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Pulmonary hypertension in smoking mice over-expressing protease-activated receptor-2

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ABSTRACT: The mechanism(s) involved in the development of pulmonary hypertension (PH) in COPD is still the object of investigation. Cigarette smoke (CS) may lead to remodelling of intrapulmonary vessels and dynamic changes in vascular function, at least in some smokers. A role for proteases in PH has been recently put forward.

We investigated, in smoking mice, the role of protease-activated receptor (PAR)-2 in the pathogenesis of PH associated with emphysema.

We demonstrated that CS exposure can modulate PAR-2 expression in mouse lung. Acute CS exposure induces in wildtype (WT) and in transgenic mice over-expressing PAR-2 ($FVB^{PAR\text{-}2\text{-}TgN}$) a similar degree of neutrophil influx in bronchoalveolar lavage fluids. After chronic CS exposure WT and $FVB^{PAR\text{-}2\text{-}TgN}$ mice show emphysema, but only transgenic mice develop muscularisation of small intrapulmonary vessels that precedes the development of PH (~45% increase) and right ventricular hypertrophy. Smoking in $FVB^{PAR\text{-}2\text{-}TgN}$ mice results in an imbalance between vasoconstrictors (especially endothelin-1) and vasodilators (*i.e.* vascular endothelial growth factor, endothelial nitric oxide synthase and inducible nitric oxide synthase) and enhanced production of growth factors involved both in fibroblast-smooth muscle cell transaction (*i.e.* platelet-derived growth factor (PDGF) and transforming growth factor β) and vascular cell proliferation (PDGF).

PAR-2 signalling can influence the production and release of many factors, which may play a role in the development of PH in smokers.

KEYWORDS: Animal model of emphysema, cigarette smoke, pulmonary hypertension, right ventricular hypertrophy, vascular remodelling

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The most important risk factor for the development of chronic obstructive pulmonary disease (COPD) is cigarette smoke (CS) [1, 2]. Inhalation of cigarette smoke causes a chronic pulmonary inflammatory infiltrate of macrophages, neutrophils and CD8+ cells that persists long after smoking cessation [3]. In susceptible individuals, this ultimately leads to emphysema characterised by irreversible destruction and dilatation of the terminal airspaces of the lung, chronic disability due to respiratory failure and premature death. One established complication of COPD is the development of pulmonary hypertension (PH). Its presence is associated with shorter survival rates and worse clinical evolution [4].

Inflammation plays a pivotal role in the pathophysiology of COPD, as it is associated to an increase of oxidant burden and an abnormal secretion/activation of proteases, which may cause the proteolytic breakdown of interstitial matrix [5]. Some of these proteases, belonging to the serine group, may represent also signalling molecules that can activate cells by cleaving specific cell surface receptors called protease-activated receptors (PARs), a family of four G-protein-coupled receptors.

After the discovery of the first PAR, the thrombin receptor PAR-1, three other receptors have been identified: PAR-2, PAR-3 and PAR-4 [6]. Among the four members so far identified, PAR-1 and PAR-2 are the most well characterised. PAR-1 is expressed on human platelets and vascular endothelial cells. PAR-2 is widely distributed, being found on vascular endothelial cells, fibroblasts,

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smooth muscle cells, bronchial endothelium, neutrophils, eosinophils, sensory neurons and cells of the gastro-intestinal tract [7, 8].

Recent studies demonstrate that PAR-2 is an integral component of the inflammatory process, and significantly, its expression by human endothelium is up-regulated by cytokines. The diffuse distribution of PAR-2 across cells from multiple systems suggests its potential role in both local and systemic inflammation [9]. Although PAR-2 activation is generally considered as pro-inflammatory, its role in the lung is controversial because there is evidence of both pro- and anti-inflammatory activities [8].

In the cardiovascular system, PAR-2 may play a role in vascular tone and blood pressure regulation as it is expressed on both vascular endothelium and smooth muscle cells [10]. Given its activation by coagulation enzymes (*i.e.* tissue factor VIIa/factor Xa complex), mast cell tryptase, epithelial proteases (*e.g.* trypsin) and enzymes released by inflammatory cells, PAR-2 could be a potential link between chronic lung inflammation and development of vascular changes in COPD [8, 11].

Chronic exposure of mice to cigarette smoke leads to lung inflammation and emphysema that, at least in part, mimic the lung changes observed in human COPD [3, 12–14]. In this study, we show that the over-expression of PAR-2 in transgenic mice ($FVB^{PAR\text{-}2\text{-}TgN}$), results in emphysema and a marked vascular remodelling of small intrapulmonary vessels in response to CS treatment. Vascular changes precede the development of PH and right ventricular hypertrophy (RVH). A series of alterations in gene expression of cytokines, growth factors and regulators of vascular tone characterises the increase of smooth muscle cells.

MATERIALS AND METHODS

An expanded materials and methods section is available in the online supplementary material.

All animal experiments were conducted in conformity with the ''Guiding Principles for Research Involving Animals and Human Beings'' and approved by the Local Ethics Committee of the University of Siena, Siena, Italy.

Experimental animals

Transgenic mice over-expressing PAR-2 ($FVB^{PAR\text{-}2\text{-}TgN}$) were supplied by Roche Bioscience (Palo Alto, CA, USA). Generation of these mice has previously been described [15]. Wild type (WT) FVB/N mice were purchased from Charles River Italia (Calco, Italy).

Experimental design

The contribution of PAR-2 to CS-induced lung changes was examined by comparing the response obtained in $FVB^{PAR\text{-}2\text{-}TgN}$ and WT mice. Four groups of mice were examined: $FVB^{PAR\text{-}2\text{-}TgN}$ and WT mice exposed to either room air or to the smoke of 3 cigarettes·day^{-1}, 5 days·week^{-1} for 1, 2, 4 and 7 months. In a preliminary experiment, we evaluated the potential role of PAR-2 in an acute smoke model of inflammation caused by the exposure of 5 cigarettes·day^{-1} for 3 consecutive days. The methodology for smoke exposure has previously been described in detail [16].

Morphology and morphometry

At 2, 4 and 7 months after chronic exposure to room air or CS, 12 animals of each group were sacrificed under anaesthesia with sodium pentobarbital and the lungs removed for morphological and morphometric assessment of emphysema. At 4 and 7 months after CS exposure measurements of right ventricular systolic pressure ($P_{rv,s}$) and assessment of RVH was performed.

Immunohistochemical analysis

The degree of muscularisation of small and medium vessels was determined after immunohistochemical staining for α-smooth muscle actin (α-SMA).

Tissue sections from mice exposed to room air or CS for 2, 4 and 7 months were also stained for transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF)-β, vascular endothelial growth factor (VEGF)-A, endothelin (ET)-1, endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and hypoxia-inducible factor 1 (HIF-1)-α protein. Some tissue sections from mice of both genotypes were also stained for PAR-2 in order to see whether CS exposure modifies the expression of the receptor.

Protein and RNA analysis

At 1 month after exposure to room air or CS, lungs from eight mice for each group were analysed by real-time RT-PCR for α-SMA, Tgfb1, Pdgfb, ET-1, Vegf-A, eNOS, iNOS, HIF-1-α and 18S rRNA.

At 4 and 7 months after exposure to room air or CS, lungs from eight mice for each group were analysed by western blots for eNOS, phospho-eNOS and VEGF.

Plasma ET-1 assay was carried out on additional eight $FVB^{PAR\text{-}2\text{-}TgN}$ and eight WT mice exposed to room air or chronic CS for 4 and 7 months.

Proliferation and apoptosis indices

At 1 month after CS exposure, SM cells of small lung vessels undergoing apoptosis or proliferation were evaluated by terminal deoxy-nucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) assay or by immunostaining with proliferating cell nuclear antigen (PCNA), respectively.

The above-mentioned methods are provided in detail in the online supplementary material.

Statistical analysis

Data are presented as mean±SD. The significance of the differences was calculated using one-way analysis of variance. A p-value <0.05 was considered significant.

RESULTS

PAR-2 hyperexpression in FVB mice did not modify the bronchoalveolar lavage fluid cell profile in response to acute cigarette smoke

In preliminary experiments, we evaluated the potential role of PAR-2 in an acute smoke model of inflammation caused by CS exposure (5 cigarettes·day^{-1} for 3 consecutive days). CS exposure induced a significant increase of neutrophils in smoking animals of both genotypes; however, we did not observe

significant differences in bronchoalveolar lavage fluid (BALF) cell counts between smoking WT and FVB$^{PAR\text{-}2\text{-}TgN}$ mice (table 1 in the online supplementary material).

Expression of PAR-2 in smoking mice

An increased expression of PAR-2 was found in lung of WT FVB mice starting from 1 month after CS exposure (fig. 1 in the online supplementary material).

Cigarette smoke causes lung emphysema in WT and FVB$^{PAR\text{-}2\text{-}TgN}$ mice

The lungs of FVB$^{PAR\text{-}2\text{-}TgN}$ and WT mice exposed to air showed a well-fixed normal parenchyma with normal airways (figs. 1a and c). 7 months after exposure to CS the lungs of both, FVB$^{PAR\text{-}2\text{-}TgN}$ and WT mice showed similar degree of emphysema as assessed by morphology (figs. 1b and d) and morphometry (table 1).

Cigarette smoke causes vascular remodelling, pulmonary hypertension and right ventricular hypertrophy only in FVB$^{PAR\text{-}2\text{-}TgN}$ mice

FVB$^{PAR\text{-}2\text{-}TgN}$ mice developed a marked lung vascular remodelling after CS exposure. The vascular changes preceded the development of PH and RVH.

The values of $P_{rv,s}$ of FVB$^{PAR\text{-}2\text{-}TgN}$ and WT mice obtained at 4 and 7 months after CS and air exposure are shown in table 1. $P_{rv,s}$ was significantly increased at 7 months in smoking FVB$^{PAR\text{-}2\text{-}TgN}$ mice (+45%) with respect to the control mice. No changes in $P_{rv,s}$ were observed between smoking and air-control FVB$^{PAR\text{-}2\text{-}TgN}$ mice at 4 months and smoking and air-control WT mice at 4 and 7 months after CS.

The presence of PH in transgenic mice is accompanied by RVH as demonstrated by the significant increase in the ratio of RV/LV+S weight at 7 months after CS exposure (table 1). RV/LV+S weight did not differ between smoking and air-exposed

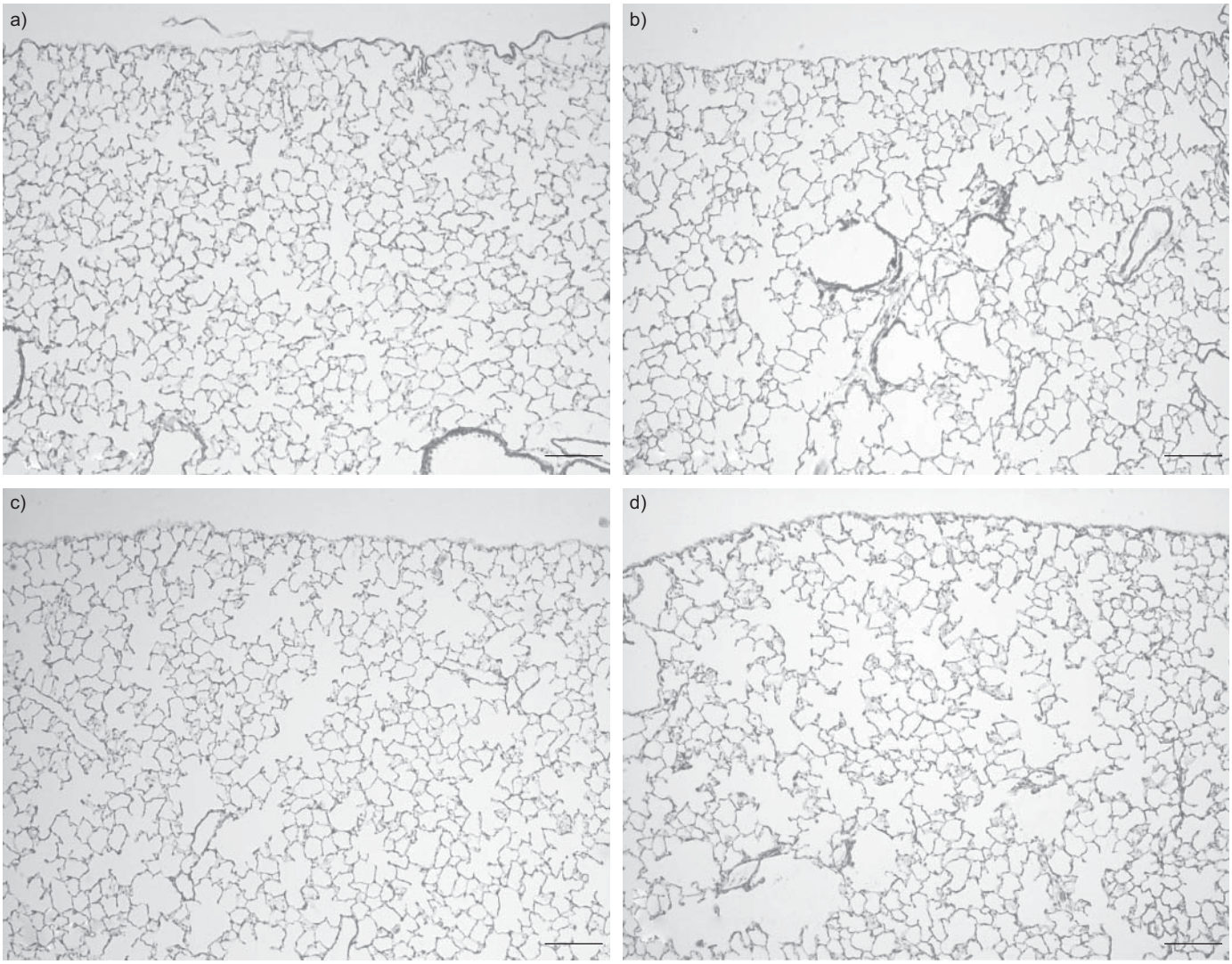

FIGURE 1. Histological sections from the lung of a) FVB$^{PAR\text{-}2\text{-}TgN}$ and c) wildtype (WT) air control mice showing a normal appearance. Lungs of b) FVB$^{PAR\text{-}2\text{-}TgN}$ and d) WT after 7 months of cigarette smoke exposure show focal areas of emphysema. Haematoxylin and eosin stain. Scale bars: 120 μm.

TABLE 1 Lung morphometry, right ventricular systolic pressure ($P_{rv,s}$) and Fulton's index determined in wildtype (WT) and $FVB^{PAR-2-TgN}$ mice at 4 and 7 months after chronic exposure to either room air or cigarette smoke

Group	Lm μm		ISA cm^2		$P_{rv,s}$ mmHg		RV/LV+septum	
	4 months	7 months	4 months	7 months	4 months	7 months	4 months	7 months
Room air								
FVB WT	34.15±1.06	36.48±1.17	1307±53	1279±49	26.4±1.3	26.7±1.7	0.201±0.017	0.197±0.014
$FVB^{PAR-2-TgN}$	34.19±0.81	35.43±0.84	1329±61	1318±58	26.1±0.9	26.2±0.7	0.230±0.014	0.232±0.012
Smoke								
FVB WT	39.23±1.60*	40.23±0.79*	1174±64*	1096±37*	26.7±1.9	26.9±1.9	0.210±0.016	0.213±0.017
$FVB^{PAR-2-TgN}$	38.71±1.40*	40.22±1.58*	1098±127*	1069±84*	27.3±0.9	38.3±0.6*	0.237±0.014	0.318±0.031*

Data are presented as mean±SD. Lm: mean linear intercept; ISA: internal surface area of the lungs; RV: right ventricle; LV: left ventricle. *: $p<0.05$ *versus* the group exposed to air.

$FVB^{PAR-2-TgN}$ mice at 4 months as well as between smoking and air-control WT mice at 4 and 7 months.

A significant vascular remodelling, characterised by an increase of smooth muscle cells in small intrapulmonary vessels precedes and accompanies the development of RVH and PH in smoking $FVB^{PAR-2-TgN}$ mice.

Plexiform or angiomatoid lesions, which usually occur in idiopathic PH in man, were not observed in smoking $FVB^{PAR-2-TgN}$ mice under our experimental conditions.

Smoking $FVB^{PAR-2-TgN}$ mice exhibited significant increase in muscularisation of small pulmonary vessels over air-control mice (fig. 2). At 2 months, in air-exposed controls of both strains, <15% of small vessels were muscularised (12±2 in WT *versus* 11±3 in $FVB^{PAR-2-TgN}$ mice, n=12) (fig. 2a). Similar values were obtained in air-exposed groups of both genotypes at 4 and 7 months. At 2, 4, and 7 months after CS exposure, 14±4%, 15±3% and 15±4% small pulmonary vessels of WT mice, and 49±5%, 51±7% and 52±5% of small vessels of $FVB^{PAR-2-TgN}$ mice, respectively, were muscularised ($p<0.01$ for the differences of muscularised vessels between genotypes).

As shown in fig. 2b, the differences in muscularisation of small pulmonary vessels between genotypes at 7 months can be accounted for by an increase in the percentage of both partially and fully muscularised vessels. In contrast, most medium vessels with a diameter of 81–150 μm were fully muscularised (87±5% in WT *versus* 90±6% in $FVB^{PAR-2-TgN}$ mice; p=NS). No increase in the percentage of muscularised medium vessels was observed in both mouse strains after CS exposure (data not shown).

At 7 months, small pulmonary vessels of smoking $FVB^{PAR-2-TgN}$ mice also showed thickening of the fully muscularised SM wall as evidenced by the increase in percent vessel wall thickness (%VWT) compared with that of their air-exposed controls (29.1±4.0 *versus* 19.2±3.1; $p>0.05$). No significant increase in %VWT was found in the smoking WT mice in respect to their respective air-exposed controls (19.0±2.9 *versus* 18.1±2.1).

Figure 2c and d illustrates representative immunohistochemistry images for α-SMA in pulmonary vessels from both genotypes 7 months after CS exposure.

Vascular remodelling in smoking $FVB^{PAR-2-TgN}$ mice is not linked to HIF-1 hyper-expression

It has recently been reported that hypoxia-inducible factor 1 (HIF-1)-α can promote proliferative response of vascular smooth muscle cells [17, 18]. In our experimental condition, we detected appreciable amount of this factor only at 7 months after CS exposure, when vascular remodelling was already evident (fig. 3). A positive reaction for this factor was evident on small (arrowheads) and medium (arrows) pulmonary vessels from both genotypes.

The combination of chronic CS exposure with over-expression of PAR-2 gene results in a vasoconstrictors/vasodilators imbalance

A series of bioactive substances such as TGF-β, PDGF-β, ET-1, VEGF, eNOS and iNOS were investigated at various time points in the two mouse genotypes.

A strong positive immunohistochemical reaction for TGF-β was found in vessels of $FVB^{PAR-2-TgN}$ mice as early as at 2 months after CS, with a maximal staining at 4 months (fig. 4b, arrow). At the same times, no immuno-reaction for this cytokine was seen on small pulmonary vessels of smoking WT mice (fig. 4d, arrow). Additionally, no reaction was found in lung vessels from the air-exposed groups of the two genotypes (fig. 4a and c). Immunohistochemical grading for TGF-β is reported in figure 4e.

With regard to PDGF-β, a progressive increased signal was seen in pulmonary vessels of smoking $FVB^{PAR-2-TgN}$ mice starting from month 4, with a maximal reaction at month 7 (fig. 4g, arrows). A positive signal for this cytokine was currently found in bronchial and bronchiolar epithelium from air- and smoke exposed mice of both genotypes, but not in lung vessels from air- and smoke-exposed WT (fig. 4h and i, arrow) and air-exposed transgenic mice (fig. 4f, arrow). The immunohistochemical-based grading analysis for this mediator is reported in figure 4j.

Lung vascular changes in smoking $FVB^{PAR-2-TgN}$ mice were also characterised by an enhanced expression of the vasoconstrictor ET-1 in endothelial and smooth muscle cells of middle and small pulmonary vessels as well as of alveolar capillaries. This reaction was clearly evident at all times after CS exposure (fig. 5b–e).

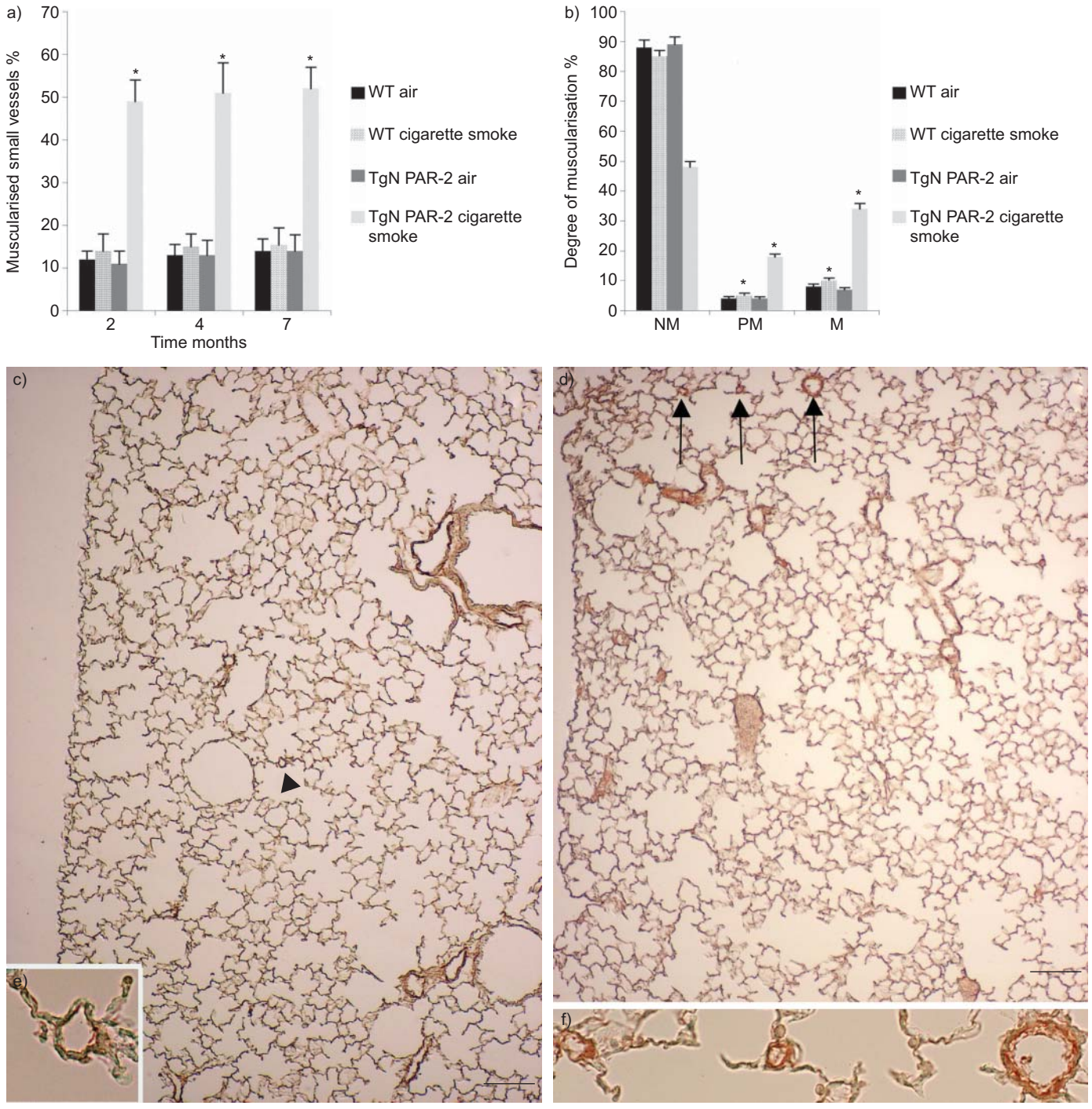

FIGURE 2. Muscularisation of small (⩽80 μm) vessels in wildtype (WT) and FVB$^{PAR\text{-}2\text{-}TgN}$ mice after exposure to cigarette smoke (CS) or air. a) Percentage of small lung vessels showing any degree of muscularisation at indicated time points. b) Percentage of nonmuscular (NM), partially muscular (PM) and fully muscular (M) small vessels at 7 months after cigarette smoke exposure. *: $p \leqslant 0.01$ *versus* air control groups. Representative immunohistochemical staining for α-smooth muscle actin (SMA) on lung parenchyma of c) WT and d) FVB$^{PAR\text{-}2\text{-}TgN}$ mice at 7 months after CS exposure. Immunostaining with anti-α-SMA antibodies shows excessive thickening of α-SMA-positive layers in small intrapulmonary vessels of FVB$^{PAR\text{-}2\text{-}TgN}$ mice (inset as f). e) A small lung vessel from WT mouse at 7 months after CS exposure is reported for comparison. Scale Bars: 150 μm.

Only a faint reaction for ET-1 was appreciated on vessels of lung sections from air control FVB$^{PAR\text{-}2\text{-}TgN}$ (fig. 5a) and from WT mice at 7 months after CS (fig. 5f). The immunohistochemical-based grading analysis for ET-1 at the various time points is shown in fig. 5g.

Of interest, the plasma ET-1 levels were significantly increased in smoking transgenic mice at 4 and 7 months (+158.2% and 291.2%, respectively) as compared with air exposed mice (181 fmol·mL^{-1}). Additionally, the mean plasma ET-1 levels were also found to be significantly increased in WT mice at the

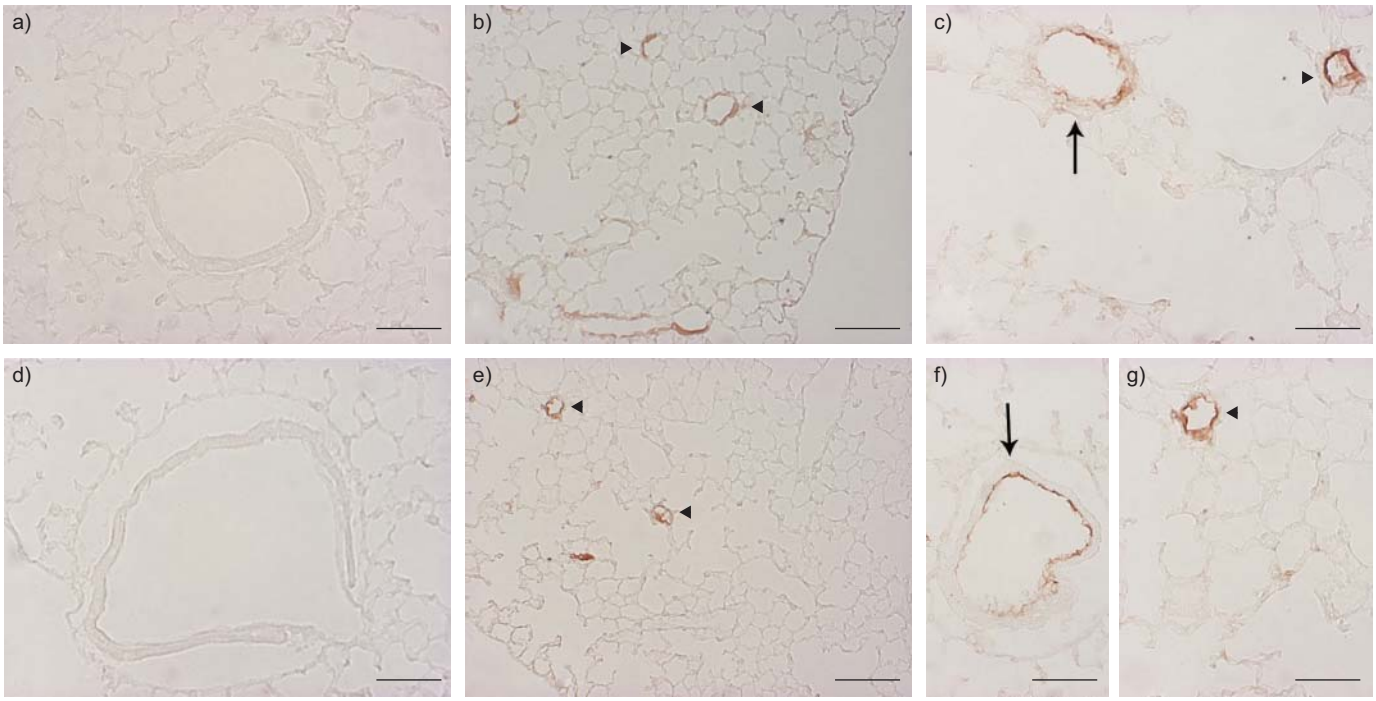

FIGURE 3. Representative immunohistochemical reaction for hypoxia-inducible factor 1-α on small (arrowheads) and medium (arrows) pulmonary vessels from $FVB^{PAR\text{-}2\text{-}TgN}$ and wildtype (WT) mice. a) Air control $FVB^{PAR\text{-}2\text{-}TgN}$ mice; b and c) $FVB^{PAR\text{-}2\text{-}TgN}$ mice after 7 months of cigarette smoke (CS) exposure; d) air control WT mice; e–g) WT mice after 7 months of CS exposure. Scale bars: 150 μm for b and e; and 75 μm for a, c, d, f and g.

same time points (+45.6% and +61.5%, respectively) with respect to their air-controls (206 $fmol \cdot mL^{-1}$), but at lower extent than those found in $FVB^{PAR\text{-}2\text{-}TgN}$ mice.

In the lungs of air-control transgenic mice, only a minimal reaction for VEGF was appreciable in the middle and small vessels, as well as in lung epithelial cells (fig. 6a). An increased VEGF expression could be observed in smoking transgenic mice only at 4 months after CS exposure (fig. 6b). A more evident VEGF signal was seen in lungs of air exposed WT mice as compared to those of transgenic mice (fig. 6c). Unlike transgenic mice, no changes of VEGF expression were found in WT animals at the different time points after CS exposure (fig. 6d). The immunohistochemical-based grading analysis for this cytokine is reported in figure 6e.

An increased VEGF expression (kDA 48) was also observed at 4 months in whole lung tissue from smoking transgenic mice after Western blotting (WB) analysis (fig. 6f).

A positive immunostaining for eNOS was seen on lung vessels of air-exposed (fig. 6g and i) and smoke-exposed transgenic and WT mice at 2 and 4 months. This reaction just returned to the control levels in smoking animals of both genotypes at 7 months (fig. 6h and j). Of interest, only in FVB $^{PAR\text{-}2\text{-}TgN}$ mice a positive staining for eNOS was evident in some small vessels at this time point (fig. 6h). In fig. 6k data on the immunohistochemical-based grading analysis for eNOS at the various time points is shown.

Using antibodies specifically directed against the Ser^{1177} phosphorylation site of the eNOS (the preferential site of activation of eNOS by VEGF) [19], we analysed the expression of active eNOS relative to whole eNOS (140 kDA). A basal phosphorylation was detectable by WB in lung tissues from both air exposed WT and transgenic mice (fig. 6l). An enhancement in phosphorylation of eNOS at Ser^{1177} was observed in smoking FVB $^{PAR\text{-}2\text{-}TgN}$ mice after 4 months, with a significant decrease of the active form at 7 months. On the contrary, at 7 months after CS exposure the protein band corresponding to phosphorylated eNOS appeared increased in WT when the band of whole eNOS was significantly decreased. Of interest, a chronological parallel between VEGF and phosphorylation of eNOS occurs in FVB $^{PAR\text{-}2\text{-}TgN}$ mice.

No detectable immuno-reaction was found for the vasodilator iNOS at any time points under our experimental conditions (data not shown).

These findings altogether suggest that an imbalance between vasoconstrictors/vasodilators occurs in the vascular bed of CS exposed FVB $^{PAR\text{-}2\text{-}TgN}$ mice.

The increase in muscularisation of lung vessels in smoking $FVB^{PAR\text{-}2\text{-}TgN}$ mice is due to an increased proliferation of SM cells

To investigate whether the increase in muscularisation of lung vessels was associated with an increase in proliferation or/and a decrease in apoptosis of SM cells, proliferation and apoptosis indexes were examined at 1 month after CS exposure. This time was chosen because muscularisation of small pulmonary arteries was readily evident at 2 months and thus the processes of proliferation or apoptosis might have already occurred in this time point.

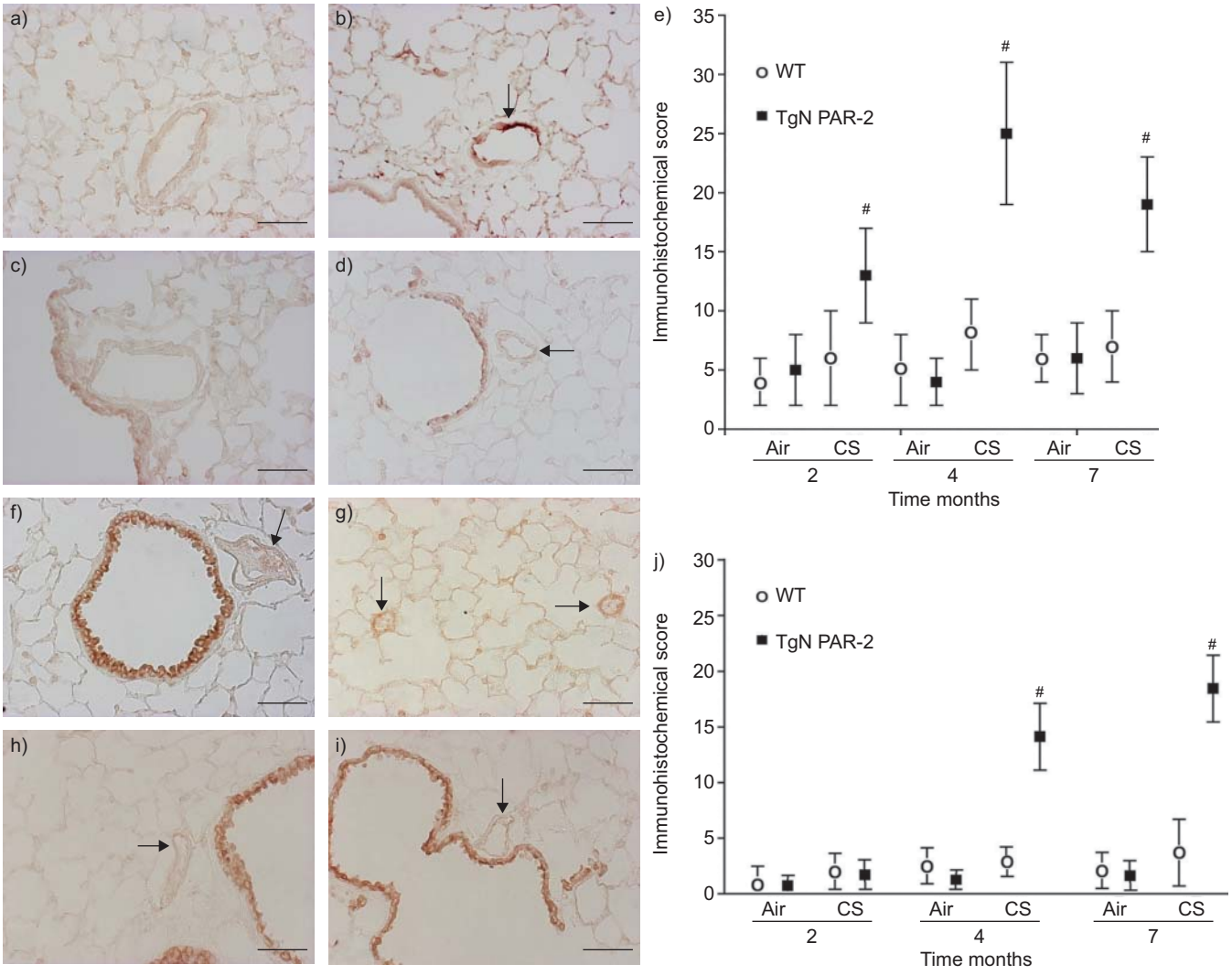

FIGURE 4. Photographic panel illustrating immunohistochemical staining for a–d) transforming growth factor (TGF)-β and f–i) platelet-derived growth factor (PDGF)-β on lung sections of $FVB^{PAR\text{-}2\text{-}TgN}$ and wildtype (WT) mice. a) TGF-β staining in air control $FVB^{PAR\text{-}2\text{-}TgN}$ mouse; b) $FVB^{PAR\text{-}2\text{-}TgN}$ mice after 4 months of cigarette smoke (CS) exposure; c) air control WT mice; and d) WT mice after 4 months of CS exposure. Arrows point to small pulmonary vessels. f) PDGF-β staining in air control $FVB^{PAR\text{-}2\text{-}TgN}$ mouse; g) $FVB^{PAR\text{-}2\text{-}TgN}$ mice after 7 months of CS exposure; h) air control WT mice; and i) WT mice after 7 months of CS exposure. Arrows point to small pulmonary vessels. Scale bars: 75 μm. Immunohistochemical-based grading analysis for e) TGF-β and j) PDGF-β, was carried out on small arteries of lungs from air- or smoke-exposed mice for each experimental group at 2, 4 and 7 months of treatment. Data are presented as mean ± SD. #: $p \leq 0.05$ compared with air-control small vessels of the same genotype at the same time point.

We found only a very small number of TUNEL-positive SM cells in the lung sections of air- and CS-exposed WT or PAR-2 transgenic mice, and no significant difference in TUNEL staining between the two genotypes at 1 month after CS (data not shown). Alternatively, the number of PCNA-positive SM cells in small distal arteries of smoking $FVB^{PAR\text{-}2\text{-}TgN}$ group appeared to be greater than that of smoking WT mice (fig. 7a–c). No differences among air-control WT, air-control PAR-2 transgenic and smoking WT mice were found in the ratio of PCNA-positive nuclei to total nuclei of SM cells (data not shown).

The increased proliferation of SM cells in small distal arteries of the lungs of smoking $FVB^{PAR\text{-}2\text{-}TgN}$ was accompanied by a significant up-regulation of mRNA for α-SMA, whereas no change in mRNA expression for α-SMA was seen in WT mice at the same time point (fig. 7f). Of interest, a strong reaction for PAR-2 was found on muscularised small vessels from WT and smoking $FVB^{PAR\text{-}2\text{-}TgN}$ mice at 7 months after CS exposure (fig. 7d and e).

The combination of CS with the hyper-expression of PAR-2 gene resulted also in up-regulation of mRNAs for TGF-β, PDGF-β, ET-1, eNOS, and VEGF (fig. 7f). Of interest, only a slight increase of mRNAs for some of these mediators was observed at RT-PCR analysis in smoking WT mice.

DISCUSSION

We report here that PAR-2 over-expression in mice did not modify the influx of inflammatory cells in BALF after acute exposure to CS. In addition, $FVB^{PAR\text{-}2\text{-}TgN}$ mice developed after chronic smoke exposure pulmonary emphysema at the same extent of that of WT mice.

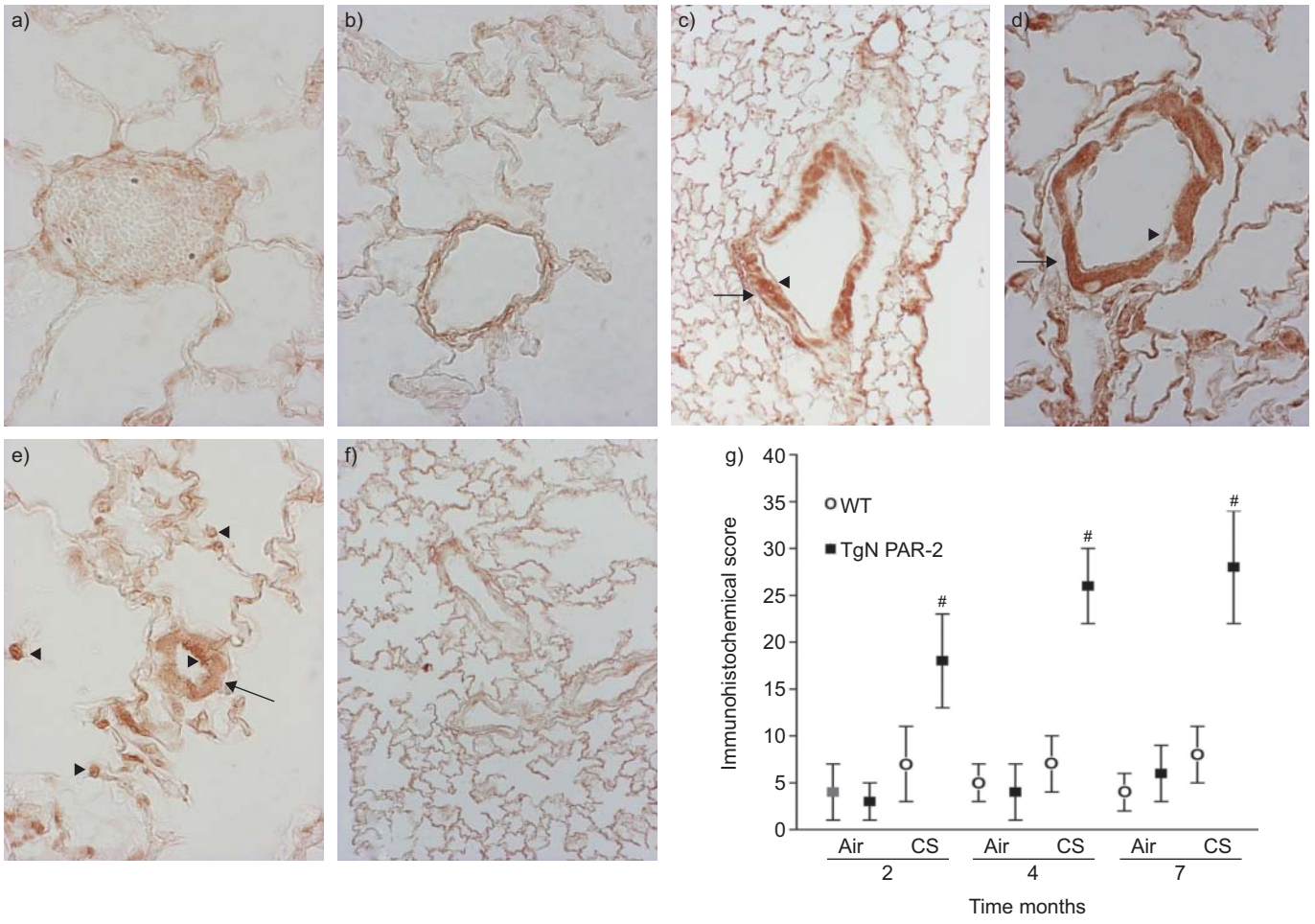

FIGURE 5. Representative immunohistochemical staining for endothelin (ET)-1 on lung sections of a) air control, and cigarette smoke (CS)-exposed $FVB^{PAR\text{-}2\text{-}TgN}$ mice at b) 2, c) 4, and d and e) 7 months after CS exposure. f) ET-1 reaction of tissue sections of wildtype (WT) mice at 7 months after CS. A positive reaction for ET-1 is found on endothelial (arrowheads) and smooth muscle cells (arrows) of middle and small pulmonary vessels as well as of alveolar capillaries of smoking transgenic mice at 4 and 7 months. Scale bars: 33 μm for a, b, d and e; and 100 μm for c and f. g) Immunohistochemical-based grading analysis for ET-1 carried out on small arteries of lungs from air- or smoke-exposed mice for each experimental group at 2, 4 and 7 months of treatment. Data are presented as mean±SD. #: $p \leqslant 0.05$ compared with air-control small vessels of the same genotype at the same time point.

However, pulmonary emphysema in mice over-expressing PAR-2 was accompanied by the development of PH, RVH and vascular changes reminiscent of human PH in COPD [20, 21]. It also appears that PAR-2 hyper-expression by itself was not sufficient to cause PH, but in combination with CS exposure it could promote the development of PH.

Unlike WT mice, $FVB^{PAR\text{-}2\text{-}TgN}$ mice showed, at 7 months after CS exposure, areas of significant emphysema associated with ~45% increase in mean $P_{rv,s}$, and a marked vascular remodelling of small pulmonary vessels. Vascular changes and RVH were not observed in WT mice exposed to CS, which developed a similar degree of emphysematous lesions.

These data altogether suggest that alveolar destruction by itself is not sufficient to cause smoking-induced cor pulmonale in FVB smoking mice.

In response to CS, the hyper-expression of the PAR-2 in FVB mice resulted in a series of alterations in gene expression of vasoconstrictors, vasodilators and growth factors involved in the process of vascular remodelling [22–26]. The increased muscularisation of small vessels was preceded by enhanced production of growth factors involved in fibroblast-SMC transaction (PDGF and TGF-β) [27, 28] and vascular cell proliferation (PDGF) [29, 30], and by an imbalance between vasoconstrictors (especially ET-1) and vasodilators (*i.e.* VEGF, eNOS and iNOS).

These events may be originated by different cell signalling pathways that follow to PAR-2 activation. In particular, activation of mitogen-activated protein kinase, protein-tyrosine phosphatase SHP2 and tyrosine kinase pathways could contribute to PAR-2-mediated cytokine production and mitogenic signalling [31].

Of interest, the imbalance between vasoconstrictors and vasodilators in transgenic mice appears to be due to a constant up-regulation of ET-1 not counteracted by a parallel increase of some important vasodilators (such as eNOS, iNOS and VEGF-A). These changes were not accompanied by an increased expression and accumulation of HIF-1α, an important mediator

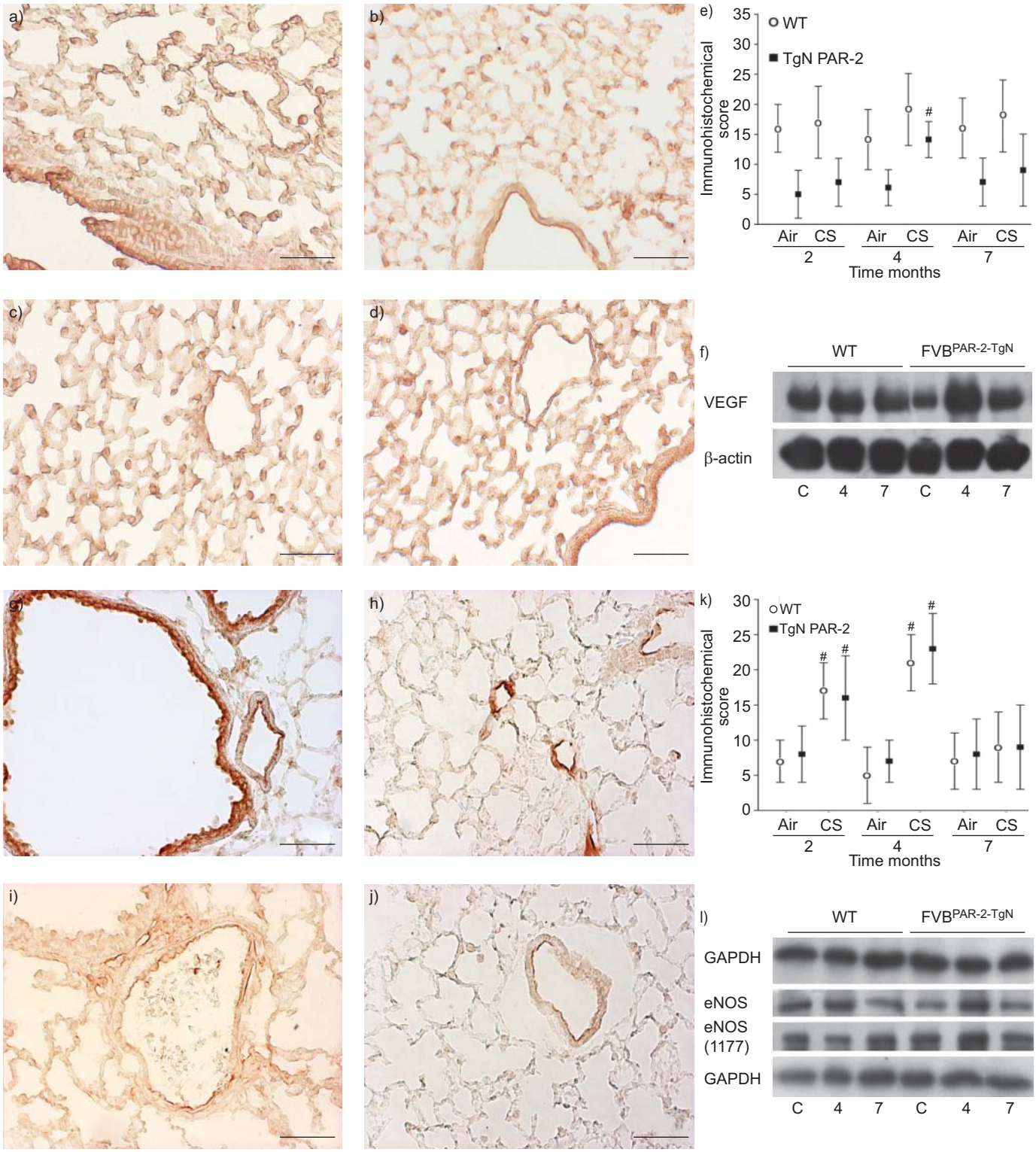

FIGURE 6. Representative immunohistochemical reactions for a–d) vascular endothelial growth factor (VEGF) and g–j) endothelial nitric oxide synthase (eNOS) on lung sections of FVB$^{PAR\text{-}2\text{-}TgN}$ and wildtype (WT) mice. a) VEGF staining in air control FVB$^{PAR\text{-}2\text{-}TgN}$ mice; b) FVB$^{PAR\text{-}2\text{-}TgN}$ mice after 4 months of cigarette smoke (CS) exposure; c) air control WT mice; and d) WT mice after 4 months of CS exposure. g) eNOS immunolocalisation in air control FVB$^{PAR\text{-}2\text{-}TgN}$ mice; h) FVB$^{PAR\text{-}2\text{-}TgN}$ mice after 7 months of CS exposure; i) air control WT mice; and j) WT mice after 7 months of CS exposure. Scale bars: 100 μm in a–d; and 75 μm in g–j. Immunohistochemical-based grading analysis for e) VEGF, and k) eNOS carried out on small arteries of lungs from air- or smoke-exposed mice for each experimental group at 2, 4 and 7 months of treatment. Data are presented as mean±SD. #: $p \leqslant 0.05$ compared with air-control small vessels of the same genotype at the same time point. Representative western blotting analysis with anti-VEGF antibody, and anti-eNOS and anti-phospho-eNOS antibodies are reported in f) and l), respectively. GAPDH: reduced glyceraldehyde phosphate dehydrogenase.

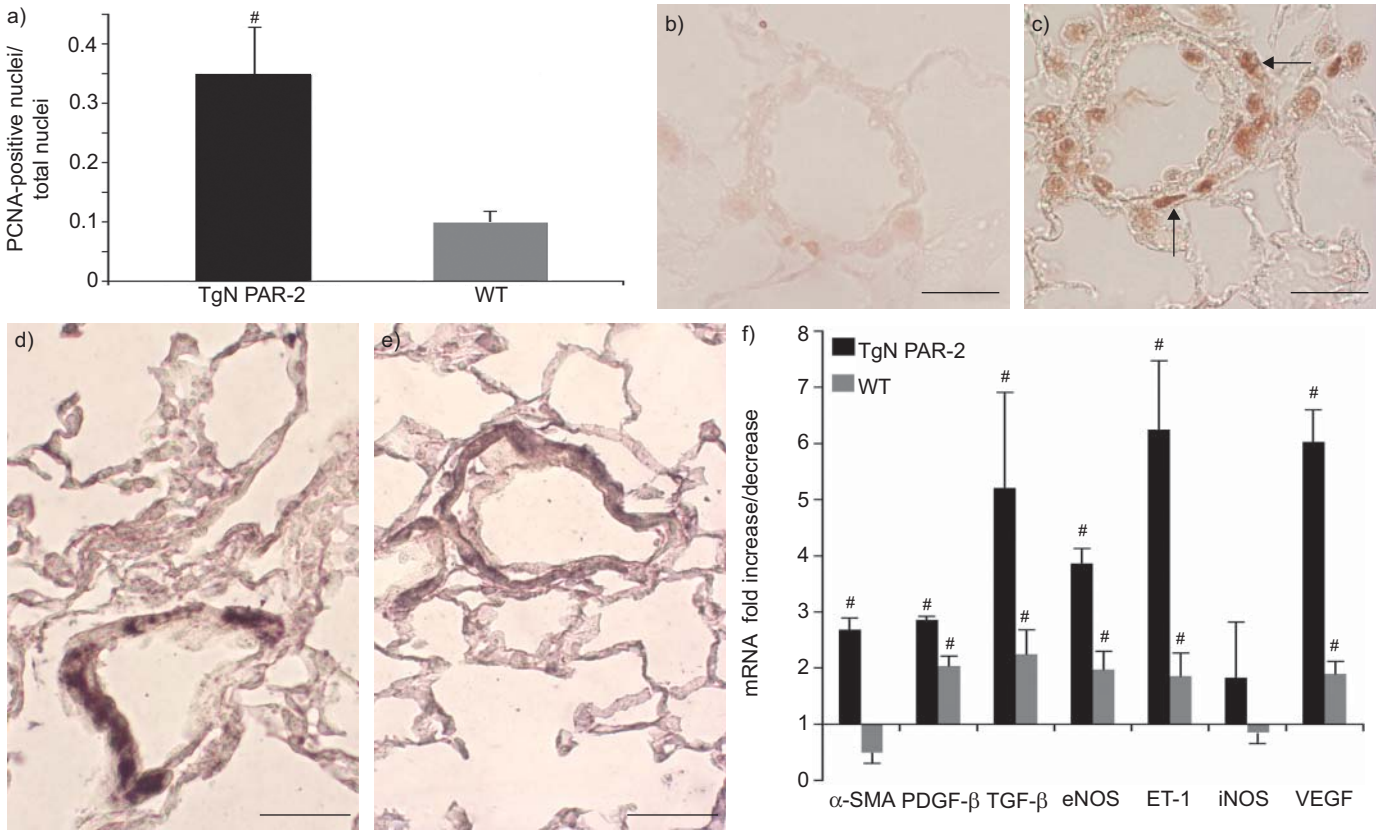

FIGURE 7. a) The ratio of proliferating cell nuclear antigen (PCNA)-positive nuclei to total nuclei of smooth muscle cells was compared between the two genotypes at 1 month after cigarette exposure. Smoking $FVB^{PAR\text{-}2\text{-}TgN}$ mice showed a significantly higher ratio of PCNA-positive smooth muscle cells ($p<0.01$, $n=8$). Representative immunohistochemical reactions for PCNA in lung sections of b) wildtype (WT) and c) $FVB^{PAR\text{-}2\text{-}TgN}$ mice at 1 month after cigarette smoke (CS) exposure are shown. Representative immunohistochemical reaction for PAR2 in small pulmonary vessels of d) $FVB^{PAR\text{-}2\text{-}TgN}$ and e) WT mice. Scale bars: 30 μm in b and c; and 50 μm for d and e. f) Real-time PCR analysis of mRNAs for α-smooth muscle actin (SMA), platelet-derived growth factor (PDGF)-β, transforming growth factor (TGF)-β, endothelial nitric oxide synthase (eNOS), endothelin (ET)-1, inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) carried out on lungs from eight mice for each experimental group at 1 month after CS exposure. Values are corrected for 18S rRNA and normalised to a median control value of 1.0. Data are presented as mean±SD. #: $p<0.05$ compared with air-control values of the same genotype.

of hypoxic response. This factor usually accumulates under hypoxic conditions and transactivates a number of genes, including ET-1, VEGF and PDGF, which have been involved in PH [32–34]. Under our experimental conditions, a positive reaction for HIF1-α was appreciated in $FVB^{PAR\text{-}2\text{-}TgN}$ mice only at 7 months after CS exposure, when vascular changes were already evident.

Comparative analysis of data between WT and $FVB^{PAR\text{-}2\text{-}TgN}$ mice exposed to CS suggests that TGF-β and PDGF are involved, through PAR-2 signalling, in lung vascular muscularisation. The presence of TGF-β in pulmonary vessels may enhance the expression of ET-1 that in turn can promote smooth muscle cell activation and vessel contraction.

These conclusions are in agreement with recent studies carried out under *in vitro* conditions that support an involvement of PAR-2 signalling in: 1) fibroblast recruitment and proliferation (through PDGF, TGF-α and MMP9 upregulation, and TGF-β activation by MMP9) [35–37], 2) fibroblast/SMC transaction (through PDGF and TGF-β hyper-expression and activation) [27, 28], 3) SMC proliferation (through PDGF) [35, 36] and 4) SMC activation and vessel contraction (through TGF-β upregulated ET-1) [38, 39].

In conclusion, the combination of CS exposure with overexpression of PAR-2 gene in FVB mice results in emphysema and vascular remodelling associated with PH and RVH. These changes are reminiscent of those characterising human COPD phenotypes with PH [20, 21]. Under our experimental conditions, PAR-2 signalling is able to influence the production and the release of many factors, which ultimately may lead to vascular remodelling and aberrant vascular physiology.

One established complication of COPD, a leading cause of morbidity and mortality worldwide [1–2], is the development of pulmonary hypertension (PH). Its presence is associated with shorter survival rates and worse clinical evolution [4]. PH in COPD tends to be of moderate severity and progresses slowly [40–42]. At the present time, there is no specific and effective treatment for this condition and current therapeutic success is unsatisfactory because of limited insight into disease mechanisms [43].

Several pathophysiological processes have been involved in the pathogenesis of PH in COPD, namely: a) an increase in vascular pulmonary resistance due to capillary loss (alveolar destruction), b) pulmonary arterial vasoconstriction secondary to alveolar hypoxia, and c) vascular remodelling and proliferation of SMC in pulmonary arterioles that are normally non-muscular [44, 45].

A number of stimuli and putative mediators in the induction of PH in COPD have also been considered [22–24]. These include hormones, growth factors, neurotransmitters, proteases and environmental stresses that induce pulmonary vascular constriction, cell proliferation and remodelling [24, 25].

Actually, the presence of PH in COPD is still an object of research. One of the major impediments for understanding the underlying pathogenic mechanisms for PH in COPD is limited access to biological samples, which are available only from lung explants and autopsy specimens at very late stage of disease.

Recent studies indicate that CS may have, at least in some individuals, a direct effect on the intrapulmonary vessels with up-regulation of mediators that lead to vascular structural remodelling and dynamic changes in vascular function [46, 47]. A role for proteases in PH has been recently put forward [46, 48].

The results reported here may explain why hypoxia by itself is not a prerequisite to cause PH in COPD patients [46, 49] and why the development of PH in human COPD is not related to the degree of alveolar destruction [43].

In this regard, a correlation between development of PH and degree of emphysema is not found under our experimental conditions. In fact, CS exposure induces in WT mice a degree of airspace enlargement similar to that observed in over-expressing PAR-2 mice with PH. The development of PH, in our study, may be related to high levels of expression of PAR-2 in transgenic mice.

Assuming that the mouse data are relevant to humans, the results of the present study indicate that an increased expression of PAR-2 in a milieu rich in proteases (such as the lungs of COPD patients) [50] would influence the development of PH. A number of serine proteases have been identified in the lung traditionally associated with COPD and PAR-2 is a target of several of these proteases, which include neutrophil elastase, cathepsin G, trypsin, mast cell tryptase and blood coagulation proteases [8, 50, 51]. Additonally, as reported in this paper and in human studies [52], cigarette smoke exposure may enhance expression of PAR2 in pulmonary structures.

The animal model reported in the current paper may represent a valuable resource by which to further our understanding of the biology of PH, and to facilitate designing and testing of new therapeutic interventions in man. In our opinion, the individual susceptibility to PH in human COPD may be influenced by several important genetic determinants such as the different levels of expression in PAR-2 gene and the different inflammatory response to the tobacco smoke. This may explain why, under our experimental conditions, vascular remodelling did occur in the wild-type FVB mice, but not at the same extent to that observed in FVB $^{PAR\text{-}2\text{-}TgN}$ mice.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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