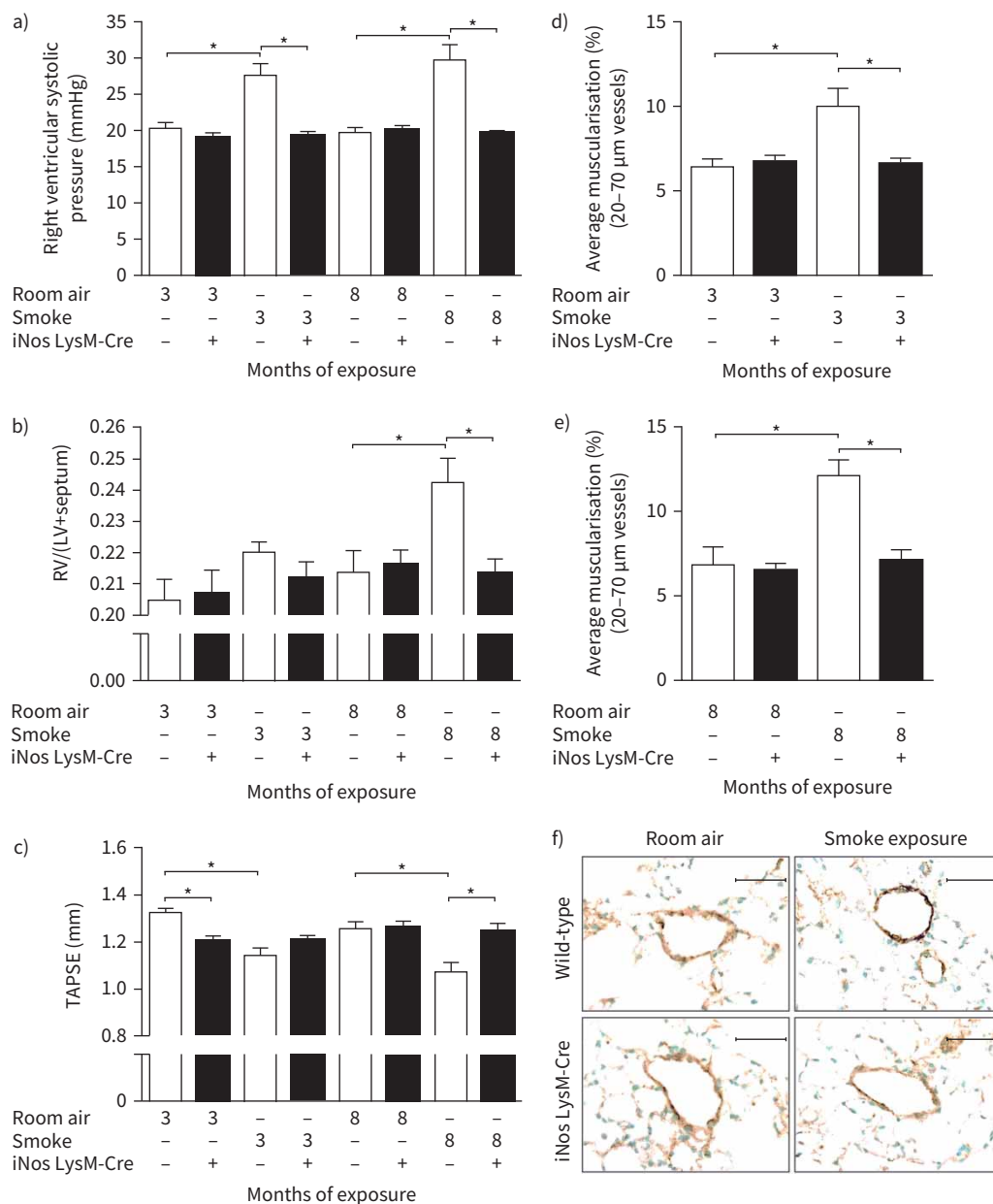
### *iNOS deletion in myeloid cells affects composition of inflammatory cells in lungs after chronic smoke exposure*

As the composition and phenotype of lung inflammatory cells are important factors in other forms of PH, we examined the effects of myeloid-cell-specific iNOS deletion on smoke-induced changes in immune cells in lung homogenate using flow cytometry. After 8 months of chronic smoke exposure, a significant increase in the portion of interstitial macrophages (characterised as shown in figure 3a) in CD45<sup>+</sup> cells was found in lung homogenates of smoke-exposed WT animals compared to respective unexposed (room air) controls, but was absent in iNOS LysM-Cre mice (figure 3b). Considering the finding that the shift towards an M2-like phenotype of macrophages contributes to pulmonary vascular remodelling in other forms of PH, we examined the expression of CD206 on interstitial macrophages found in the lung homogenate after chronic smoke exposure. This analysis revealed in WT animals a significant increase in the expression of CD206, suggesting a smoke-induced shift of interstitial macrophages towards an M2-like phenotype. Importantly, CD206 expression on interstitial macrophages in lungs of iNOS LysM-Cre mice remained unchanged (figure 3c, d).

Additionally, we found that the smoke-induced increase in the proportion of CD4<sup>+</sup> T-cells in CD45<sup>+</sup> cells in lung homogenate was significantly higher in WT compared to iNOS LysM-Cre animals. Similarly, there was a tendency towards a higher smoke-induced increase in both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in bronchoalveolar lavage of WT compared to iNOS LysM-Cre mice (supplementary figure S3a, b).

### *iNOS deletion in myeloid cells does not affect development of hypoxia-induced PH and right ventricular hypertrophy*

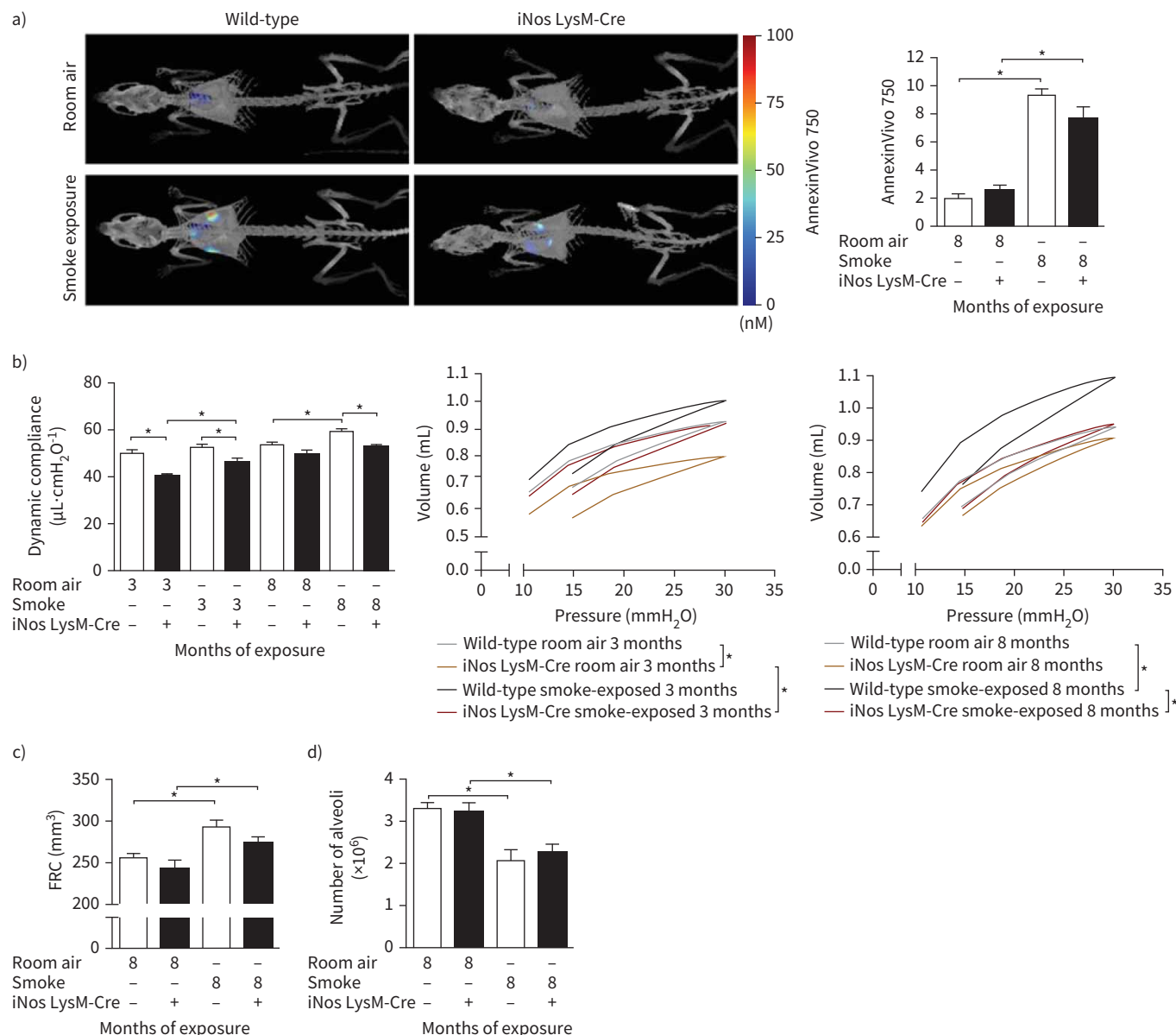
As alveolar hypoxia caused by impairment of respiratory function can contribute to vascular remodelling in COPD [17], and the M2 phenotype of macrophages is important for development of hypoxia-induced PH [26], we investigated whether iNOS deletion in myeloid cells can also protect mice against PH after exposure to chronic hypoxia. However, myeloid-cell-specific iNOS knockout mice developed hypoxia-induced PH to the same level as observed in WT mice. This was evident from haemodynamic measurements (figure 4a), determination of RV hypertrophy and the decrease of RV function (figure 4b, c). Chronic hypoxia led to a similar increase in the degree of muscularisation of small pulmonary vessels in both genotypes (figure 4d).



**FIGURE 1** Myeloid cell type-specific inducible nitric oxide synthase (iNOS) deletion prevents development of cigarette-smoke-induced pulmonary hypertension in mice. Mice were exposed either to cigarette smoke or room air for 3 and 8 months. **a)** Right ventricular systolic pressure (n=9–12). **b)** Changes in the right ventricular structure shown as the ratio of the right ventricular (RV) and the left ventricular plus septum (LV+septum) mass (n=9–12). **c)** Echocardiographic assessment of right ventricular systolic function (n=11–13) depicted by means of tricuspid annular plane systolic excursion (TAPSE). **d–f)** Remodelling of the small pulmonary vessels (20–70 µm outer diameter) presented as the average muscularisation of total vessel count after **d)** 3 months (n=4–5) or **e)** 8 months of smoke exposure (n=5–7); and **f)** representative images of lung sections from mice exposed to cigarette smoke for 8 months and respective room air controls, co-stained against α-smooth muscle actin (purple) and von Willebrand factor (brown). Scale bars=50 µm. Data are presented as mean±SEM. \*: p<0.05. Two-way ANOVA (with Tukey's multiple comparison *post hoc* test) was used for statistical analysis.

### Cigarette smoke extract treatment of M2 macrophages increases proliferation and migration of co-cultured pulmonary artery smooth muscle cells in an iNOS-dependent manner

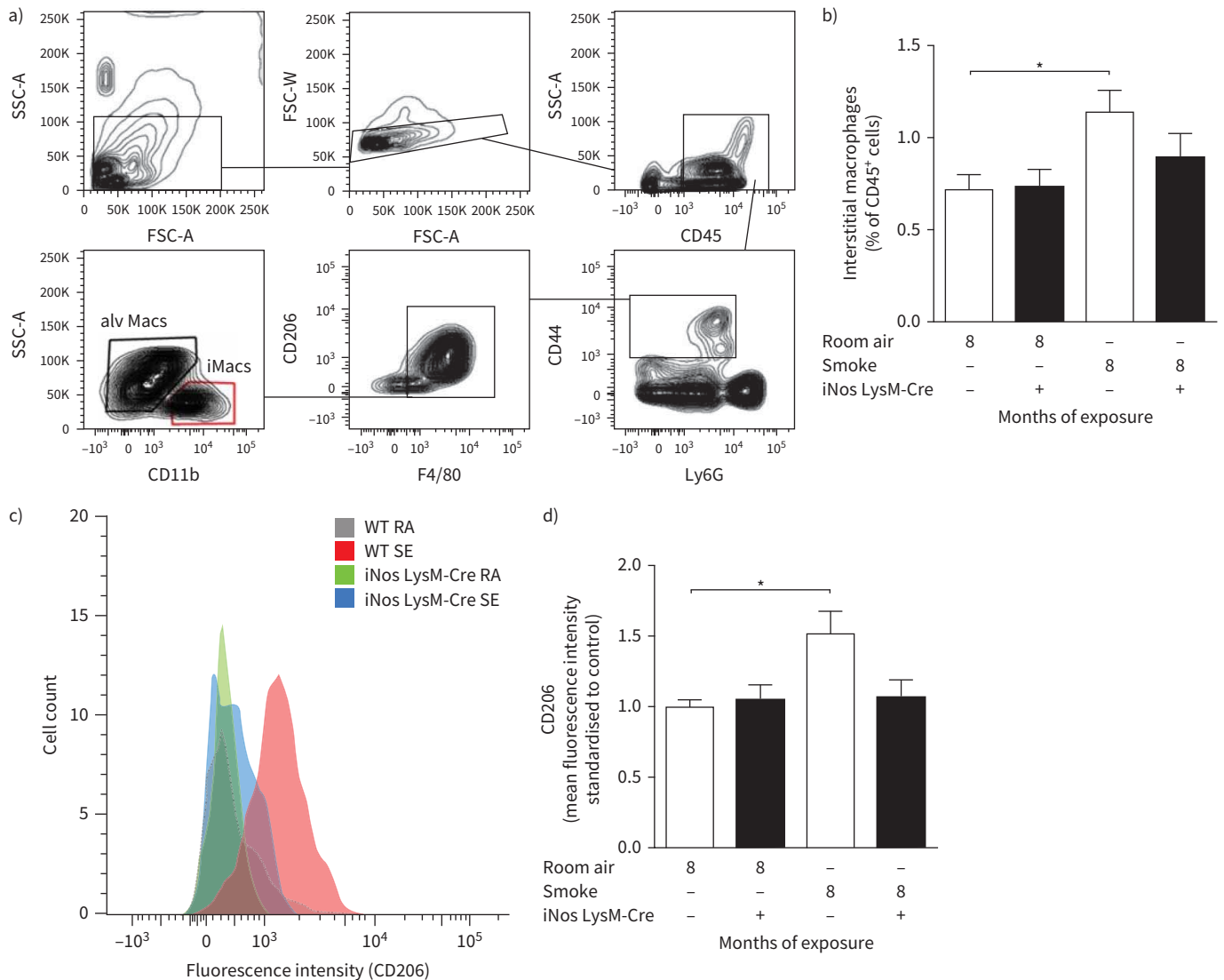
Based on the prominent iNOS-dependent changes in the portion and phenotype of interstitial macrophages in lungs upon smoke exposure, we investigated the proliferation of murine primary pulmonary artery smooth muscle cells (PASMCs) co-cultured with bone-marrow-derived and cigarette smoke extract



**FIGURE 2** Chronic smoke exposure leads to emphysema development in myeloid-cell-specific inducible nitric oxide synthase (iNOS) knockout mice. Mice were exposed to either cigarette smoke or room air for 3 and 8 months. **a)** *In vivo* quantification of apoptosis in lungs (n=6–7), detected by a fluorescence molecular tomography imaging system, using the AnnexinVivo 750 probe. **b)** Lung function (n=8–11) presented as dynamic compliance and respiratory pressure–volume loops. **c)** Functional residual capacity (FRC) assessed by micro-computed tomography (n=11–13). **d)** Number of alveoli (n=7–9) estimated by design-based stereology. Data are presented as mean±SEM. \*: p<0.05. Two-way ANOVA (with Tukey’s multiple comparison *post hoc* test) was used for statistical analysis.

(CSE)-stimulated macrophages by the use of a Transwell system (figure 5a). For the treatment, we chose a concentration of CSE that did not affect viability/metabolic activity of macrophages even during prolonged exposure (supplementary figure S4a). Pre-treatment of M2, but not of M1 or naïve macrophages with CSE increased proliferation of PASCs in co-culture after 6 h and 24 h (figure 5a). Intriguingly, deletion of iNOS in M2 macrophages abolished the CSE-dependent increase of PASC proliferation (figure 5a). In addition, this effect was dependent on the communication between the co-cultured cell types, because it was absent when PASCs were not co-cultured but were treated with macrophage culture-conditioned medium (CM) (supplementary figure S4b).

Moreover, we examined the effects of the cross-talk between macrophages and PASCs on migration and apoptosis of PASCs by treatment of PASCs in monoculture with co-culture CM. As in the case of

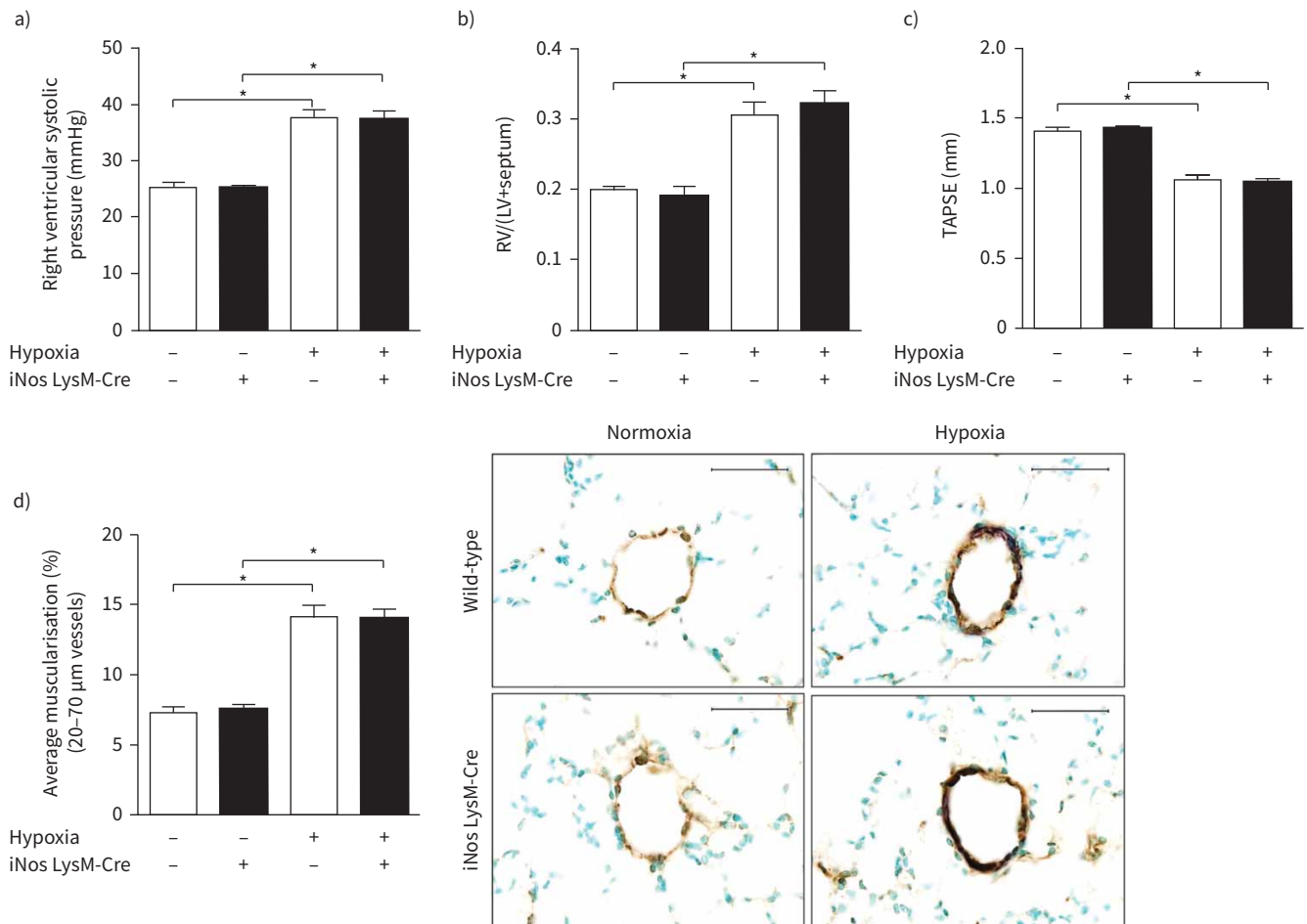


**FIGURE 3** Myeloid-cell-specific inducible nitric oxide synthase (iNOS) deletion prevents smoke-induced accumulation of interstitial macrophages and upregulation of CD206 (the marker of “M2-like” polarisation) on these cells. Mice were exposed either to smoke (SE) or room air (RA) for 8 months and macrophages in lung homogenates were analysed by flow cytometry. **a)** Representative flow cytometry gating scheme, used to separate alveolar and interstitial macrophages. **b)** Content of interstitial macrophages in the lungs of smoke-exposed mice and respective unexposed (room air) controls (n=4–5), assessed by flow cytometry and given as the percentage of CD45<sup>+</sup> cells. **c)** Representative histogram showing CD206<sup>+</sup> cells in lungs of wild-type (WT) and myeloid-cell-specific iNOS knockout mice. **d)** CD206 expression on interstitial macrophages (n=4–5), assessed by flow cytometry and given as the mean fluorescence intensity standardised to control. Data are presented as mean±SEM. SSC: side scatter; FSC: forward scatter. \*: p<0.05. Two-way ANOVA (with Tukey’s multiple comparison *post hoc* test) was used for statistical analysis.

proliferation of co-cultured (figure 5a) or co-culture CM-treated PASCs, the pro-migratory effect of co-culture CM on PASCs was increased by CSE treatment of M2 macrophages and abolished by iNOS inhibition in these cells (supplementary figure S5a). However, CM from co-cultures decreased PASC apoptosis and that effect was not dependent on CSE-treatment of M2 macrophages nor iNOS activity in these cells (supplementary figure S5b).

***IL-4 and mitogen-activated protein kinase signalling might contribute to CSE-induced iNOS-dependent proliferation of PASCs in co-cultures***

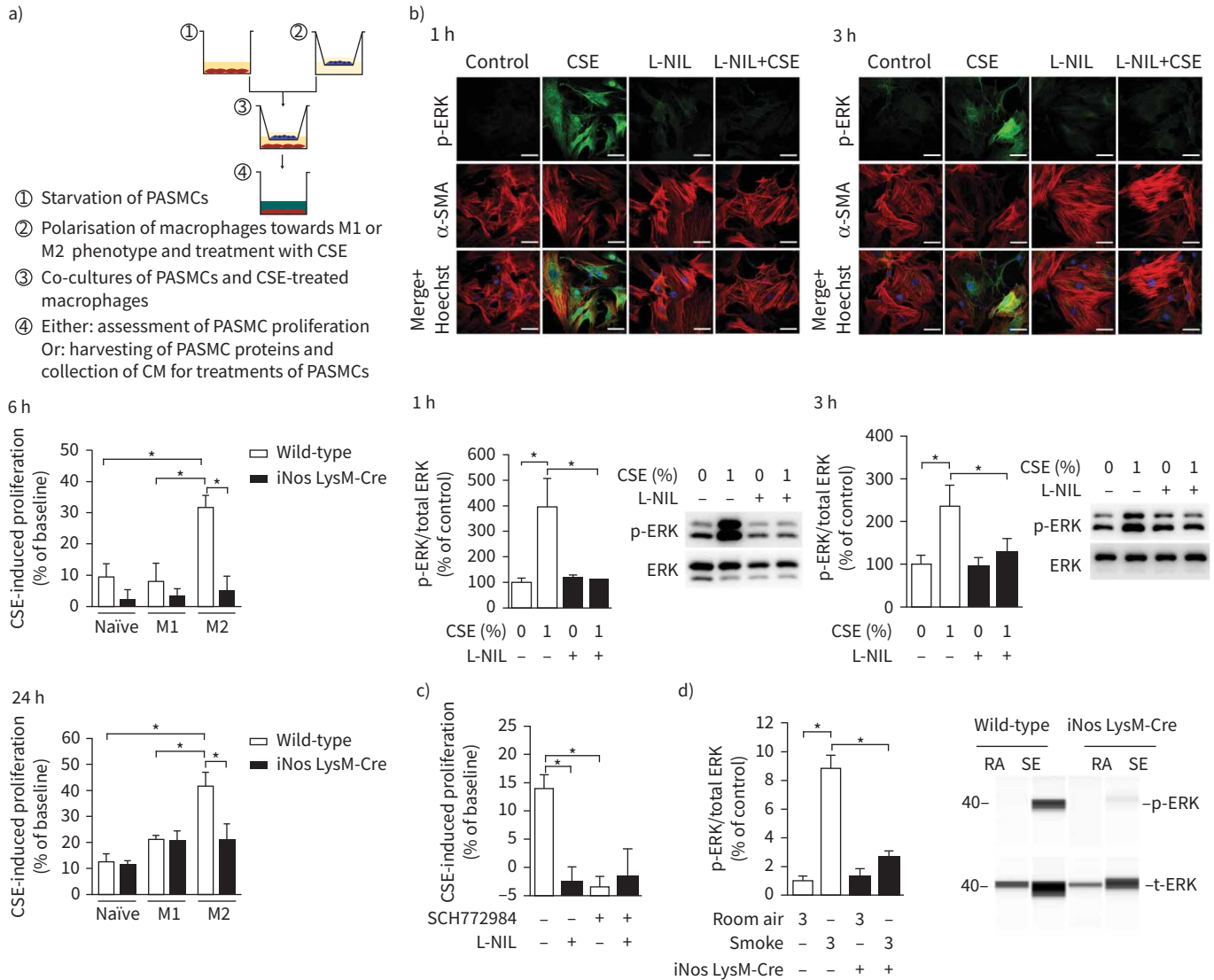
Next, we examined the mechanism by which M2 macrophages in co-culture trigger the proliferation of PASCs. These investigations revealed an increase in phosphorylation of extracellular signal-regulated



**FIGURE 4** Myeloid-cell-specific inducible nitric oxide synthase (iNOS) knockout does not protect mice against hypoxia-induced pulmonary hypertension (PH). Mice were exposed to either normobaric normoxia or hypoxia for 28 days. **a)** Right ventricular systolic pressure (n=6–8). **b)** Changes in right ventricular structure shown as the ratio of the right ventricle (RV) and the left ventricular plus septum (LV+septum) mass (n=6–8). **c)** Echocardiographic assessment of RV systolic function depicted by means of tricuspid annular plane systolic excursion (TAPSE) (n=6–8). **d)** Remodelling of the small pulmonary vessels (20–70 μm outer diameter), presented as the average muscularisation of total vessel count after chronic hypoxia exposure (n=6–8) and representative images of lung sections from mice exposed to chronic hypoxia and respective normoxic controls, co-stained against α-smooth muscle actin (purple) and von Willebrand factor (brown). Scale bars=50 μm. Data are presented as mean±SEM. \*: p<0.05. Two-way ANOVA (with Tukey's multiple comparison *post hoc* test) was used for statistical analysis.

kinase (ERK) in PASMCs at early time points (1 h, 3 h and 6 h) after co-culturing. Such an increase of phospho-ERK was absent upon L-NIL treatment of the phagocytic cells (figure 5b and supplementary figure S6a). The fact that the pattern of ERK phosphorylation resembled PASMC proliferation suggested a functional role of ERK phosphorylation in the CSE-M2-macrophage driven proliferation. To prove this suggestion, we investigated whether ERK inhibition affected PASMC proliferation when treated with co-culture CM. Indeed, the ERK1/2 inhibitor SCH772984 inhibited the increase in PASMC proliferation induced by CM from PASMC-M2 (CSE-pre-treated) macrophage co-cultures (figure 5c and supplementary figure S6c). This effect was similar to the effect of iNOS inhibition (figure 5c). Crucially, these results are consistent with our *in vivo* data, where smoke exposure increases ERK1/2 phosphorylation in pulmonary vessels (figure 5d) and in lung homogenates (supplementary figure S6c) of WT but not of knockout animals.

Additionally, cytokine profiling in the co-culture medium revealed decreased concentrations of IL-4 in co-cultures of PASMCs with M2 macrophages when treated with L-NIL (figure 6a, b). Functionally, the application of recombinant IL-4 in co-cultures of PASMCs with M2 macrophages reversed the reduction of CSE-induced proliferation caused by L-NIL-treatment of M2 macrophages (figure 6c).

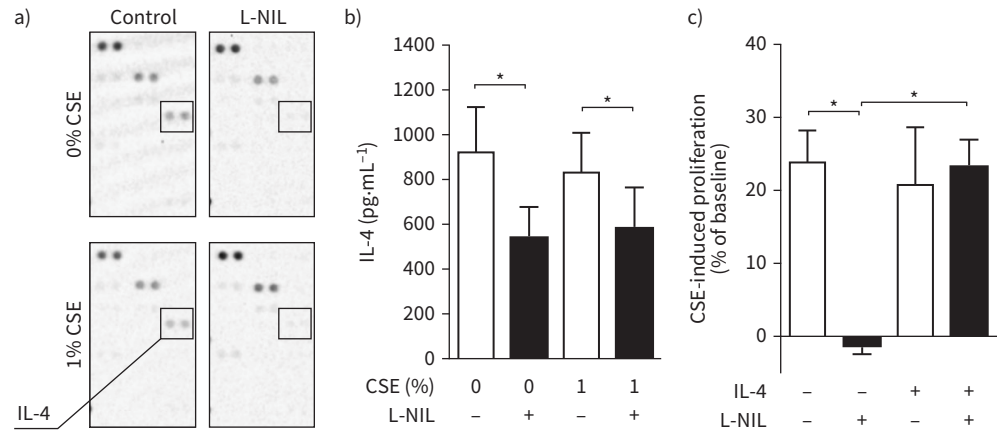


**FIGURE 5** Inducible nitric oxide synthase (iNOS) activity in macrophages increases proliferation of adjacent pulmonary artery smooth muscle cells (PASMCs) through the activation of the extracellular signal-regulated kinase (ERK) pathway. PASMCs were co-cultured with bone-marrow-derived naïve, M1- or M2-polarised macrophages pre-treated for 3 h with 1% cigarette smoke extract (CSE). **a)** Schematic depiction of the experimental setup for co-culturing bone-marrow-derived macrophages with PSMCs, and proliferation of PSMCs in co-cultures with macrophages assessed by BrdU assay after 6 h (n=4) and 24 h (n=5) standardised to control PSMCs. Data are given as the difference from the co-culturing with non-CSE exposed controls. **b)** Representative photos and Western blot analysis (n=5) of the ERK phosphorylation in PASMCs co-cultured with CSE- and/or L-NIL treated M2 cells for 1 h and 3 h. Data are given as the ratio between phosphorylated and total ERK and standardised to control co-cultures (unexposed to CSE and untreated with L-NIL). PASMCs shown in the upper panel are stained for phosphorylated ERK (green), α-smooth muscle actin (SMA) (red) and counterstained with Hoechst (blue). Scale bars=50 μm. **c)** Proliferation of PASMCs treated with the ERK inhibitor SCH772984 and conditioned medium (CM) from co-cultures of PASMCs and CSE- and/or L-NIL treated M2 macrophages, assessed by BrdU assay. The proliferation is standardised to the control (treatment with PSMC CM). Data are given as the difference between the effects of CM from CSE-exposed and unexposed co-cultures. **d)** Western blot analysis of the ERK phosphorylation in pulmonary vessels captured by laser microdissection from lungs of animals exposed to smoke for 3 months and respective unexposed controls (n=3). Data are given as the ratio between phosphorylated and total ERK, standardised to control (unexposed wild-type). Data are presented as mean±SEM. \*: p<0.05. Two-way ANOVA (with Sidak's multiple comparison *post hoc* test for *in vitro* and Tukey's multiple comparison *post hoc* test for *in vivo* experiments) was used for statistical analysis.

***iNOS<sup>+</sup> and CD206<sup>+</sup> macrophages accumulate in close proximity of remodelled vessels in lungs of COPD patients***

To investigate the relevance of the described findings for human COPD, we performed double staining of human lung sections for the macrophage marker CD68 and iNOS and found an increased number of





**FIGURE 6** Interleukin (IL-4) is implicated in cigarette smoke extract (CSE)-induced pro-proliferative signalling in co-cultures of M2 macrophages and pulmonary artery smooth muscle cells (PASCs). IL-4 in the conditioned medium from co-cultures of M2 macrophages with PASCs, assessed by **a)** cytokine array and **b)** ELISA assay (n=7). **c)** CSE-induced proliferation of PASCs in co-cultures with control or L-NIL-treated M2 macrophages, in the absence or presence of IL-4 in the co-culture medium (n=6). Proliferation was assessed by BrdU assay and standardised to the control PASCs. Data are given as the difference from the co-culturing with non-CSE exposed controls. Data are presented as mean±SEM. \*: p<0.05. Two-way ANOVA (with Sidak's multiple comparison *post hoc* test) was used for statistical analysis.

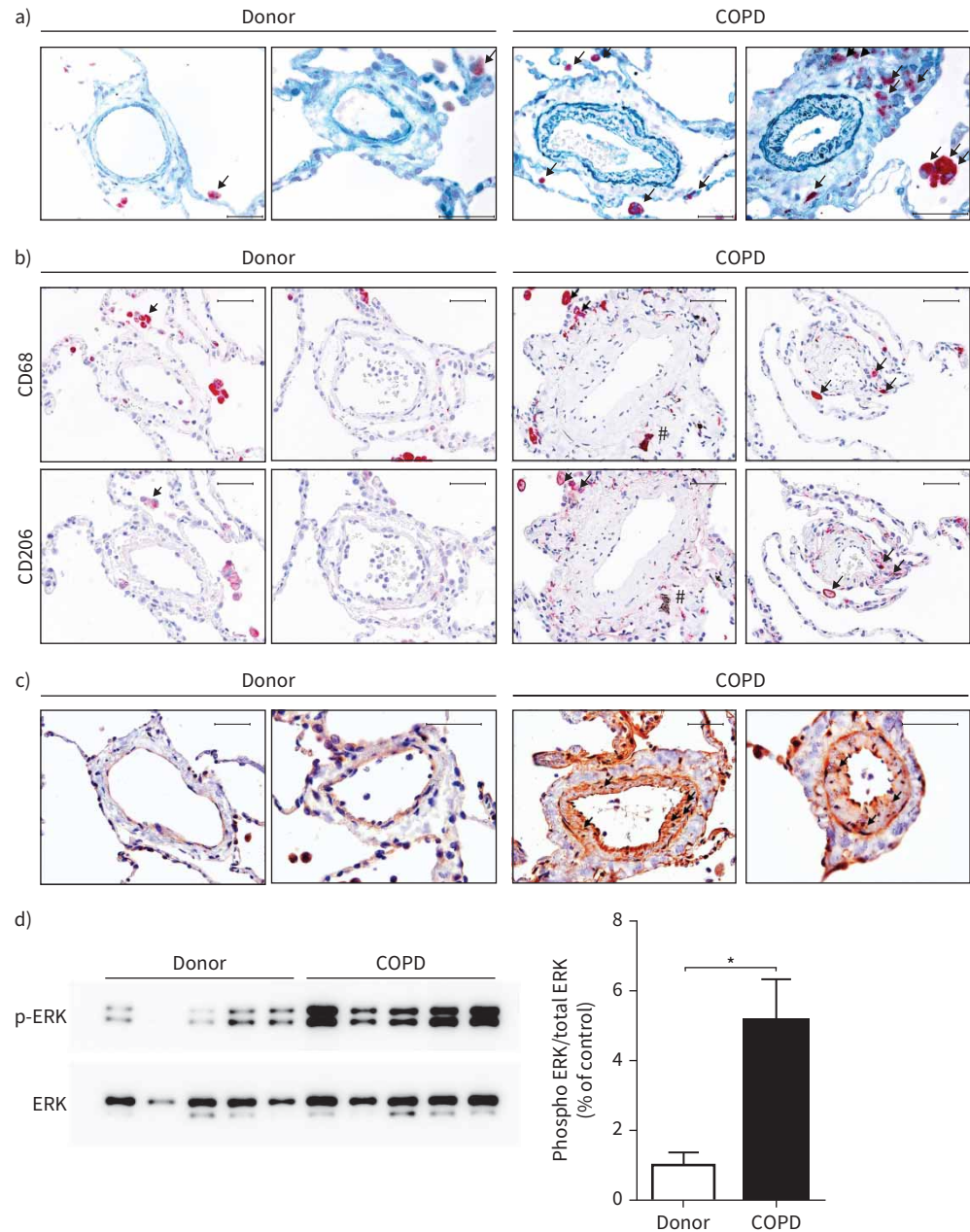
iNOS-expressing macrophages in the proximity of the remodelled vessels in COPD patients when compared to donors (figure 7a). Additionally, we stained human COPD lungs for CD68 and CD206, a protein highly expressed by alternatively activated M2-like macrophages, and found numerous double-positive cells around the remodelled vessels in COPD lungs (figure 7b). Similar to our findings in mouse lungs, immunohistochemical staining localised a high expression of phosphorylated ERK1/2 in the vessel media of COPD lungs compared to healthy donor controls (figure 7c). Increase in ERK1/2 phosphorylation in lungs of COPD patients compared to healthy donors was also confirmed by Western blot analysis (figure 7d). These data support our conclusion of an important role of M2 macrophages for pulmonary vascular remodelling.

## Discussion

Our study revealed that myeloid-cell-specific deletion of iNOS 1) prevents the development of smoke-induced PH, but not emphysema; 2) in contrast, it does not prevent hypoxia-induced PH. Myeloid-cell-specific deletion of iNOS 3) counteracts the increase in expression of the marker of M2 polarisation (CD206) on interstitial macrophages in smoke-exposed lungs. Moreover, we provided evidence for 4) the iNOS-dependent crosstalk between M2-polarised macrophages and PASCs that drives proliferation of PASCs in cigarette smoke-induced PH, and 5) the involvement of phospho-ERK and IL-4 in the downstream signalling processes (figure 8). Similar recruitment of M2-like, iNOS-containing macrophages as in our mouse model occurred in human COPD lungs in close proximity to pulmonary vessels.

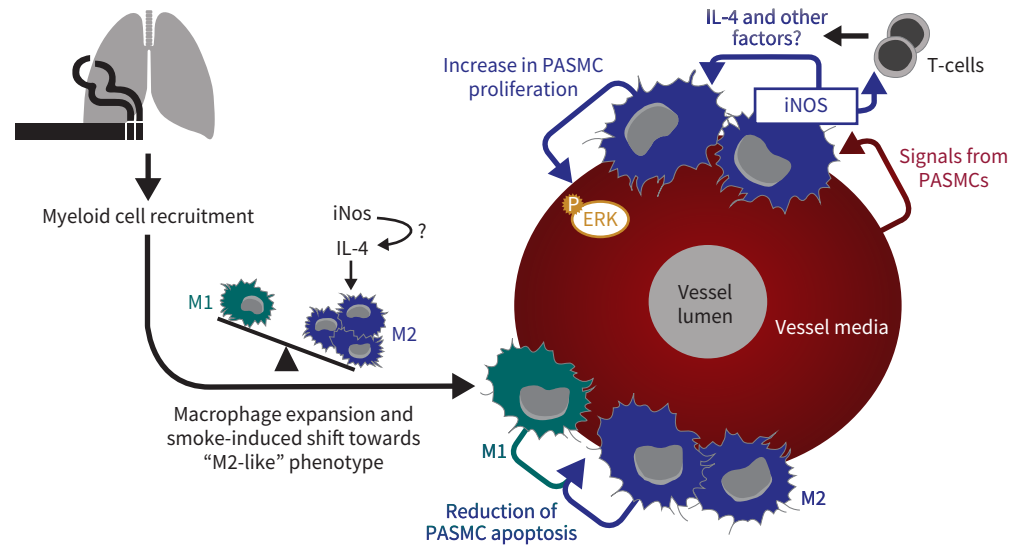
Since pulmonary vascular remodelling is an early hallmark of COPD pathology [9], it was hypothesised that this remodelling can contribute to or even drive emphysema development. However, the existence of emphysema in myeloid-cell-specific iNOS knockout animals upon smoke exposure supports our previous finding that PH and parenchymal destruction can occur independently [5]. Nevertheless, there is a possibility that molecular alterations in the vasculature (and not remodelling *per se*) are driving parenchymal destruction. Such a situation would explain emphysema development in the absence of pulmonary vascular remodelling but (co-)driven by vascular molecular alterations, as iNOS upregulation primarily in the vascular compartment of the lungs leads to parenchymal destruction [5].

Although the pivotal role of inflammatory cells and their mediators in other forms of PH is well substantiated by experimental evidence in rodent models and patients [21–23], only a few studies, mentioned in the introduction, have addressed the role of inflammation in COPD-PH. However, to the best of our knowledge, our study is the first to implicate macrophages as the inflammatory cell-type driving



**FIGURE 7** Inducible nitric oxide synthase (iNOS)<sup>+</sup> and CD206<sup>+</sup> macrophages accumulate in close proximity of remodelled vessels in lungs from COPD patients. Representative images of lung sections from donors and COPD patients. **a)** Co-staining of iNOS (green) and CD68 (red). Arrow: CD68<sup>+</sup>iNOS<sup>+</sup> cells. **b)** Staining of serial sections for CD68 (red, upper panel) and CD206 (red, lower panel). Arrow: CD68<sup>+</sup>CD206<sup>+</sup> cells; #: CD68<sup>+</sup>CD206<sup>-</sup> cells. **c)** Staining for phosphorylated extracellular signal-regulated kinase (ERK) (brownish red). Arrow: positive signal. Scale bars=50  $\mu$ m. **d)** Western blot analysis (n=10 per group) of ERK phosphorylation in lung homogenates of COPD patients and donors. Data are given as the ratio between phosphorylated and total ERK and are standardised to control (donor group). Data are presented as mean $\pm$ SEM. \*: p<0.05. A t-test was used for statistical analysis.

smoke-induced PASM C proliferation and consequent pulmonary vascular remodelling in an iNOS-dependent manner. We used a driver line targeting all myeloid cells, which by its specificity goes beyond previous studies from bone-marrow transplantation experiments and rules out shortcomings of these previous experimental approaches such as the effects of radiation and reconstitution [5, 27–29]. Our experiments revealed that macrophages seem the most important candidates of the myeloid cell lineage,



**FIGURE 8** Scheme of the proposed pathological mechanism underlying smoke-induced vascular remodelling in COPD. Communication between M2 macrophages and pulmonary artery smooth muscle cells (PASMCs) that leads to extracellular signal-regulated kinase (ERK) activation and PASMC proliferation is potentiated by smoke exposure of macrophages. It depends on inducible nitric oxide synthase (iNOS) expression in those cells and the interleukin (IL)-4 concentration in the vascular environment.

1) because iNOS plays an important role in these cells [30, 31]; 2) because of their presence in pulmonary vessels upon smoke exposure; and 3) because of their effect on PASMC proliferation in our *in vitro* experiments. Intriguingly, we demonstrated that there is a cross-talk between macrophages and PASMCs, which drives proliferation of those vascular cells and can be prevented by inhibiting or deleting M2 macrophage-derived iNOS. In agreement with our findings, a recent study reported a two-way communication between M2 macrophages and PASMCs, which relies on both CCR2 and CCR5 and drives proliferation of PASMCs *in vitro* and pulmonary vascular remodelling in idiopathic pulmonary arterial hypertension and animal hypoxia and SU5416/hypoxia models of PH *in vivo* [22]. However, no data exist for group 3 PH [16]; nor has the role of iNOS in these cells been addressed in the context of group 3 PH. Although our data support the concept that M2 macrophage-derived signals are important contributors to pulmonary vascular remodelling, we could not find smoke- or iNOS-dependent changes in the levels of CCR2 and CCR5 (unpublished observations), suggesting that there is indeed a unique molecular signature of the vasculature in COPD-PH, as previously suggested by our laboratory [5]. This conclusion is supported by our finding that iNOS deletion in myeloid cells protects against smoke-induced, but not hypoxia-induced PH (for a detailed discussion please refer to the supplementary material).

There are two important effects of myeloid-cell-specific iNOS deletion on the composition and the phenotype of lung inflammatory cells upon smoke exposure, which may amplify *in vivo* the antiproliferative effect of iNOS inhibition in M2 cells observed in our co-culture system.

First, the smoke-induced increase in the expression of CD206 on interstitial macrophages was not observed in our knockout mice, suggesting protection against the smoke-induced shift towards the M2-like phenotype. This is of even more interest, as iNOS is considered to be a classical marker of M1 polarisation. However, recent and partially contradicting reports suggest that this pleiotropic enzyme can influence polarisation of expressing macrophages. VAN DEN BOSSCHE *et al.* [32] reported that iNOS expression prevented repolarisation of macrophages from M1 to M2 phenotype, presumably through the inhibitory effect on oxidative phosphorylation. Conversely, LU *et al.* [33] showed that myeloid cell-derived iNOS suppressed M1 macrophage polarisation and speculated that it supports dedifferentiation and phenotypic plasticity of these cells, without affecting the M2 macrophage population. Although the concept that iNOS is a regulator of gene expression and phenotype of macrophages can be easily applied to our findings, none of the described scenarios can completely explain protection of myeloid-cell-specific iNOS knockout against smoke-induced PH or differential smoke-induced effects of WT and iNOS-deficient M2 macrophages on PASMC proliferation in co-culture. Due to the dynamic nature of

macrophage phenotypes, it is likely that micro-environmental factors such as smoke and signals from PSMCs *in vitro*, and additionally the age of animals, apoptosis of lung epithelial cells and presence of other inflammatory cell types and mediators *in vivo* contribute to the specific behaviour of macrophages observed in our models upon smoke exposure.

The second important consequence of myeloid-cell-specific iNOS deletion on the composition of inflammatory cell infiltrates is the preserved proportion of T-cells in the population of lung immune cells (discussed in more detail in the supplementary material).

Regarding the potential molecular mechanisms, our results indicate a regulatory role of iNOS in macrophage polarisation, in the response of M2 macrophages to smoke and in the pro-proliferative communication between PSMCs and M2 macrophages. Since such effects were not observed for M1 cells, mechanistically they are probably not caused by the production of large amounts of nitric oxide and consequent nitrosative stress, but rather by protein nitration as a regulatory post-translational modification. Such a situation was observed before for the regulation of Janus tyrosine kinase-2 downstream of the IL-12 receptor and retinoic acid receptor-related orphan receptor- $\gamma$ T, in the processes of functional maturation of natural killer and T-cells, respectively [34, 35]. The specificity of the iNOS-dependent regulation observed in our smoke-challenged M2 macrophages is further supported by the finding that deletion of this enzyme abolished their smoke-induced proliferative and pro-migratory signalling to PSMCs in co-culture, but did not influence anti-apoptotic effects that conditioned medium from such co-cultures exerted on PSMCs. Intriguingly, judging from our *in vitro* experiments, the regulatory role of iNOS in pro-proliferative signalling of M2 cells is unique for specific conditions, which include both exposure of macrophages to smoke and communication between macrophages and PSMCs. Importantly, immunohistochemical staining of human lungs suggests that similar conditions exist in COPD, as the macrophages that accumulated in close vicinity to remodelled vessels in the lungs of COPD patients were positive for iNOS and CD206.

Focusing on the iNOS-dependent signalling events, our experiments revealed that ERK signalling was upregulated in PSMCs co-cultured with M2 macrophages and in lungs of smoke-exposed WT animals. Functionally, inhibition of this pathway in PSMCs successfully counteracted pro-proliferative signals from M2 macrophages in our co-culture model. We demonstrated the relevance for human COPD, as our immunohistochemical analysis for phosphorylated ERK revealed a prominent upregulation, located in the remodelled vessels in the lungs of COPD patients. Although activation of this pathway in COPD lungs was previously investigated in the context of other anatomic lesions [36, 37], to the best of our knowledge our study is the first to provide evidence for the involvement of the ERK pathway in remodelling of the pulmonary vessel media in human COPD. Taken together, these results demonstrate that ERK signalling, implicated in other forms of PH [38–40], might also be important for smoke-induced vascular remodelling and is driven by iNOS. Furthermore, our results suggest that activation of the ERK pathway and consequent PASM proliferation is an early event after the contact with smoke-challenged M2 macrophages.

In addition, our findings suggest IL-4 as the factor that controls the responsiveness of M2 macrophages to CSE stimulation and thus regulates their pro-proliferative signalling to PSMCs. Along these lines, KUMAR *et al.* [41] demonstrated that IL-4, acting in concert with IL-13, plays a key pathogenic role in transforming growth factor (TGF)- $\beta$ -mediated *Schistosoma mansoni*-induced pulmonary arterial hypertension. In accordance with our findings (data not given), the authors did not find increased levels of total TGF- $\beta$ ; instead, they propose that M2 macrophages receiving IL-4/IL-13 signals activate a latent form of this growth factor *via* a mechanism that has yet to be elucidated. Interestingly, TGF- $\beta$  is known to induce vascular smooth muscle cell proliferation through activation of the ERK pathway [42, 43]. However, the connection between iNOS and IL-4 needs further investigation. As iNOS is known to promote glycolysis [32, 44] and lactate stimulates IL-4 and IL-13 production [45], it is conceivable that metabolic changes are the missing link connecting these two important players in CSE-induced PASM proliferation.

### Conclusions

Our data demonstrate that iNOS deletion in myeloid cells protects mice against smoke-induced PH; that M2 macrophages are the most likely candidates of the myeloid cell line driving pulmonary vascular remodelling upon smoke exposure; and that cross-talk between M2 macrophages and PSMCs is an essential process in this regard. iNOS dependent ERK- and IL-4-signalling have been identified as mechanistic downstream signalling (figure 8). Similar processes as in our mouse model occur in human COPD. Our data further support the concept that pulmonary vascular remodelling and emphysema can

occur independently, but does not rule out that parenchymal destruction can be driven by molecular alterations in vascular cell types.

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