Connexin-43 is a promising target for pulmonary hypertension due to hypoxaemic lung disease

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Connexin (Cx)-43, part of intercellular channels, is increased in patients with chronic hypoxia-induced pulmonary hypertension (CH-PH). It is crucial in lung inflammation and pulmonary artery remodelling in mice with CH-PH, suggesting Cx43 as a therapeutic option http://bit.ly/35zNkGm

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ABSTRACT The mechanisms underlying pulmonary hypertension (PH) are complex and multifactorial, and involve different cell types that are interconnected through gap junctional channels. Although connexin (Cx)-43 is the most abundant gap junction protein in the heart and lungs, and critically governs intercellular signalling communication, its contribution to PH remains unknown. The focus of the present study is thus to evaluate Cx43 as a potential new target in PH.

Expressions of Cx37, Cx40 and Cx43 were studied in lung specimens from patients with idiopathic pulmonary arterial hypertension (IPAH) or PH associated with chronic hypoxaemic lung diseases (chronic hypoxia-induced pulmonary hypertension (CH-PH)). Heterozygous Cx43 knockdown CD1 ($Cx43^{+/-}$) and wild-type littermate ($Cx43^{+/+}$) mice at 12 weeks of age were randomly divided into two groups, one of which was maintained in room air and the other exposed to hypoxia (10% oxygen) for 3 weeks. We evaluated pulmonary haemodynamics, remodelling processes in cardiac tissues and pulmonary arteries (PAs), lung inflammation and PA vasoreactivity.

Cx43 levels were increased in PAs from CH-PH patients and decreased in PAs from IPAH patients; however, no difference in Cx37 or Cx40 levels was noted. Upon hypoxia treatment, the $Cx43^{+/-}$ mice were partially protected against CH-PH when compared to $Cx43^{+/+}$ mice, with reduced pulmonary arterial muscularisation and inflammatory infiltration. Interestingly, the adaptive changes in cardiac remodelling in $Cx43^{+/-}$ mice were not affected. PA contraction due to endothelin-1 (ET-1) was increased in $Cx43^{+/-}$ mice under normoxic and hypoxic conditions.

Taken together, these results indicate that targeting Cx43 may have beneficial therapeutic effects in PH without affecting compensatory cardiac hypertrophy.

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Introduction

Pulmonary hypertension (PH) encompasses a group of devastating cardiovascular diseases with high morbidity and mortality, comprising idiopathic pulmonary arterial hypertension (IPAH) and forms of PH associated with cardiopulmonary diseases such as hypoxaemic chronic lung diseases (chronic hypoxia-induced pulmonary hypertension (CH-PH)) [1]. All forms of PH are characterised by progressive remodelling of distal pulmonary arteries (PAs) and vasoconstriction/relaxation imbalance leading to sustained elevated mean pulmonary arterial pressure (PAP) over 25 mmHg at rest. As a consequence, hypertrophic and failing right heart is responsible for premature patient death [2, 3]. Recent evidence also points out the importance of inflammation in all forms of PH [4, 5]. PH is still incurable and lung transplantation is too often the only therapeutic option with a survival rate of 52–75% at 5 years after surgery [1]. Moreover, treatment for CH-PH is currently based on drugs developed for IPAH without clear evidence of their efficacy on CH-PH [1]. PH and CH-PH thus remain an important challenge and development of new drugs specific to different PH forms may be a key point.

In PH, two essential actors are involved in PA dysfunction: pulmonary artery endothelial cells (PA-ECs) and pulmonary artery smooth muscle cells (PA-SMCs) [6]. Under physiological conditions, PA-ECs and PA-SMCs are responsible for fine control of PA tone through subtle crosstalk involving direct myoendothelial communications (gap junctions) [7]. Gap junctions are clusters of intercellular channels composed of various structural proteins (connexins (Cx)) also essential for spreading cellular signalling [7, 8]. Our group and others have previously shown that Cx37, Cx40 and Cx43 are expressed in rat, mouse and human PAs and such expression is modified in experimental models of PH as well as in patients with IPAH; however, disparities have been observed between the different studies [8–14]. Interestingly, Cx43 is known to be involved in tone regulation, cell proliferation and inflammatory infiltration in vessels, which are the main hallmarks of PH [7, 8, 10, 15–17]. Cx40, Cx43 and Cx45 are also expressed in cardiac tissue, with Cx43 being the most abundant. Moreover, Cx43 expression and localisation are modified in hypertrophic cardiac pathologies, including in right-ventricle hypertrophy in the well-established monocrotaline-induced rat model of PH [18–20]. However, cardiac Cx43 expression has not been studied in CH-PH so far.

We hypothesised that Cx43 may contribute to PH and especially CH-PH. Using heterozygous Cx43 knock-down mice ($Cx43^{+/-}$), we addressed the expression and the role of Cx43 on PA reactivity, remodelling processes in cardiac tissues and PA and lung inflammation in an experimental model of CH-PH. From a translational point of view, we also evaluated Cx43 expression in human PH, such as CH-PH as well as IPAH, in order to examine the relative specificity of changes in these two severe clinical entities.

Methods

Further details and a full description of all methods are provided in the supplementary material.

Human material

For the *in vitro* and *in situ* studies we used lung specimens obtained during lung transplantation from patients with IPAH or CH-PH. The six patients with CH-PH had the following diseases associated to PH: emphysema, Kartagener syndrome, cystic fibrosis, familial IPAH (with severe hypoxaemia, oxygen tension (P_{O_2})=59 mmHg), pulmonary veno-occlusive disease and cystic lung disease. The mean age of the patients was 51.3±6.6 years and the group included three women and three men. The mean of the mean PAP was 43.5±2.6 mmHg. Vessels used were intrapulmonary arteries of the second and third order. Control lung tissue (intrapulmonary arteries) was obtained during lobectomy or pneumonectomy for localised lung cancer. Preoperative cardiological evaluation including echocardiography was performed in the controls to rule out PH and the lung specimens from the controls were collected at a distance from tumour foci. PA-SMCs exposed to *in vitro* chronic hypoxia (CH) (supplemental figure S2) were obtained from extrapulmonary arteries of healthy lung donors during lung transplantation. The study was approved by the local ethics committees (Comité de Protection des Personnes, Sud-Ouest et Outre-mer III, Bordeaux,

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France; Comité de Protection des Personnes, Ile de France VII, Le Kremlin-Bicêtre, France) and informed consent was obtained from each individual patient.

Isolation, culture and treatment of human pulmonary vascular cells

Human pulmonary microvascular endothelial cells (PM-ECs) and PA-SMCs were obtained and cultured as previously described [5, 21–23]. PA-SMCs were exposed to CH conditions *in vitro* (1% oxygen, the "CH group") as previously described [24]. PA-SMCs of the control group were exposed to normoxia (N) conditions (21% oxygen, 74% nitrogen and 5% carbon dioxide, the "N group"). Cells were used between passages three and six. Further details are provided in the supplementary material.

Animal experiments

All animal studies conformed to the Declaration of Helsinki conventions for the use and care of animals. Agreement (number A33-063-907) was obtained from the French authorities and all protocols used were approved by the local ethics committee (Comité d'éthique de Bordeaux no. 50, protocol number APAFIS#9212-2017031018562273 v5). Genetically modified adult male CD1 mice (8–12 weeks, $Gja1^{tm1Kdr}$, Jackson Laboratory, Bar Harbor, ME, USA) were used and compared to their wild-type litter mates. Mutation is due to the in-frame insertion of a promoterless neomycin (Neo) gene into exon 2 of the Cx43 (Gja1) gene [25]. $Gja1^{tm1Kdr}$ homozygous ($Cx43^{-/-}$) mice die at birth due to a severe heart defect [25]. Consequently, only heterozygous ($Cx43^{+/-}$) and wild-type ($Cx43^{+/+}$) mice were used for the study. PH was induced by exposing mice to CH conditions in a hypobaric chamber (380 mmHg) over 21 days, while control animals were kept under normobaric N conditions (room air). The number of mice used is specified in the legend of the figures for each set of experiments. Further details are provided in the supplementary material.

Statistical analysis

All data are expressed as mean±SEM of n independent observations. Two-way ANOVA was used to compare concentration–response curves and a Mann–Whitney test was used when comparing two groups. For all other parameters, one-way ANOVA was used to assess differences among groups, followed by adapted *post hoc* tests (Dunn tests). Values of p less than 0.05 were considered significant. Analyses were performed using GraphPad Prism version 6.07 (Graphpad Software, La Jolla, CA, USA).

Results

Cx43 expression varies in the PA vascular bed in experimental and human PH

Cx43 protein levels were increased in PAs from patients with CH-PH (figure 1a). Interestingly, Cx43 expression was inversely decreased in human PA-SMCs whereas it was not modified in PM-ECs from patients with IPAH compared to control patients (figures 1b and 1c, respectively). Consistent with these

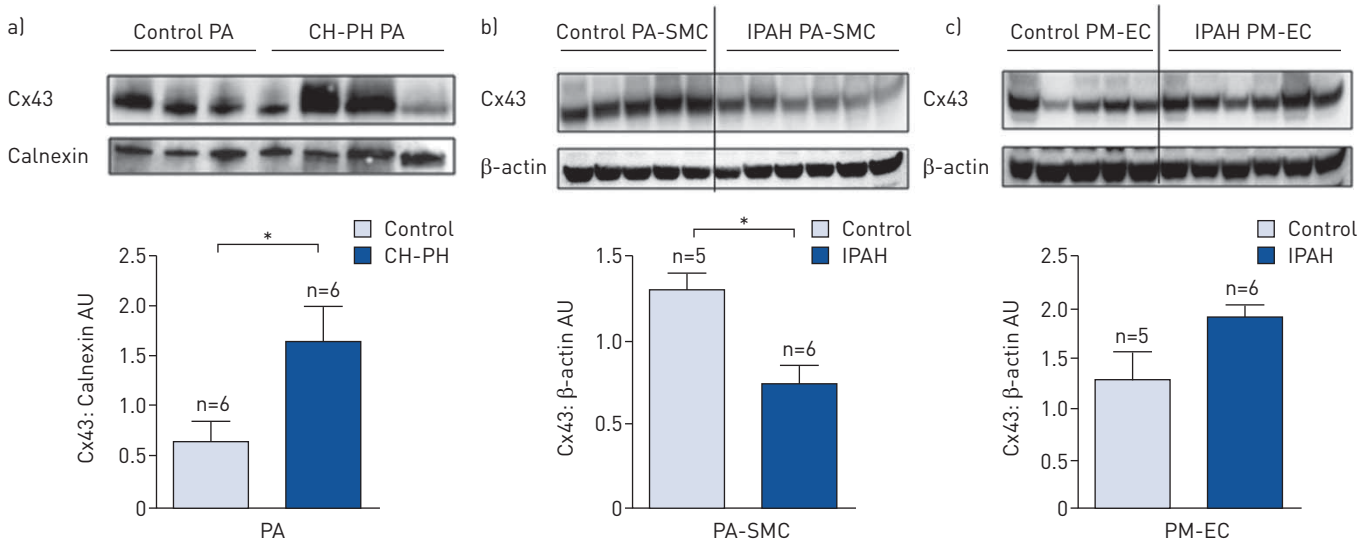

FIGURE 1 Expression of connexin-43 (Cx43) in human pulmonary hypertension (PH). Panels are as follows: (a) expression of Cx43 assessed by Western Blot analysis in pulmonary arteries (PAs) from patients with hypoxia-induced pulmonary hypertension (CH-PH) compared with control patients; (b) and (c) expression of Cx43 assessed by Western Blot analysis in pulmonary artery smooth muscle cells (PA-SMCs) and pulmonary microvascular endothelial cells (PM-ECs) from patients with idiopathic pulmonary arterial hypertension (IPAH) and control patients, respectively. Cx43 expression was normalised to calnexin ((a)) or β-actin ((b) and (c)). Data presented are mean±SEM. n: number of patients. *: p<0.05.

findings, lung section staining showed a strong increase in Cx43 expression in the smooth muscle of PAs from patients with CH-PH compared to patients with IPAH or to control patients (figure 2 and supplementary figure S1). Moreover, when PA-SMCs from control patients were exposed to CH (1% oxygen for 48 h), Cx43 mRNA levels were increased (supplemental figure S2) suggesting that hypoxia may be responsible for the increased Cx43 expression in PAs from CH-PH patients.

Cx40 protein expression seemed unchanged in CH-PH and IPAH patients (supplemental figure S3, right-hand images), whereas Cx37 protein expression appeared to be decreased in PA-SMCs from IPAH patients and unchanged in PA-SMCs from CH-PH patients (supplemental figure S3, left-hand images).

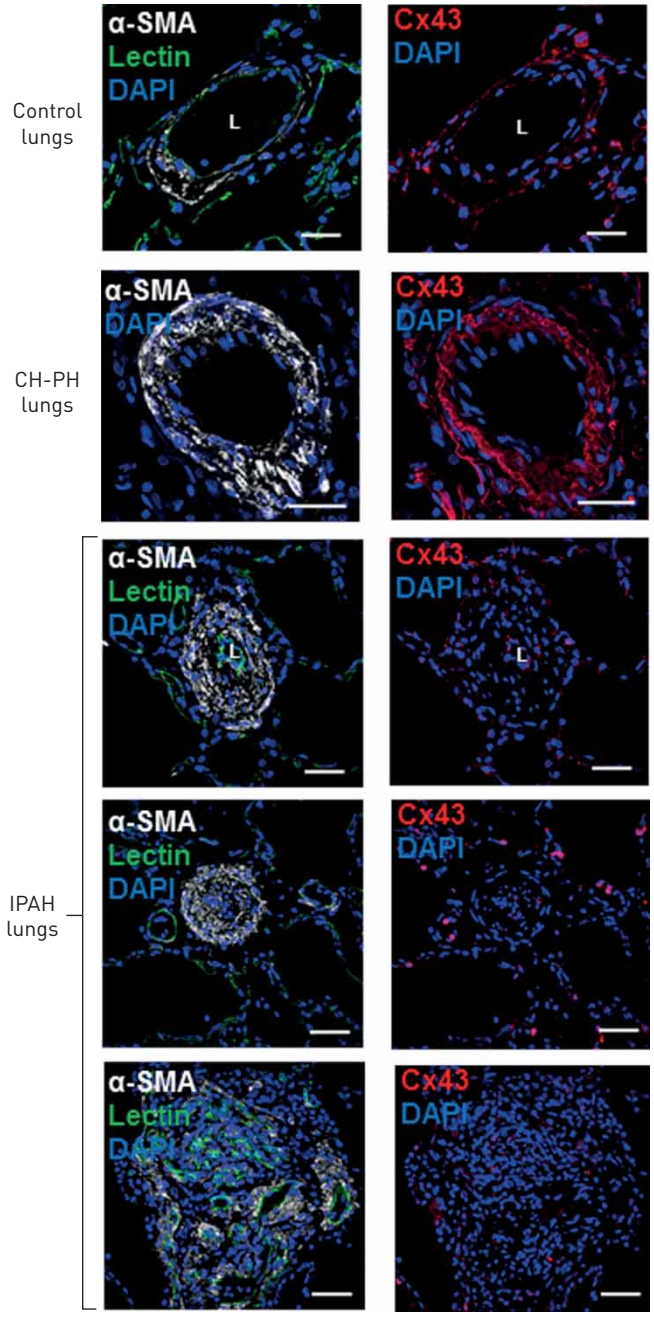

FIGURE 2 Localisation and expression of connexin-43 (Cx43) in human pulmonary hypertension (PH) as assessed by immunofluorescent staining in sections of intrapulmo-nary arteries from lungs of patients with hypoxia-induced pulmonary hypertension (CH-PH) or idiopathic pulmonary arterial hypertension (IPAH) compared with control patients. Cx43 is labelled in red, pulmonary artery (PA) media is labelled in white using an antibody against α-smooth muscle actin (α-SMA), endothelium is labelled in green with a lectin and nuclei are labelled in blue with 4′,6-diamidino-2-phenylindole (DAPI). Scale bars= 20 µm. L: PA lumen.

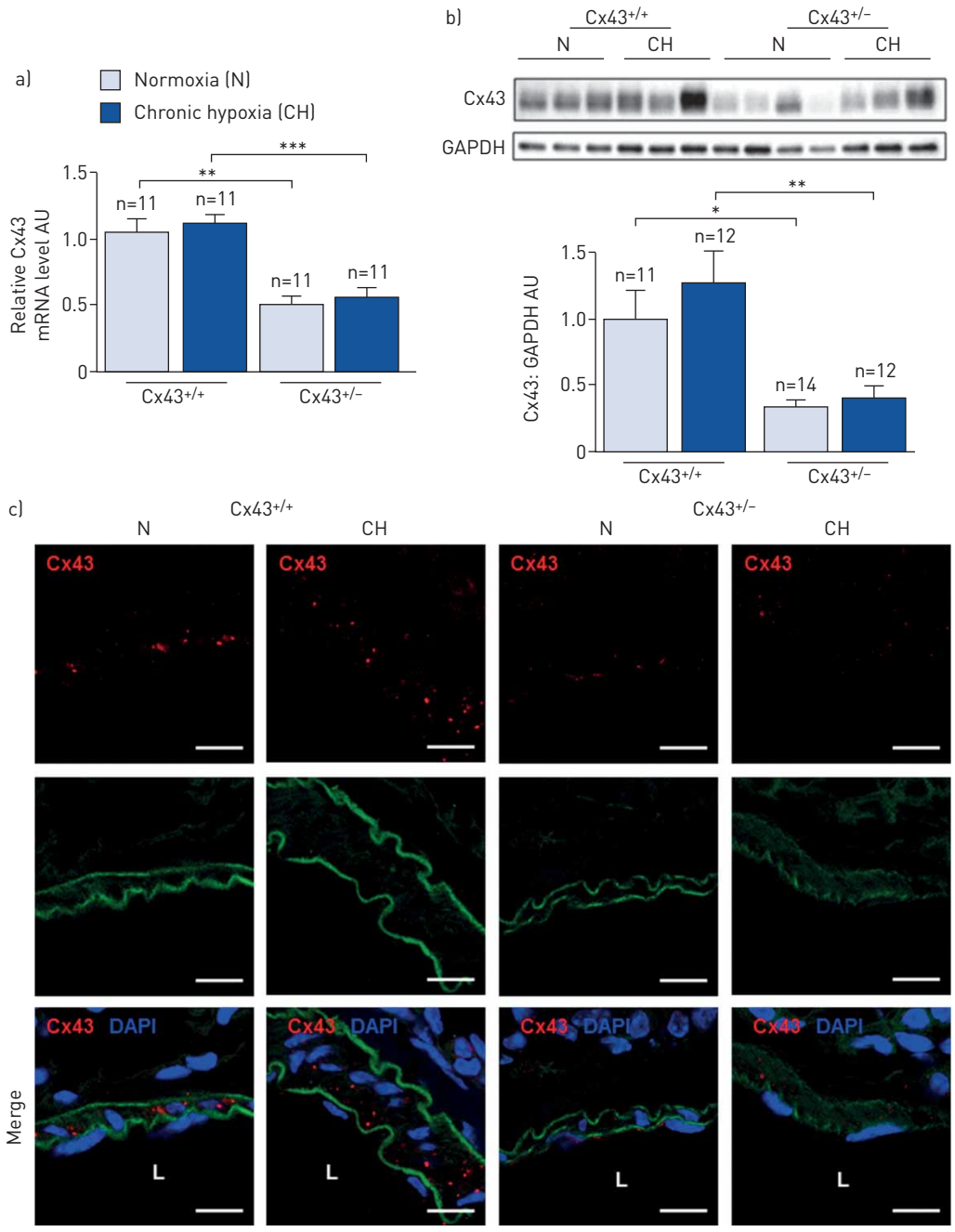

FIGURE 3 Localisation and expression of connexin-43 (Cx43) in intrapulmonary arteries from mice with hypoxia-induced pulmonary hypertension (CH-PH), comparing $Cx43^{+/-}$ mice with $Cx43^{+/+}$ mice under conditions of normoxia (N) and chronic hypoxia (CH). Cx43 mRNA and protein levels were assessed by quantitative PCR and Western Blot analysis (panels (a) and (b), respectively). Cx43 protein expression was normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Panel (c) shows expression and localisation of Cx43 protein as assessed by immunofluorescent staining in sections of intrapulmonary arteries from the mice lungs from different experimental groups. Cx43 is labelled in red, nuclei are labelled in blue using 4′,6-diamidino-2-phenylindole (DAPI) and elastic lamina autofluorescence is labelled in green. Data presented are mean±SEM. Scale bars=10 µm. n: number of mice; L: pulmonary artery (PA) lumen. *: p<0.05; **: p<0.01; ***: p<0.001.

In $Cx43^{+/+}$ and $Cx43^{+/-}$ mice, expression of both Cx43 mRNA and protein was similar in PAs from N mice compared to CH mice (figures 3a and 3b). Interestingly, Cx43 expression seems to be more abundant close to the plasma membrane in CH $Cx43^{+/+}$ mice compared to N $Cx43^{+/+}$ mice (supplemental figure S4). As expected, expression of both Cx43 mRNA and protein was significantly decreased under N as well as CH conditions in $Cx43^{+/-}$ mice (figures 3a and 3b). Immunofluorescence assays revealed that,

in $Cx43^{+/+}$ mice, Cx43 was expressed in the media between the two elastic lamina shown in green (figure 3c and supplemental figure S5) under both N and CH conditions. These experiments also confirmed the decrease of Cx43 in $Cx43^{+/-}$ mice (figure 3c and supplemental figure S5). In accordance with the previous results, mRNA levels for Cx37 and Cx40 were not significantly different between the four experimental groups of mice (supplemental figures S6a and S6b, respectively), thus suggesting that the decrease in Cx43 in $Cx43^{+/-}$ mice is not compensated by any modification of Cx37 and/or Cx40 expression.

Role of Cx43 in pulmonary vascular remodelling and right-ventricular hypertrophy in mice chronically exposed to hypoxia

By using haematoxylin–eosin staining, we observed a significant increase in pulmonary arterial wall thickness in $Cx43^{+/+}$ mice with CH-PH that was not present in $Cx43^{+/-}$ mice with CH-PH (figure 4a and 4b). Proliferating cell nuclear antigen (PCNA) expression, which labels proliferating cells, was consistently increased only in PAs (smooth muscle cells and endothelial cells) from $Cx43^{+/+}$ mice with CH-PH (figure 4c and 4d). No TUNEL-positive cells were detected in PAs from lung sections of the four experimental groups of mice, whereas few TUNEL-positive cells were detected in mouse colon used as a positive control (figure 4e). Thus, $Cx43^{+/-}$ mice were protected against cell proliferation and intrapulmonary artery remodelling associated to CH-PH.

In contrast, in the heart, right-ventricular systolic pressure was significantly increased in both $Cx43^{+/+}$ and $Cx43^{+/-}$ mice with CH-PH ($p<0.001$, figure 4f). Moreover, right-ventricular hypertrophy (as assessed by the Fulton index), a hallmark of right-ventricular remodelling in PH, was also present in CH-PH in both $Cx43^{+/+}$ and $Cx43^{+/-}$ mice ($p<0.001$, figure 4g). Interestingly, its magnitude was statistically significantly smaller in $Cx43^{+/-}$ mice compared to $Cx43^{+/+}$ mice ($p<0.05$, figure 4g). Pulmonary artery acceleration time (PAAT), as assessed by echocardiography, is similarly decreased in $Cx43^{+/+}$ and $Cx43^{+/-}$ mice under CH conditions (supplemental figures S7a and S7b), confirming that right-ventricular function is similarly modified in $Cx43^{+/+}$ and $Cx43^{+/-}$ mice under CH conditions (figures 4f and 4g). Heart rate (HR) measured by pulsed-wave Doppler was similar in all groups of mice (supplemental figure S7c). CD31 immunofluorescent staining and TUNEL experiments were also performed, to measure capillary density and apoptosis respectively, in hearts from all groups of mice (supplemental figure S8). In all groups of mice, capillary density was similar (supplemental figures S8a and S8b) and right-heart apoptosis was absent (supplemental figure S8c), suggesting that, interestingly, heart modifications observed in $Cx43^{+/-}$ mice are not strongly deleterious.

Knocking down of Cx43 prevents hypoxia-induced lung inflammation in mice

Inflammatory cell infiltration was estimated by CD45 expression and assessed using Western Blot and immunofluorescent labelling. CD45 expression was significantly increased in $Cx43^{+/+}$ mice with CH-PH ($p<0.001$), whereas it was almost absent in $Cx43^{+/-}$ mice (figure 5a and supplemental figure S9). Consistent with this finding, the number of inflammatory cells was increased in $Cx43^{+/+}$ mice with CH-PH (figure 5b, expansions 5 and 6, and supplemental figure S9) while such an inflammatory process was not present in $Cx43^{+/-}$ mice with CH-PH (figure 5b, expansions 7 and 8, and supplemental figure S9). Interestingly, CD45 immunofluorescent staining was localised at the perivascular level of the lung sections (supplemental figure S9). Altogether, these findings indicate that $Cx43^{+/-}$ mice with CH-PH are protected from lung inflammation induced by CH.

Pulmonary arterial contraction and relaxation in $Cx43^{+/+}$ and $Cx43^{+/-}$ mice

Pulmonary arterial reactivity to contractile and relaxant agonists was examined in PAs from $Cx43^{+/+}$ and $Cx43^{+/-}$ mice under N and CH conditions. In $Cx43^{+/-}$ mice, the contraction response curve to endothelin-1 (ET-1) was shifted to the left under N conditions compared to $Cx43^{+/+}$ mice and the maximal response was increased under CH conditions (figure 6a). Moreover, following blockade of endothelin receptors with bosentan (20 $mg{\cdot}kg^{-1}$ *in vivo*), carbachol (Carb, 10 μM) had a significant effect only in $Cx43^{+/-}$ mice under CH conditions, confirming that the ET-1 contribution to vasotonus is more important in $Cx43^{+/-}$ mice compared to $Cx43^{+/+}$ mice under CH conditions (supplemental figure S10).

Contraction response curves to serotonin (5-HT) were identical in $Cx43^{+/+}$ and $Cx43^{+/-}$ mice under both N and CH conditions (figure 6b). The maximal contractile response to phenylephrine (Phe) was significantly decreased in $Cx43^{+/-}$ mice compared to $Cx43^{+/+}$ mice under N conditions ($p<0.01$, figure 6c), whereas it was significantly increased in $Cx43^{+/-}$ mice compared to $Cx43^{+/+}$ mice under CH conditions ($p<0.05$, figure 6c). Finally, relaxation to Carb was identical in $Cx43^{+/-}$ and $Cx43^{+/+}$ mice under N and CH conditions (figure 6d). Interestingly, CH conditions strongly reduced relaxation in both $Cx43^{+/-}$ and $Cx43^{+/+}$ mice compared to N conditions, suggesting the existence of an endothelial dysfunction in CH-PH as previously described [26]. Labelling with von Willebrand factor (vWF), a marker of endothelium, did

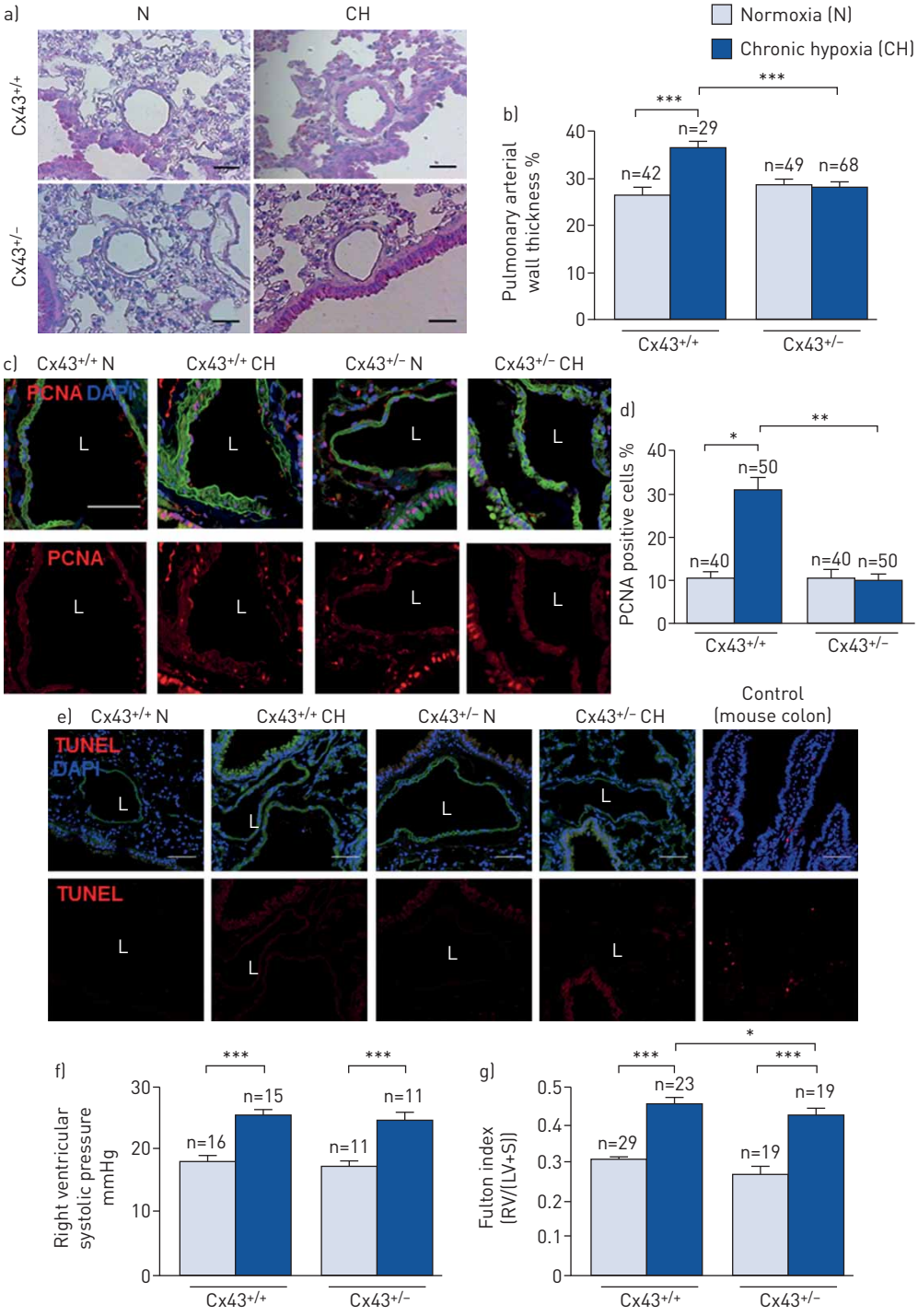

FIGURE 4 Remodelling of intrapulmonary arteries and the right ventricle in hypoxia-induced pulmonary hypertension (CH-PH) in mice, comparing $Cx43^{+/-}$ mice and $Cx43^{+/+}$ mice under conditions of normoxia (N) and chronic hypoxia (CH). Panels are as follows: (a) representative sections of intrapulmonary arteries (with haematoxylin and eosin staining); (b) percentage of the intrapulmonary arterial wall thickness in the different experimental groups as shown in (a); (c) proliferating cell nuclear antigen (PCNA) expression as assessed by immunofluorescent staining; (d) percentage of PCNA positive cells in the different experimental groups as shown in (c); (e) detection of apoptotic cells in intrapulmonary arteries from lung sections using TUNEL methodology (mouse colon was used as a positive control). Nuclei of proliferating or apoptotic cells were labelled in red ((c) and (e), respectively), nuclei of all cells were labelled in blue with 4′,6-diamidino-2-phenylindole (DAPI) and autofluorescence of the elastic lamina was labelled in green; (f) right-ventricular systolic pressure recordings; (g) right-ventricular remodelling (assessed by the Fulton index). Data presented are mean±SEM. Scale bars=15 µm for (a) and 50 µm for (c) and (e); n: number of vessels from four to five mice per group for (b) and (d), and number of mice for (f) and (g); L: pulmonary artery (PA) lumen. *: p<0.05; **: p<0.01; ***:p<0.001.

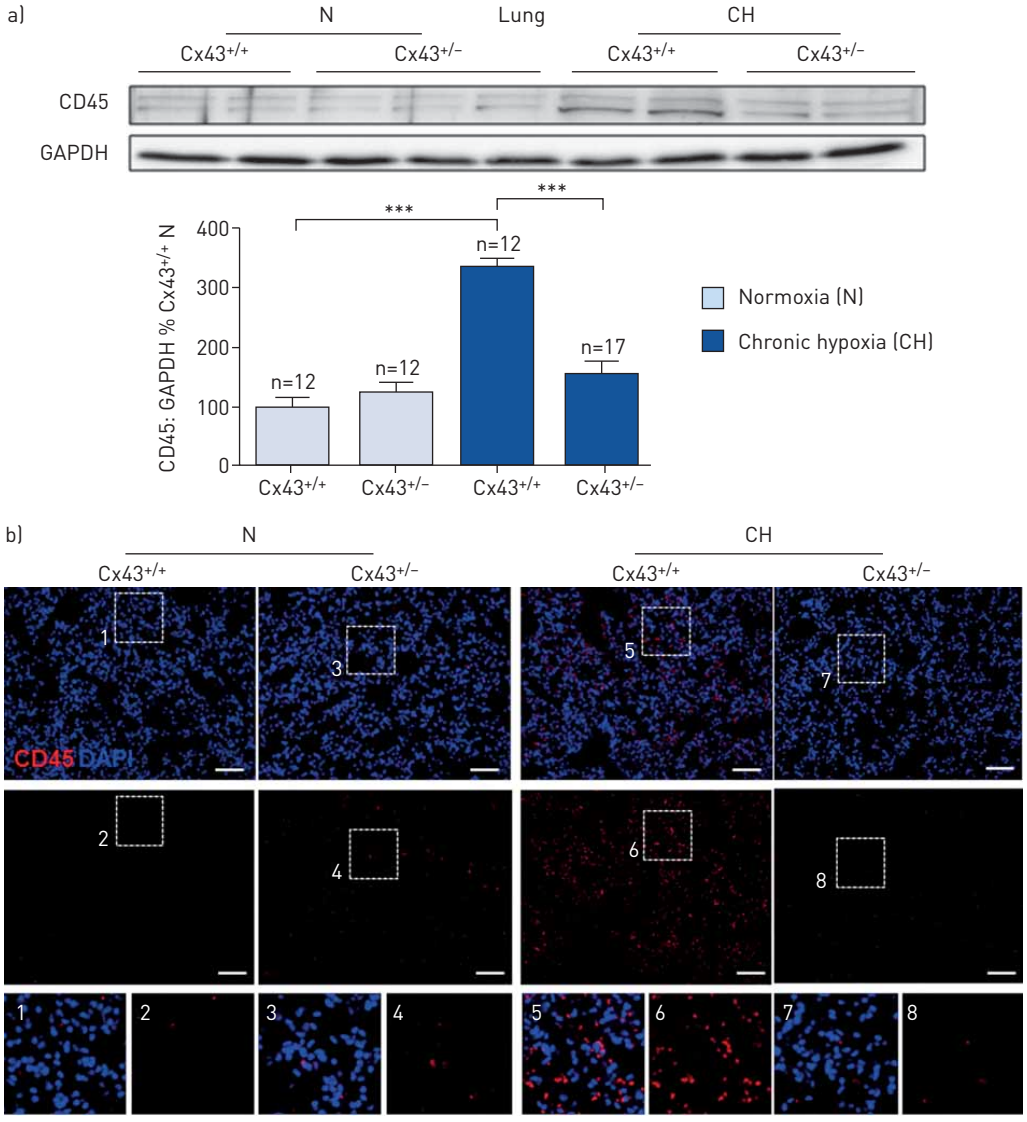

FIGURE 5 Lung inflammation in $Cx43^{+/-}$ mice compared with $Cx43^{+/+}$ mice under conditions of normoxia (N) and chronic hypoxia (CH). Expression of CD45 (a leukocyte marker) was assessed by (a) Western Blot analysis in whole lung homogenates and by (b) immunofluorescent labelling in lung sections (red labelling). For Western Blot experiments, CD45 expression was normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as a percentage of CD45 expression in lungs from $Cx43^{+/+}$ mice under N conditions. For immunofluorescent labelling, nuclei were labelled in blue with 4′,6-diamidino-2-phenylindole (DAPI). Images 1–8 at the bottom of panel (b) are expansions of the similarly marked squares in the respective main images. Data presented are mean±SEM. Scale bars=100 µm. n: number of mice. ***: p<0.001.

not show any obvious endothelial damage in PAs from all groups of mice, indicating that endothelium damage cannot be used to explain this dysfunction.

Cx43 expression in right and left ventricles in mice

Cx43 expression in right-heart *versus* left-heart ventricles was compared for all experimental groups of mice. Both Cx43 mRNA (figures 7a and 7b) and protein (figures 7c and 7d) were similarly expressed in right-heart and left-heart ventricles from $Cx43^{+/+}$ mice under N and CH conditions. As expected, Cx43 mRNA and protein were significantly decreased in $Cx43^{+/-}$ mice compared to $Cx43^{+/+}$ mice under both N and CH conditions. However, as in $Cx43^{+/+}$ mice, both Cx43 mRNA and protein were similarly expressed in right-heart and left-heart ventricles from $Cx43^{+/-}$ mice under N and CH conditions. Moreover, mRNA levels for Cx37 and Cx40 were not significantly different between the four experimental groups of mice for both right-heart and left-heart ventricles (supplemental figures S11a and S11b), confirming that as in PAs there was no compensation in the heart for the partial Cx43 deletion by Cx37 or Cx40 altered expression.

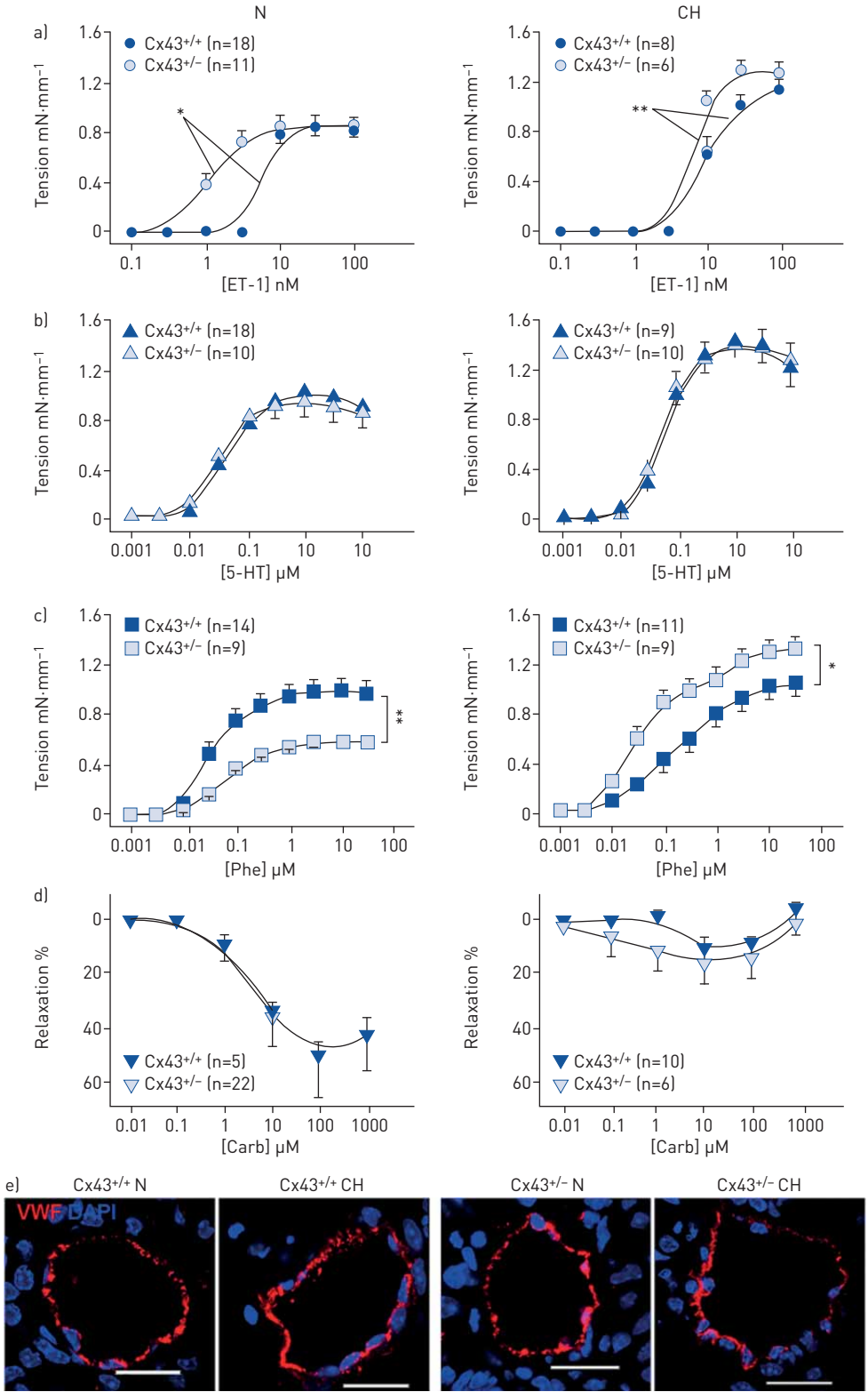

FIGURE 6 Pulmonary arterial reactivity in $Cx43^{+/-}$ mice compared with $Cx43^{+/+}$ mice under conditions of normoxia (N) and chronic hypoxia (CH). Contraction of intrapulmonary arterial rings was induced by cumulative concentrations of either (a) endothelin-1 (ET-1; 0.1–100 nM), (b) serotonin (5-HT; 0.001–10 µM) or (c) phenylephrine (Phe; 0.001 to 30 µM). Relaxation of intrapulmonary arterial rings was induced by cumulative concentrations of carbachol (Carb; 0.01 to 1000 µM) (d) on vessels pre-contracted with Phe (1 µM). Panel (e) shows endothelium labelling in red with von Willebrand Factor (vWF). Nuclei are labelled in blue with 4′,6-diamidino-2-phenylindole (DAPI). Data presented are mean±SEM. Scale bars=20 µm. n: number of vessels from at least three mice per condition. *:p<0.05; **: p<0.01.

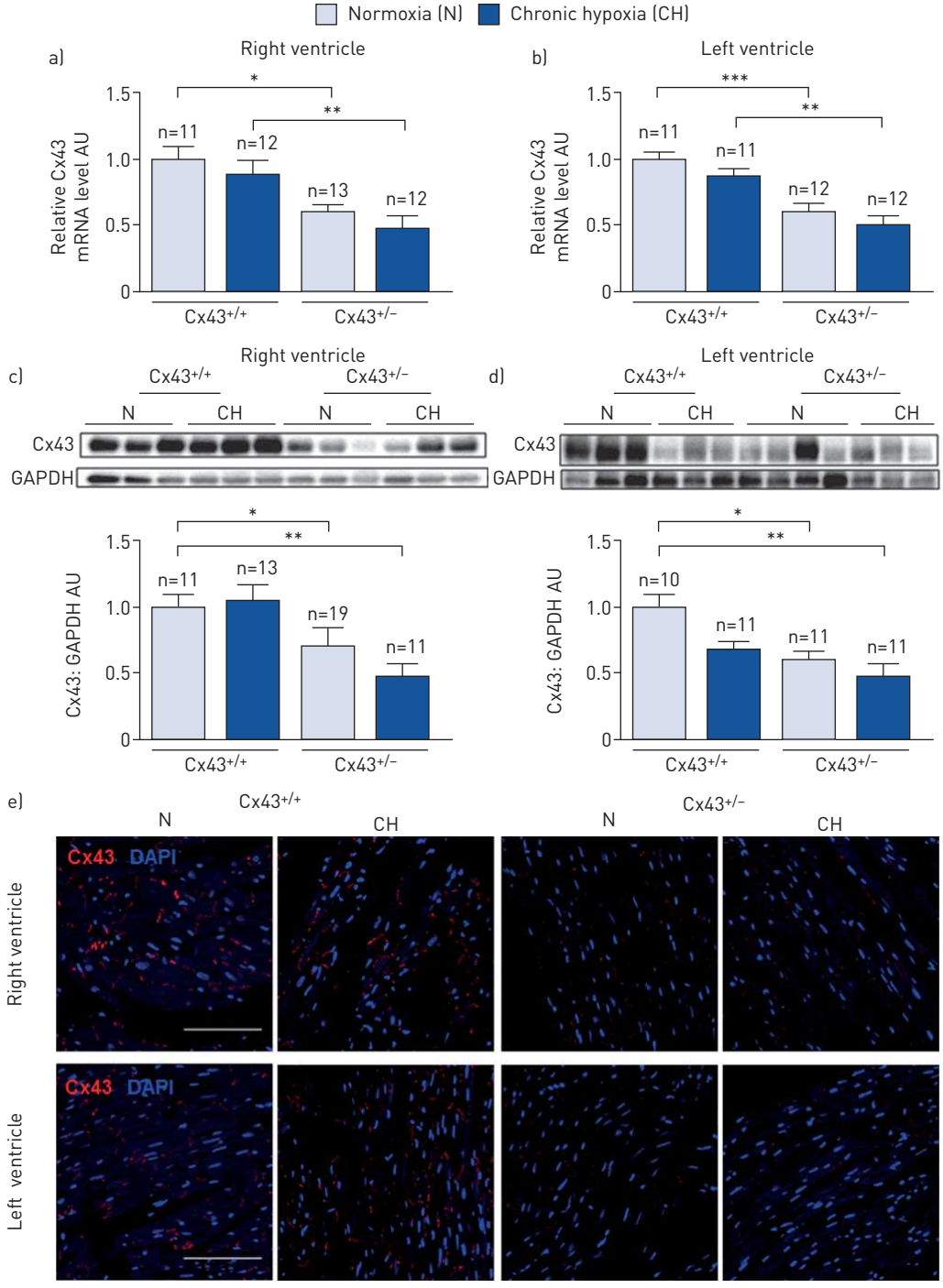

FIGURE 7 Expression and localisation of Cx43 in right-heart *versus* left-heart ventricles from $Cx43^{+/-}$ mice compared with $Cx43^{+/+}$ mice under conditions of normoxia (N) and chronic hypoxia (CH). Cx43 mRNA levels ((a) and (b)) and protein levels ((c) and (d)) were assessed by quantitative PCR and Western Blot analysis. Cx43 protein expression was normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Localisation of Cx43 protein as assessed by immunofluorescent staining in sections of right-heart or left-heart ventricles is shown in panel (e). Cx43 is labelled in red and nuclei are labelled in blue using 4′,6-diamidino-2-phenylindole (DAPI). Data presented are mean±SEM. Scale bars=100 µm. n: number of mice. *: p<0.05; **: p<0.01; ***: p<0.001.

Discussion

In the present study, the first complete description of the deleterious effects of Cx43 in CH-PH in mice is provided. Decrease in Cx43 expression is shown to attenuate CH-induced PA remodelling and lung inflammation. Partial decrease of Cx43 protein expression also modifies PA vasoreactivity, another

important characteristic of PH. Likewise, in human CH-PH, Cx43 protein expression varies and this variation is specific to the PH form (*i.e.* CH-PH *versus* IPAH), supporting a specific role of Cx43 according to the PH form. It is thus suggested that Cx43 may be a promising therapeutic option to consider, particularly for CH-PH that lacks specific treatments [1]. Further work will need to be performed in animal models *in vivo* in order to confirm the beneficial role of blocking Cx43.

In patients with CH-PH, an important increase in Cx43 protein levels was clearly demonstrated for the first time. In patients with IPAH, Cx43 expression was decreased in PA-SMCs whereas it was not changed in PM-ECs, supporting the idea that the vascular Cx43 protein level is a distinctive characteristic of these two severe forms of PH. Although such experiments have never been performed previously on these human cells, it has been consistently shown that Cx43 protein is decreased in blood-derived endothelial-like cells in patients with IPAH [27]. Likewise, the expression of Cx37 and Cx40 (two other Cx commonly expressed in resistant vessels [28]) is also decreased in lung tissues and in PA-ECs from patients with IPAH [12]. For Cx37 expression, a tendency to decrease was consistently observed in PA-SMCs from patients with IPAH. In addition, the present study showed that 1% oxygen over 48 h induced a five-fold increase in the amount of Cx43 mRNA in cultured PA-SMCs from control patients, indicating that hypoxia may be responsible for Cx43 protein increase in PAs from patients with CH-PH. Such Cx43 sensitivity to hypoxia has already been shown in the systemic circulation, specifically in cultured smooth muscle cells from rat thoracic aorta exposed to 2% oxygen over 6 h [29]. Therefore, our study indicates that hypoxia may explain the difference in Cx43 PA expression between patients with CH-PH *versus* IPAH. In the present study we have not addressed the mechanisms involved in hypoxia-induced Cx43 regulation; however, a previous study has shown that HIF-1α is able to increase Cx43 expression, through the activation of HRE-5 present in the Cx43 promoter region in human melanoma cell lines [30]. We can thus speculate that such a mechanism could be responsible for the hypoxia-induced Cx43 increase in human PAs (figures 1a and 2, and supplemental figures S1 and S2).

Some of our results regarding Cx expression conflict with previous studies. For instance, we observed similar Cx43 expression under N and CH conditions and similar Cx37 and Cx40 mRNA levels in $Cx43^{+/+}$ *versus* $Cx43^{+/-}$ mice independent of the conditions tested in both PAs and hearts (supplementary figures S6 and S11), indicating that Cx43 knock-down was not compensated by variations in expression of Cx with a homology close to Cx43 and expressed in PAs of mice. However, Htet *et al.* [11] demonstrated 1) a decrease in Cx43 mRNA expression in PAs from both $Cx43^{+/+}$ and $Cx43^{+/-}$ mice with CH-PH compared to N mice; and 2) a decrease in Cx37 and Cx40 mRNA expression in N $Cx43^{+/-}$ mice compared to N $Cx43^{+/+}$ mice. It should be noted however that they used a C57BL6 mouse strain whereas we used a CD1 mouse strain. Furthermore, they induced CH over 14 days whereas we induced CH over 21 days.

Moreover, it should be noted that although Cx43 expression is increased in human PAs (figures 1a and 2 and supplementary figures S1 and S2) this is not the case in PAs from mice (figure 3). However, Cx43 protein expression has been quantified by performing Western Blot experiments on whole intrapulmonary arteries from mice and therefore we detected Cx43 from cytosol as well as from all cellular membranes. Interestingly, Cx43 expression seems to be more abundant close to the plasma membrane in CH-PH $Cx43^{+/+}$ mice compared to N $Cx43^{+/+}$ mice (supplementary figure S4). Such results could explain the discrepancy between Western Blot experiments on human PAs and mice PAs. Moreover, functional Cx43 is phosphorylated and we can thus speculate that although the total amount of Cx43 is similar under normoxic and hypoxic conditions, the phosphorylation state of Cx43 could be higher in mice under hypoxic conditions.

In $Cx43^{+/+}$ mice, PA remodelling characterised by smooth muscle and endothelial cell proliferation, and inflammation revealed by immune cell infiltration, were observed under CH conditions (figures 4 and 5). In contrast, such processes did not appear in $Cx43^{+/-}$ mice with CH-PH, suggesting that blocking Cx43 would have a beneficial effect in CH-PH. Since Cx43 is known to be decreased in the hypertrophic right ventricle in a classical monocrotaline PH model in rats [19, 20, 31] and $Cx43^{-/-}$ mice are born with a lethal defect (due to an abnormal right-ventricular outflow tract from the heart [25]), one would expect a harmful effect on the heart when blocking Cx43. In fact, both right-ventricular systolic pressure and hypertrophy were similarly increased in $Cx43^{+/+}$ and $Cx43^{+/-}$ mice with CH-PH (figures 4f and 4g). However, it should be noted that the increase in right-ventricular hypertrophy (as assessed by the Fulton index) was statistically significantly smaller in $Cx43^{+/-}$ mice compared to $Cx43^{+/+}$ mice with CH-PH. Consequently, $Cx43^{+/-}$ mice with CH-PH were still alive and, although high, right-ventricular systolic pressure and Fulton index were not higher than in $Cx43^{+/+}$ mice with CH-PH. Interestingly, in all groups of mice, HR and heart capillary density were similar and cardiomyocytes apoptosis was absent (supplementary figures S7c and S8). Therefore, blocking Cx43 functions or reducing Cx43 expression level should not worsen PH cardiac symptoms. Intratracheal instillation of ^{43}Gap26, a peptide that specifically inhibits Cx43, had a consistently beneficial effect on lipopolysaccharide (LPS)-induced lung inflammation in mice without any obvious

deleterious side effects [32]. Importantly, Cx43 decrease in the heart following PH has only been previously shown in a monocrotaline PH model, considered to be closer to IPAH, but not in CH-PH models. Altogether, the present results should strengthen the interest in targeting Cx43 in CH-PH.

The role of Cx43 in PA vasoreactivity, another important feature that participates in PH development [33], was also addressed. PA vasoreactivity was tested in response to ET-1 and 5-HT, whose circulating concentrations are known to increase in PH [34–39]. PA vasoreactivity to ET-1 was increased in $Cx43^{+/-}$ mice compared to $Cx43^{+/+}$ mice, under both N and CH conditions (figure 6a), whereas PA vasoreactivity to 5-HT was identical in all groups of mice (figure 6b). Such results are consistent with those observed by Htet *et al.* [11] in $Cx43^{+/-}$ C57BL6 mice. Such a role for Cx43 on PA vasoreactivity to ET-1 may explain why, despite PA remodelling and inflammation being absent, right-ventricular systolic pressure and Fulton index remained high in $Cx43^{+/-}$ mice with CH-PH compared to $Cx43^{+/+}$ mice with CH-PH. Such ET-1 induced PA hyper-reactivity in $Cx43^{+/-}$ mice can be considered as a minor secondary effect since it could be treated with endothelin receptor antagonists that have already been shown to be of benefit for patients [1]. Collectively, these results indicate that Cx43 plays an important role in PA vasoreactivity, under both physiological and pathophysiological conditions (N and CH-PH, respectively), as already shown in rat and C57BL6 mouse pulmonary circulation [7, 8, 11].

In conclusion, the present study demonstrates for the first time that partial decrease of Cx43 expression suppresses PA remodelling and inflammation in mice with CH-PH. It also shows that even in the absence of pulmonary vascular remodelling, right-ventricular pressures and hypertrophy remain high in $Cx43^{+/-}$ mice with CH-PH. Importantly, a slight reduction in cardiac hypertrophy is observed, suggesting that blocking of Cx43 might have some beneficial effect on the heart. Since decrease in Cx43 expression also enhances PA vasoreactivity, we suggest that both Cx43 and ET-1 pathway inhibitors could be a new therapeutic option that might be further explored for PH. Finally, since Cx43 was increased in PAs from patients with CH-PH but not in patients with IPAH, we suggest that a treatment combining Cx43 and ET-1 blockers could be considered especially for patients with CH-PH.

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