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Recombinant CCN1 Prevents Hyperoxia-induced Lung Injury in Neonatal Rats

Ruben Vaidya¹, Ronald Zambrano¹, Julia K Hummler¹, Shihua Luo¹, Matthew R Duncan¹, Karen Young¹, Lester F. Lau², and Shu Wu¹

¹Division of Neonatology, Batchelor Children Research Institute, University of Miami Miller School of Medicine, Miami, FL

²Department of Biochemistry, University of Illinois at Chicago, Chicago, IL

Abstract

Background—Cystein rich protein 61 (Cyr61/CCN1) is a member of the CCN family of matricellular proteins that plays an important role in tissue development and remodeling. However, the role of CCN1 in the pathogenesis of bronchopulmonary dysplasia (BPD) is unknown. Accordingly, we have investigated the effects of CCN1 on a hyperoxia-induced lung injury model in neonatal rats.

Methods—In experiment 1: newborn rats were randomized to room air (RA) or 85% oxygen (O₂) for 7 or 14 days and we assessed the expression of CCN1. In experiment 2: rat pups were exposed to RA or O₂ and received placebo or recombinant CCN1 by daily ip injection for 10 days. The effects of CCN1 on hyperoxia-induced lung inflammation, alveolar and vascular development, vascular remodeling, and right ventricular hypertrophy (RVH).

Main Results—In experiment 1, hyperoxia down-regulated CCN1 expression. In experiment 2, treatment with recombinant CCN1 significantly decreased macrophage and neutrophil infiltration, reduced inflammasome activation, increased alveolar and vascular development, reduced vascular remodeling and RVH in the hyperoxic animals.

Conclusion—These results demonstrate that hyperoxia-induced lung injury is associated with down-regulated basal CCN1 expression, and treatment with CCN1 can largely reverse hyperoxic injury.

INTRODUCTION

Bronchopulmonary dysplasia (BPD) continues to be one of the most common long-term pulmonary complication associated with preterm birth (1, 2). Lung injury from antenatal/postnatal infection, oxygen toxicity and mechanical ventilation leads to lung inflammation. The role of inflammation in the pathogenesis of BPD has been firmly established (3). Inflammation results in accumulation of inflammatory cells, activation of inflammasomes,

Corresponding author: Shu Wu, MD. Department of Pediatrics/Division of Neonatology, University of Miami Miller School of Medicine, PO Box 016960, Miami, FL 33101, swu2@med.miami.edu.

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increased pro-inflammatory cytokines and production of reactive oxygen species, which likely result in the pathological changes seen in BPD, characterized by alveolar simplification, reduced vascular growth and variable interstitial fibrosis (4, 5). Severe BPD is often complicated by pulmonary hypertension (PH) that significantly increases mortality.

The CCN (cyr61, ctgf, nov) proteins belong to an important family of matricellular regulatory factors involved in internal and external cell signaling and play a crucial role in regulation of tissue regeneration and inflammation (6). The CCN family of proteins consists of six members and despite similar structures, CCN proteins have a diverse variety of biological functions, which are highly dependent on cellular context (6). For example, CCN1 (Cyr61) and CCN2, also known as connective tissue growth factor (CTGF), are structurally related but functionally distinct and are expressed in many organs and tissues only during specific developmental or pathological events (7).

CCN2 has pro-inflammatory, pro-fibrotic and anti-angiogenic activities, and its crucial role as an inducer of the pathogenesis of various forms of adult pulmonary fibrosis and vascular diseases is firmly established (8, 9). Recent studies on the role of CCN2 in BPD showed that mechanical ventilation and hyperoxia exposure induced CCN2 overexpression in neonatal rat lungs (10, 11) and conditional overexpression of CCN2 in airway and alveolar type II epithelial cells severely disrupted alveolarization and vascular development (12, 13). Furthermore, CCN2 overexpression has been demonstrated in the postmortem lungs of preterm BPD infants as well as in the lungs of hyperoxia-exposed neonatal rats (14). Moreover, treatment with FG-3149, a monoclonal neutralizing CCN2 antibody prevented hyperoxia-induced alveolar damage in neonatal rats (14).

On the other hand, most studies show that CCN1 has anti-inflammatory, anti-fibrotic and pro-angiogenic activities during tissue development and injury repair (15-18), although some studies conversely suggest a pro-inflammatory/pro-fibrotic activity for CCN1 (19, 20). CCN1 largely exerts its anti-fibrotic effect by promoting cellular senescence and apoptosis and by attenuating TGF- β signaling (15, 17, 21). In addition, it has been recently demonstrated that CCN1 plays an important down-regulatory role in the early inflammatory phase of wound healing by stimulating the clearance of neutrophils via the process of efferocytosis (16). And CCN1 promotes angiogenesis by increasing VEGF receptor 2 (VEGFR2) expression, enhancing endothelial cell adhesion, migration and survival (18, 22). However, the role of CCN1 in BPD pathogenesis is unknown.

We hypothesized that CCN1 should play a protective role in BPD development and progression by attenuating inflammation, promoting alveolarization and angiogenesis, and decreasing PH. We thus evaluated the therapeutic potential of recombinant CCN1 protein in the prevention of hyperoxia-induced lung injury in neonatal rats, an experimental model of BPD. Given the increasingly recognized importance of the inflammasome in innate immune responses, organ injury and BPD pathogenesis (23, 24), we also evaluated the effects of CCN1 therapy on inflammasome expression and activation. Nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3 (NLRP3), NLRP1, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), active caspase-1 and active IL-1 β are key components of the inflammasome cascade. Our

results demonstrate that hyperoxia down-regulated CCN1 expression in neonatal rat lungs and treatment with recombinant CCN1 protein suppressed hyperoxia-induced activation of inflammasome, attenuated inflammation, improved alveolar and vascular development, decreased vascular remodeling and PH in neonatal rats. These findings provide new insights into understanding the role of CCN1 in the pathogenesis of BPD, and additionally suggest that CCN1 protein may have therapeutic potential in BPD prevention or treatment in neonates.

MATERIALS AND METHODS

Detailed descriptions of the materials and methods are provided in online supplement.

Animal Model and Experimental Protocol

The study protocol was approved by the University of Miami Institutional Animal Care and Use Committee. Experiment 1: To evaluate the temporal and spatial effects of hyperoxia on CCN1 expression, newborn Sprague-Dawley rats were randomized on postnatal day 1 to receive room air (RA) or 85% O₂ for 7 or 14 days and animals were sacrificed after 7 or 14 days of hyperoxia. Experiment 2: To study the efficacy of recombinant CCN1 in the prevention of hyperoxia-induced lung injury, newborn Sprague-Dawley rats were randomized on postnatal day 1 into 3 groups: RA + placebo (PL, normal saline); O₂ + PL; RA + CCN1; and O₂ + CCN1. Recombinant CCN1 (1 mg/kg) or placebo (equal volume) was administered by intraperitoneal (ip) injection on day 1, day 4, day 7 and day 9 during continuous room air or hyperoxia exposure. Murine recombinant CCN1 was produced using a baculovirus expression system and chromatographically purified (16) and the dose was used as referenced previously (17). Animals were sacrificed on day 11.

Assessments of CCN1 Protein Expression

Expression of CCN1 protein was assessed by Western blot analysis as previously described (13, 14).

Assessments of Lung Inflammation

Immunostaining with Mac3, a macrophage marker was performed, and the numbers of Mac3-positive cells in the alveolar airspaces were counted in 10 random images on each lung section for determining macrophage infiltration. To assess neutrophil infiltration, immunostaining with an anti-neutrophil elastase antibody was performed. Infiltrated neutrophils were counted from 10 random images on each lung section. Expression of inflammasome component proteins, NLRP-1, ASC, active caspase-1 and active IL-1 β were determined by Western blot analysis.

Lung Histology and Morphometry

Lungs were infused with 4% paraformaldehyde via a tracheal catheter at 20 cm H₂O pressure for 5 minutes, fixed overnight, and paraffin embedded. Hematoxylin and eosin-stained tissue sections were used to measure radial alveolar count (RAC) as previously described (13, 14).

Pulmonary Vascular Morphometry

Lung tissue sections were stained for von Willebrand factor (vWF), an endothelial marker to assess vascular density. The average number of vWF stained vessels ($< 50 \mu\text{m}$ in diameter) was counted from 5 random images on each lung section (13, 14).

Assessment of Pulmonary Vascular Remodeling

Lung tissue sections were double immunofluorescence stained for α -smooth muscle actin (α -SMA) and vWF to assess the extent of muscularization. The percentage of peripheral vessels ($< 50 \mu\text{m}$ in diameter) that were stained with α -SMA ($> 50\%$ circumference) was determined from 10 random images on each lung section (13, 14). To assess vascular smooth muscle cell proliferation, double immunofluorescence with an anti-Ki67 antibody (nuclear proliferating antigen) and α -SMA antibody was performed. The percentage of vessels with at least one positive Ki67 nuclei was determined.

Assessment of Right Ventricular Hypertrophy (RVH)

RVH (Fulton's index) was utilized as an index for PH. Hearts were dissected and the weight ratio of RV to left ventricle plus septum (LV+S) (13, 14) was determined.

Data Management and Statistical Analysis

Data were expressed as means $\pm$ SD, and comparisons were performed by two-way ANOVA followed by post hoc analysis (Student-Newman Keuls). A P value of less than 0.05 was considered statistically significant.

RESULTS

Hyperoxia Down-regulates CCN1 Expression in Neonatal Lungs

We evaluated the expression of CCN1 in lungs by Western blot analysis at day 7 and day 14 after continuous hyperoxia exposure. As demonstrated in Figure 1, quantitative densitometry analysis demonstrated that hyperoxia exposure resulted in significant suppression of CCN1 expression on both 7 days (1.73 ± 0.33 vs. 0.4 ± 0.45 , $P < 0.001$, RA vs. O_2) and 14 days (2.25 ± 0.64 vs. 0.79 ± 0.28 , $P < 0.01$, RA vs. O_2) (Figures 1a and 1b). These data suggest that CCN1 may play a role in hyperoxia-induced neonatal lung injury.

CCN1 Therapy Suppresses Hyperoxia-induced Lung Inflammation and Inflammasome Activation

We assessed the effects of CCN1 therapy on lung inflammation by quantifying macrophage and neutrophil infiltration. The macrophage counts were significantly elevated in the O_2 + PL group in comparison to the RA + PL group (8.0 ± 4.63 vs. 1.6 ± 0.47 , $P < 0.001$, O_2 + PL vs. RA + PL; Figures 2a and Figure 2b). Similarly, neutrophil counts were also significantly elevated with hyperoxia exposure compared to room air (7.4 ± 4.11 vs. 1.0 ± 0.35 , $P < 0.001$, O_2 + PL vs. RA + PL; Figures 2c and Figure 2d). However, administration of CCN1 resulted in significant decreases in both the macrophage and neutrophil counts induced by hyperoxia exposure (macrophage: 3.1 ± 0.64 vs. 8.0 ± 4.63 , P

< 0.01 , $O_2 + \text{CCN1}$ vs. $O_2 + \text{PL}$; Figure 2a and Figure 2b; neutrophil: 2.6 ± 1.33 vs. 7.4 ± 4.11 , $P < 0.001$, $O_2 + \text{CCN1}$ vs. $O_2 + \text{PL}$; Figure 2c and Figure 2d).

We further evaluated the effects of recombinant CCN1 on lung inflammation by measuring expression of inflammasome component proteins and active IL-1 β production. Hyperoxia plus placebo group had significantly increased expression of NLRP1, ASC and active caspase-1, compared to the room air group lungs (Figure 2e-2i). Hyperoxia-induced inflammasome protein expression appeared to be accompanied by inflammasome activation as we observed a significant elevation of active IL-1 β in hyperoxia exposed lungs compared to room air group lungs (1.27 ± 0.37 vs. 0.46 ± 0.21 , RA + PL vs. $O_2 + \text{PL}$, $P < 0.001$, Figure 2e and 2i). Treatment with recombinant CCN1 resulted in significant reductions in all 3 elevated inflammasome proteins during hyperoxia (Figure 2e-2h). And similarly CCN1 treatment resulted in a significant decrease in active IL-1 β expression in hyperoxia exposed lungs (0.52 ± 0.11 vs. 1.27 ± 0.37 , $P < 0.001$, $O_2 + \text{CCN1}$ vs. $O_2 + \text{PL}$, Figure 2eA and 2i). These results suggest a crucial role for CCN1 in protecting newborn lungs against the hyperoxia-induced inflammatory response via down-regulation of the inflammasome-IL-1 β cascade.

Treatment with CCN1 Improves Hyperoxia-suppressed Alveolar Development

We next evaluated the effects of CCN1 on alveolar development by measuring RAC. Compared to room air exposed rats, the lungs from hyperoxia and placebo exposed rats had significantly reduced RAC, suggesting poor alveolar development (6.06 ± 0.4 vs. 8.93 ± 1.03 , $P < 0.001$, $O_2 + \text{PL}$ vs. RA + PL, Figure 3a and 3b). Treatment with CCN1 resulted in attenuation of the alveolar injury induced by hyperoxia as demonstrated by increased RAC (6.06 ± 0.4 vs 7.97 ± 2.11 ; $P < 0.01$, $O_2 + \text{PL}$ vs. $O_2 + \text{CCN1}$, Figure 3a and 3b). Thus, CCN1 improves alveolarization during hyperoxia.

Treatment with CCN1 Improves Hyperoxia-suppressed Vascular Development

Pulmonary vascularization was assessed by measuring the vascular density of vWF-positive vessels ($< 50 \mu\text{m}$ in diameter) in lung tissue sections. As seen in Figure 4, hyperoxia exposure resulted in a significant reduction in vascular density compared to room air (5.30 ± 1.03 vs. 11.10 ± 2.87 , $P < 0.001$, $O_2 + \text{PL}$ vs. RA + PL, Figures 4a and 4b). In contrast, treatment with CCN1 significantly increased hypoxia-reduced vascular density (7.88 ± 0.57 vs. 5.30 ± 1.03 , $P < 0.05$, $O_2 + \text{CCN1}$ vs. $O_2 + \text{PL}$, Figures 4a and 4b). These results suggest that CCN1 improves vascular development in hyperoxia exposed neonatal rat lungs.

Administration of CCN1 Reduces Hyperoxia-induced Pulmonary Vascular Muscularization

To assess whether CCN1 affects pulmonary vascular remodeling during hyperoxia, we measured the extent of muscularization of peripheral pulmonary vessels that are less than $50 \mu\text{m}$ in diameters and with more than 50% muscularization by double immunofluorescent vWF and α -SMA staining of lung sections. The percentage of muscularized vessels was significantly increased in the hyperoxia group compared to the room air group (56% vs. 18%, $P < 0.001$, $O_2 + \text{PL}$ vs. RA + PL, Figure 5a and 5b). And, the percentage of muscularized vessel was significantly decreased by treatment with CCN1 during hyperoxia

(56% vs. 33%, $P < 0.01$, O₂ + PL vs. O₂ + CCN1, Figure 5a and 5b). Thus, CCN1 treatment decreases hyperoxia-induced pulmonary vascular remodeling.

We also assessed the effects of CCN1 on vascular smooth muscle cell proliferation. With exposure to hyperoxia, there was a significant increase in peripheral vessels with proliferating smooth muscle cells (55% vs. 19%, $P < 0.001$, O₂ + PL vs. RA + PL, Figure 5c and 6d). However, treatment with CCN1 protein resulted in a significant decrease in the percentage of proliferating peripheral vessels induced by hyperoxia exposure, (55% vs. 36%, $P < 0.001$, O₂ + PL vs. O₂ + CCN1, Figure 5c and 5d).

Treatment with CCN1 Decreases Hyperoxia-induced RVH

To evaluate for the degree of PH we measured RVH (Fulton index). Hyperoxia exposure resulted in a significant increase in RVH compared to room air exposure (0.41 ± 0.04 vs. 0.31 ± 0.02 , $P < 0.001$, O₂ + PL vs. RA + PL, Figure 6) and treatment with CCN1 resulted in a significant reduction in the elevated Fulton index induced by hyperoxia (0.31 ± 0.04 vs. 0.41 ± 0.04 , $P < 0.001$, O₂ + CCN1 vs. O₂ + PL, Figure 6). These results suggest that CCN1 can prevent hyperoxia induced RVH in neonatal rats.

DISCUSSION

In this study, we report that hyperoxia down-regulates CCN1 in newborn rat lungs. Moreover, we found evidence for a protective role for CCN1 in hyperoxia-induced neonatal lung injury by demonstrating that treatment with recombinant CCN1 decreases lung inflammation, improves alveolarization and vascular development, decreases pulmonary vascular remodeling and decreases RVH, all of which are key components of BPD pathology. These findings provide new insights into understanding the role of CCN1 in the pathogenesis of BPD and if future studies show CCN1 is also down regulated in BPD patients, then CCN1 has the potential to be a novel agent for the prevention or treatment of BPD in preterm infants.

Although there are many studies examining the expression pattern of CCN1, no previous studies have focused on the neonatal lung. We showed that high levels of CCN1 is expressed during normal neonatal rat lung development and that hyperoxia down-regulates CCN1 expression in the neonatal rat lungs. This expression pattern is in a sharp contrast to CCN2 expression which is low during normal lung development and up-regulated by hyperoxia (14). These results suggest that CCN1 and CCN2 may play different and/or opposing roles in lung development and injury repair in neonates.

Likewise, prior studies employing hyperoxia-models in adult rodents support our finding that enhancing CCN1 levels has an anti-inflammatory protective effect against hyperoxia-induced lung injury. For example, Moon et al have reported that endogenous lung epithelial cell-produced CCN1 exerted anti-inflammatory activity by promoting IL-10 production and by inhibiting multiple pro-inflammatory cytokines and neutrophil infiltration into the lung (25). Further, Jin et al demonstrated that suppressing CCN1 expression by siRNA accelerated lung epithelial cell death after hyperoxia, and conversely that overexpressing CCN1, conferred increased resistance to hyperoxia-induced cell death (26). While these

reports are in agreement with our findings here that CCN1 treatment had an anti-inflammatory and protective effect on hyperoxia-induced lung inflammation and damage in neonatal rats, the work of Perkowski et al conflicts with our finding that hyperoxia decreases lung CCN1 expression as they report hyperoxia increased lung CCN1 mRNA and protein expression (27). But, it is of note that the models used were significantly different from our model. They used adult mice and exposed them to >95% O₂ for short period of time (24-48 hours) to induce acute lung injury. On the contrary, we used a neonatal rat model, using 85% O₂ hyperoxia exposure for a longer period of 7-14 days to simulate chronic lung disease. Similarly other studies suggesting CCN1 is pro-inflammatory/fibrotic in mouse bleomycin models of lung fibrosis were performed using 8 week or 6 month old mice (19). Our differing results would seem to support the hypothesis that the differential physiologic function of CCN1 in cell survival/death are dependent on cell and organ types, type of cellular stimuli and the duration of inflammation.

Our results indicate that CCN1 therapy significantly reduced the neutrophil and macrophage counts in hyperoxia exposed rat lungs. And this may be partially related to the ability of CCN1 to increase efferocytosis of neutrophils, as has been described for wound tissue (16). But, to further investigate CCN's anti-inflammatory activity we also examined lung levels of inflammasome-related proteins. Studies have shown that cyclic stretch activates NLRP3 inflammasomes and induces the release of active IL-1 β in mouse alveolar macrophages (23). Studies by Liao et al have shown that the NLRP3 inflammasome is associated with the development of BPD and that hyperoxia-exposed neonatal mice lungs have increased caspase-1 and IL-1 β activation (24). Our recent studies have demonstrated that hyperoxia activates NLRP1 inflammasome and inhibition of Rac1 signaling down-regulates NLRP1 inflammasome and decreases lung injury (28). In this study, we did not find significant changes in NLRP3 expression, however, we did find that hyperoxia up-regulated expression of NLRP1, ASC, active caspase-1, and production of active IL-1 β and that recombinant CCN1 treatment resulted in significant down-regulation of all four hyperoxia-elevated proteins, suggesting CCN1's anti-inflammatory activity may be mediated via attenuated inflammasome expression. Whether this attenuated inflammasome expression is due to decreased protein synthesis in lung resident macrophages and neutrophils or the result of CCN1 decreasing infiltrating neutrophil and macrophages counts, awaits further investigation. These results suggest a crucial role for inflammasomes in hyperoxia-induced neonatal lung injury and possibly also in BPD pathogenesis.

This study also demonstrated that CCN1 dramatically improved alveolarization in hyperoxia exposed neonatal lungs. This could be secondary to the decreased inflammation induced by CCN1 treatment. Previous *in vitro* studies have shown that CCN1 protects against hyperoxia-induced lung epithelial cell death by activating cytoprotective signaling pathways (26, 29, 30). Thus, additional future studies are needed to investigate the potential mechanisms that are responsible for CCN1 protection of alveolar structure. Angiogenesis play a crucial role in the pathogenesis of BPD and it has been hypothesized that disruption of angiogenesis during critical periods of lung growth can impair alveolarization and contribute to lung hypoplasia in BPD (31). Previous studies have demonstrated that treatment with recombinant VEGF, an important angiogenic factor promotes angiogenesis and alveolarization in hyperoxia-exposed neonatal rats (32). CCN1 has been shown to play a

role in inducing angiogenesis and CCN1 knockout mice display severe defects in angiogenesis during embryo development and commonly die from placental vascular inefficiency due to compromised blood vessels (18, 33-35). In agreement with these prior studies, we have demonstrated here that hyperoxia resulted in poor vascular development which was associated with low CCN1 expression and that treatment of hyperoxic animals with CCN1 resulted in improved vascular density. These results suggest that CCN1 might also play a critical role in vascular development in hyperoxia-induced neonatal lung injury.

We have showed that CCN1 therapy was associated with a reduction of pulmonary vascular remodeling induced by hyperoxia exposure, characterized by a decreased percentage of peripheral muscularized and proliferating vessels in the CCN1 treated hyperoxia group compared to the group exposed to hyperoxia plus placebo. While the cellular mechanisms responsible for our observed reduction in pulmonary vascular remodeling by CCN1 treatment were not examined, previous studies on the role of CCN1 in cutaneous wound healing suggest CCN1 dampens and resolves fibrosis during wound healing by inducing myofibroblast senescence and upregulates the expression of antifibrotic genes to restrict fibrosis during tissue repair (36). Such mechanisms might explain the decrease in vascular remodeling we observed with CCN1 treatment. We also demonstrated that CCN1 therapy resulted in decreased RVH in hyperoxia exposed rat pups, which likely is a direct reflection of improved vascular development and reduced pulmonary vascular remodeling. Lee et al have shown that CCN1 suppresses hypoxia-induced pulmonary vascular smooth muscle contraction *in vitro* and it also decreases right ventricular pressure in hypoxia as well as monocrotaline induced PH in mice (37). These results highlight an important role of CCN1 in regulating vascular remodeling and PH.

There are potential limitations of this study. BPD is a multifactorial disease with risk factors including lung immaturity, prenatal/postnatal infection, traumatic ventilation and oxygen toxicity. Although the current study focuses on oxygen-induced lung injury which has phenotypic features similar to BPD, future studies are needed to investigate the role of CCN1 in the pathogenesis of BPD induced by other risk factors. In addition, more advanced stereological and three dimensional approaches to assess lung alveolar structure have been recently reported (38) and these techniques will provide new insights into architectural changes in experimental models of BPD.

In conclusion, this study demonstrates the beneficial effects of CCN1 therapy on preventing lung inflammation and inflammasome activation, improving alveolarization and vascularization, and reducing pulmonary vascular remodeling and RVH, all of which are key components of BPD pathology. These findings provide new insights into understanding the role of CCN1 in the pathogenesis of BPD and additionally identify CCN1 as a potential novel therapeutic target for this disease.

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Figure 1. Hyperoxia down-regulates CCN1 expression

Newborn rats were exposed to room air (RA, open bar) or to hyperoxia (85% O₂, solid bar) for 7 (**a**) or 14 (**b**) days and CCN1 expression in lung extracts was quantitated by Western blot densitometry analysis after normalization to house keeping gene β-actin. Representative Western blot photo images are shown. **C.** Hyperoxia exposure down-regulated CCN1 expression at both 7 days (* $P < 0.001$) and 14 days (** $P < 0.01$) as compared to room air.

Figure 2. Treatment with CCN1 decreases hyperoxia-induced inflammation and inflammasome activation

Immunostaining for Mac3 was performed on lung tissue sections (a) and the average numbers of macrophages in alveolar airspaces were counted from 10 random images, taken under the high power view (HPV, 200x) on each lung section (b). The O₂ + PL lungs showed increased macrophage counts compared to RA + PL lungs which were decreased by administration of CCN1 (* $P < 0.001$: RA + PL vs. O₂ + PL; † $P < 0.01$: O₂ + PL vs. O₂ + CCN1). $n = 6$ /group. Scale bar: 100 μ m. Immunostaining with an anti-neutrophil elastase

antibody was performed on lung tissue sections (e) and the average numbers of neutrophils in alveolar airspaces were counted from 10 random images, taken under the high power view on each lung section (d). Exposure to hyperoxia in the presence of the placebo increased neutrophil infiltration into the alveolar airspaces, while treatment with CCN1 significantly decreased neutrophil infiltration during hyperoxia (* $P < 0.001$: RA + PL vs. O₂ + PL; $^{\dagger}P < 0.001$: O₂ + PL vs. O₂ + CCN1; ** $P < 0.05$: RA + CCN1 vs. O₂ + CCN1). n = 6/group. Scale bar: 100 μ m. (e). Representative Western blot images for NLRP1, ASC, active caspase-1 (aCasp-1), active IL-1 β (aIL-1 β) and β -actin. The relative expression levels of NLRP1 (f), ASC (g), active caspase 1 (h) and active IL-1 β (i) were analyzed by densitometry and normalized to β -actin. All three inflammasome proteins and active IL-1 β were increased by hyperoxia in the placebo group as compared to room air group [$*P < 0.001$ (NLRP-1); $*P < 0.001$ (ASC); $*P < 0.001$ (Caspase-1); $*P < 0.001$ (IL-1 β)]. However, treatment with CCN1 during hyperoxia decreased expression of all three inflammasome proteins and active IL-1 β as compared to hyperoxia plus placebo group [$^{\dagger}P < 0.001$ (NLRP-1); $^{\dagger}P < 0.001$ (ASC); $^{\dagger}P < 0.001$ (Caspase-1); $^{\dagger}P < 0.001$ (IL- β)]. ** $P < 0.05$: O₂ + CCN1 vs. RA + CCN1. n = 6/group. Open bar: room air. Solid bar: hyperoxia.

Figure 3. Treatment with CCN1 improves hyperoxia-suppressed alveolarization

(a). Histological examination of O₂ + PL lung sections revealed larger and simplified alveoli in comparison to RA + PL lungs, which showed more numerous and smaller alveoli. CCN1 treatment reversed the effects of hypoxia as O₂ + CCN1 lungs showed more alveolarization. Scale bar: 50 μm. (b). Morphometric analysis demonstrated that hyperoxia exposure decreased radial alveolar count (RAC) in placebo-treated rats, which was significantly reversed by administration of CCN1 (* $P < 0.001$: RA + PL vs. O₂ + PL; † $P < 0.01$: O₂ + PL vs. O₂ + CCN1). $n = 6$ /group. Open bar: room air. Solid bar: hyperoxia.

Figure 4. CCN1 administration improves hyperoxia-suppressed vascular development

a: Immunofluorescence staining with an anti-vWF antibody (green signal) and DAPI nuclear staining (blue signal) was performed on lung tissue sections. Scale bar: 50 μm. **b:** Vascular density was determined by counting vWF positive vessels (< 50 μm) on 5 random images from each lung section. The vascular density was significantly decreased in O₂ + PL lungs compared to normoxic lungs (**P* < 0.001: RA + PL vs. O₂ + PL). Treatment with CCN1 significantly increased vascular density in hyperoxia exposed animals (†*P* < 0.05: O₂ + PL vs. O₂ + CCN1). ***P* < 0.05: RA + CCN1 vs. O₂ + CCN1. n = 6/group. Open bar: room air. Solid bar: hyperoxia.

Figure 5. Treatment with CCN1 decreases hyperoxia-induced pulmonary vascular remodeling
a: Double-immunofluorescence staining for vWF (green signal) and α -SMA (red signal) and DAPI nuclear staining (blue signal). Scale bar: 50 μ m. **b:** The percentage of < 50 μ m in diameter muscularized peripheral pulmonary vessels (> 50% of circumference α -SMA positive) was significantly increased in lungs from the O₂ + PL group. Administration of CCN1 significantly decreased vascular muscularization in hyperoxia-exposed animals (* P < 0.001: RA + PL vs. O₂ + PL; ** P < 0.01: RA + CCN1 vs. O₂ + CCN1; † P < 0.01: O₂ + PL vs. O₂ + CCN1). n = 6/group. **c:** Double immunofluorescence staining with Ki67 (red signal) and α -SMA (green signal) and DAPI nuclear stain (blue signal) was performed to assess vascular smooth muscle cell proliferation. Pink signals indicates Ki67 positive nuclei. Scale bar: 50 μ m. **d:** The percentage of vessels (< 50 μ m in diameter) with at least 1 Ki67 positive nuclei on each vessel was determined. O₂ + PL lungs had increased proliferating vessels compared to room air lungs. Treatment with CCN1 decreased vascular proliferation (* P < 0.001: RA + PL vs. O₂ + PL; ** P < 0.001: RA + CCN1 vs. O₂ + CCN1; † P < 0.001: O₂ + PL vs. O₂ + CCN1). n = 6/group. Open bar: room air. Solid bar: hyperoxia.

Figure 6. Effects of CCN1 on hyperoxia-induced RVH

Hyperoxia exposure in the presence of the placebo resulted in an increase in Fulton index (RV/LV + S), indicating RVH and PH. Administration of CCN1 significantly decreased RVH during hyperoxia (* $P < 0.001$: RA + PL vs. O₂ + PL; † $P < 0.001$: O₂ + PL vs. O₂ + CCN1). n = 6/group. Open bar: room air. Solid bar: hyperoxia.