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Downregulation of Inflammasome Activation to Attenuate Lung Injury in Neonatal Rats Exposed to Hyperoxia

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CONTENTS

1 ABBREVIATIONS

ASC	Adaptor protein (apoptosis-associated speck-like protein containing a caspase activation recruitment domain)
α-SMA	α-smooth muscle actin
BPD	Bronchopulmonary dysplasia
Caspase	Cyteinyl-aspartate specific protease
CCN	(**C**yr61, **c**tgf and **n**ov + Wnt induced secreted proteins) family of matricellular proteins
CCN1	Cystein-rich protein 61 (Cyr61/CCN1)
CCN2	= CTGF
CTGF	Connective tissue growth factor
IL-1β	Interleukin- 1β
Mac3	Monoclonal antibody to macrophage antigens
MLI	Mean linear intercept
NLRP-1	NOD-like receptor
NSC23766	specific Rac1 inhibitor
P2	Postnatal day 2
P12	Postnatal day 12
PDA	Patent ductus arteriosus
PH	Pulmonary hypertension
PL	Placebo
RAC	Radial alveolar count
Rac1	Ras-related C3 botulinum toxin substrate 1
RDS	Respiratory Distress Syndrome
RVH	Right ventricular hypertrophy
RVSP	Right ventricular systolic pressure
RV: LV + S	Right ventricle/left ventricle plus septum weight ratio
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

2 PUBLICATION LIST

2.1 Publication I

Inhibition of Rac1 Signaling Downregulates Inflammasome Activation and Attenuates Lung Injury in Neonatal Rats Exposed to Hyperoxia

Julia K Hummler, Fredrick Dapaah-Siakwan, Ruben Vaidya, Ronald Zambrano, Siwei Luo, Shaoyi Chen, Nadine Kerr, Juan Pablo de Rivero Vaccari, Robert W Keane, W Dalton Dietrich, Eduardo Bancalari, Karen C Young, Shu Wu

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2.2 Publication II

Recombinant CCN1 Prevents Hyperoxia-Induced Lung Injury in Neonatal Rats

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3 INTRODUCTION

3.1 General introduction: bronchopulmonary dysplasia (BPD)

Bronchopulmonary dysplasia (BPD) was first described in 1967 by Northway et al. after histological evaluation of premature babies who were dying from a characteristic and severe respiratory failure (1). The pathology of this nowadays so called 'old BPD' described severe alveolar emphysema and atelectasis, inflammation and fibrosis associated with prominent airway disruption as a consequence of mechanical ventilation (1).
Due to advances in neonatal critical care including prevention options and new ventilation strategies (2) the pathophysiology and the definition has changed. The 'new BPD' predominantly shows disrupted lung development with less cystic and fibrotic lung changes (3, 4) and mostly involves extremely premature infants (3, 5, 6). Pathology is characterized by enlarged and simplified terminal respiratory units with less alveolization and a variable degree of inflammation and fibrosis (7). Impaired alveolar development and angiogenesis are closely linked, which explains vascular rarefication in experimental BPD resulting in a reduced cross-sectional vascular density (7, 8). The 'new BPD' has a multi-factorial pathogenesis, including a 'combination of prenatal and postnatal risk factors' (9), and involving a complex interaction between altered alveolar and vascular development (5-7, 10-12).

BPD incidence correlates with immaturity explaining the fact that the most common form of BPD nowadays is 'characterized by chronic respiratory insufficiency in extremely preterm infants' (13) (5, 7, 10, 11). Northway originally described a severe lung injury in relatively mature preterm infants (1). Successful approaches to prevent or treat RDS such as antenatal steroid therapy, exogenous surfactant application, less invasive mechanical ventilation couldn't reduce the incidence of BPD, as a result of improved survival of extremely premature infants (14, 15). Besides asthma and cystic fibrosis bronchopulmonary dysplasia associated with preterm birth remains one of the most common chronic lung diseases in pediatric care.
The most commonly used diagnostic criteria assess infants at 36 weeks of postmenstrual age (16, 17) and has many shortcomings, such as the unability to classify infants dying from respiratory failure prior to this time point, and has a limited

value for an early description of severity and prediction of long-term respiratory outcomes (13, 18), and is inappropriate for on time treatment. Current potential therapies, such as postnatal corticosteroids, fluid management, improved nutrition, diuretics, patent ductus arteriosus (PDA) treatment have often only short-term physiological effects and are often unsatisfying (19-21) and correlated with side effects (4, 22). BPD leads to prolonged hospitalization, is associated with a higher risk of comorbidities like retinopathy of prematurity (23, 24) and impaired neurological development (7, 25) and is associated with increased respiratory and cardiovascular risk in adolescence and adulthood (26). Hence, 'novel therapeutic strategies to prevent and treat preterm infants with BPD are urgently needed' (27).
The ongoing challenges include but are not limited to defining accurate diagnostic criteria for BPD useful for daily practice, finding reliable predictors for early identification and long-term outcome, as well as developing successful treatment strategies. Research and clinical trials evaluating innovative target options are in progress – and curing preterm infants of bronchopulmonary dysplasia will be an ongoing challenge in the future, as long as we are not able to eliminate prematurity.

Understanding the multifactorial pathogenesis and complex definition research is focusing on prevention strategies and management of BPD at different stages of the disease progress (5, 28, 29). Although the detailed pathogenesis of BPD is still not completely understood, postnatal oxygen supplementation and infections are blamed to contribute to the development of this disease (3, 4).
Small animal models exposed to the known risk factors play an important role in translational research. 'The hyperoxia-induced neonatal lung injury model is widely used as an experimental model' (27) because chronic exposure of neonatal rats to high concentrations of oxygen induces the pathological hallmarks of BPD (30, 31). Considering that oxygen levels and duration of exposure influences the histological results, limitations of this animal model needs to be memorized (29, 32, 33).

The aim of this thesis was to investigate the effects of different mediators involved in inflammasome activation on a hyperoxia - induced lung injury model in neonatal rats.

3.2 Inhibition of Rac1 signaling downregulates inflammasome activation and attenuates lung injury in neonatal rats exposed to hyperoxia

As a member of the Rho family of small GTPases Rac1 mediates inflammasome activation (34, 35) and regulates migration of inflammatory cells into lung tissue (36, 37). Treatment with NSC23766, a specific Rac1 inhibitor showed decreased infiltration of leukocytes and lung injury in animal models (38, 39).
This study tested the hypothesis, that inhibition of Rac1 signaling prevents lung injury in an animal model of BPD. Newborn rat pubs were randomized into groups exposed either to room air or hyperoxia (85% oxygen) and received daily intraperitoneal injections of placebo (normal saline) or NSC23766 for ten days. Hyperoxia exposure upregulated Rac1, that was accompanied by increased expression of inflammasome proteins. The treatment with NSC23766 showed significant enhancement in inhibition of inflammasome activation when compared with placebo treated rats. In hyperoxia exposed and NSC23766 treated rats we demonstrated decreased macrophage infiltration, improved alveolar and vascular development and reduced pulmonary vascular remodeling. We concluded that Rac1 regulates inflammasome expression and plays a pivotal role in the pathogenesis of hyperoxia-induced neonatal lung injury. We speculate that Rac1 inhibition might be an efficient new therapeutic strategy. However the validity of Rac1 signaling as a potential target against BPD has to be evaluated in further studies.

3.3 Recombinant CCN1 prevents hyperoxia-induced lung injury in neonatal rats

The CCN family of proteins consists of six members and despite equal structures they have various functions (40-43). Cystein-rich protein 61 (Cyr61) also called CCN1 has an important role in tissue development and remodeling (44). Connective tissue growth factor (CTGF/ CCN2) is structurally related to CCN1 but functionally distinct and is expressed in many organs only during specific developmental or pathological events (44) Earlier studies have shown that CTGF disrupts postnatal pulmonary development (45, 46), is upregulated in lung fibrosis (47-49), and by

CTGF- inhibition, hyperoxia-induced lung injury in neonatal rats is attenuated (50, 51). ‘However, the role of CCN1 in the pathogenesis of bronchopulmonary dysplasia is not defined. We hypothesized that CCN1 has a protective role in BPD development and progression’ (52). In experiment 1 we randomly exposed rats to room air or hyperoxia (85% oxygen) and assessed the expression of CCN1. In experiment 2 we evaluated the therapeutic potential of recombinant CCN1 protein in prevention of hyperoxia-induced lung injury in neonatal rats. Our results demonstrate that hyperoxia downregulated the expression levels of CCN1 in neonatal rat lungs. Treatment with CCN1 protein suppressed the activation of inflammasome, which was associated with attenuated inflammation, improved alveolar and vascular development and decreased vascular remodeling and pulmonary hypertension (PH) in neonatal rats.

Our data thus not only provides new insights into the pathogenesis of bronchopulmonary dysplasia, but also depicted feasible perspectives for further research studies to understand the role of CCN1 and develop future treatment options.

3.4 Contribution to completion of publications

As a medical student I had the opportunity and great pleasure to work with the research team in New Born Neonatal Developmental Biology Laboratory at the Batchelor Children’s Research Institute at University of Miami School of Medicine, Miami, Florida. My mentor Shu Wu, M.D. supervised me during my nine-month stay to gain insight into basic science research techniques and to conduct my own project. First, I undertook a required course in laboratory animal welfare. The modules taught me basic care of rats in research, including but not limited to physical examination of the animals’ clinical condition, administering parenteral drugs via injections, how supportive care is provided and how these animals are monitored for their wellbeing and the variables of interest. I obtained specific skills about measuring specific endpoints, doing procedures, as well as euthanasia and small animal surgery to collect samples.

During my research activities I took an active role in designing and executing my own project. I worked on my independent project to investigate the role of Rac 1 in the pathogenesis of hyperoxia-induced neonatal lung injury, a widely used experimental

model of BPD. We randomized newborn rats into four groups and exposed them either to room air or hyperoxia (85% FiO_2) and we applied daily intraperitoneal injections of placebo or a specific inhibitor for ten days. Sacrificing of animals had to be coordinated, because several team members were necessary to collect data and samples simultaneously. I was responsible for preparation, counting and labeling of samples, as well as for all important documentation during procedures. During animal surgery I provided sedation to rat pups by intraperitoneal injection and measured heart weight ratio after thoracotomy and heart dissection.
For histologic examination, I analyzed lung tissue sections under a field of equally spaced horizontal lines to count mean linear intercept (MLI) on random picked images. I learned research skills such as pulmonary vascular morphometry as well as immunostaining.
To accomplish accurate results, I had to pay special attention to many details and ensure all the necessary documentation. I always conferred with my teammates and especially Shu Wu to discuss interim reports. Finally, I analyzed the data to obtain descriptive and statistical results and wrote the manuscript for publication. Under supervision of Dr. Shu Wu I submitted successfully my first own manuscript and therefore I am first author of this peer-reviewed publication.
During Pediatric Academic Societies (PAS) 2015 Annual Meeting in San Diego, California I presented my project and received one of the Medical Student Research Awards for this work.

Participants of neonatal perinatal fellowship at Jackson Health System in affiliation with the University of Miami Jackson Memorial Medical Center are next to patient care trained to set up a research project. Each fellow conducts her/his own clinical or translational experiment, learns how to analyze and interpret the data and possibly presents and publishes the results.
Usually fellows start while another one almost finishes the project to learn skills and understand basic procedures. As a medical student from Germany I started the same way and was then assigned to my own project while being full-time engaged in the laboratory. So I was able to help with other neonatology fellows' projects, while those were on clinical duty. For the second publication I supported Ruben Vaidya in his own project, showed him skills I had learned earlier und helped him with animal care. After euthanasia of the animals I collected data, for example by assessment of

alveolar morphometry while being blinded of group assignment. Moreover, I was involved in research of related literature and correction of his final manuscript. This explains my position as a co-author in the second publication.

4 SUMMARY OF PUBLICATIONS

4.1 Zusammenfassung

Die Bronchopulmale Dysplasie (BPD) gehört weiterhin zu den häufigsten chronischen Lungenerkrankungen des Frühgeborenen (16, 29, 53, 54). Derzeitige Behandlungsmöglichkeiten sind aufgrund der begrenzten Effektivität nicht zufriedenstellend (9, 29).
Das Ziel dieser Doktorarbeit war es, verschiedene neue Therapieansätze in der Unterdrückung der Entzündungskaskade im Tiermodell zu untersuchen.
Neugeborene Ratten zeigen nach Exposition von hohen Sauerstoffkonzentrationen erhebliche Lungenschäden, die vergleichbar mit den pathogenetischen Prozessen bei BPD und deshalb für wissenschaftliche Untersuchungen geeignet sind.
Durch das ‚targeting' verschiedener Signalwege, konnten wir neue Einblicke in die entzündlichen Prozesse der Pathogenese der BPD erzielen. Die Studien untersuchten verschiedene therapeutische Ansatzpunkte in der Entzündungskaskade und konnten eine signifikant reduzierte Lungenschädigung darstellen.
In histologisch untersuchten Lungenschnitten konnten wir eine reduzierte Zahl von Entzündungszellen, eine verbesserte Alveolarisierung und Gefäßentwicklung, sowie einen reduzierten Gefäßumbau mit verringerter Rechtsherzhypertrophie nachweisen.
Die zielgerichtete Inhibition des Rac1 Entzündungsweges, sowie der Einsatz von rekombinantem CCN1 könnten neue Therapieansätze in der Prävention und Therapie der bronchopulmonalen Dysplasie beim Frühgeborenen darstellen. Weitere experimentelle und klinische Studien zur Überprüfung der möglichen Therapieansätze in der BPD sind dafür aber notwendig.
Diese kumulative Dissertation basiert auf den folgenden beiden Originalveröffentlichungen.

4.2 Abstract

Bronchopulmonary dysplasia (BPD) remains one of the most common pulmonary long-term complications of premature infants (16, 29, 53, 54). Current therapeutic strategies are unsatisfying, due to their limited efficacy (9, 29).

The aim of this doctoral thesis was to evaluate novel signaling pathways by ‘inhibiting inflammasome activation in a newborn rat model of BPD induced by hyperoxia’ (27). By targeting different signaling pathways, we obtained novel insights into the inflammation cascade leading to the pathological hallmarks of bronchopulmonary dysplasia. The studies tested various pathways of downregulation of inflammasome activation, resulting in decreased lung inflammation displaying less accumulation of inflammatory cells, improved alveolar and vascular development and reduced vascular remodeling and right ventricular hypertrophy (RVH) in hyperoxic animals. Therefore, targeting Rac 1 signaling as well as treatment with recombinant CNN1 may provide novel strategies to prevent and reduce hyperoxic injury in preterm infants. Further additional experimental and clinical studies are necessary to demonstrate the beneficial effects on the pathogenesis of BPD.
This cumulative doctoral thesis is based on the following two original publications.

5 PUBLICATION I

Original Paper

Neonatology

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Inhibition of Rac1 Signaling Downregulates Inflammasome Activation and Attenuates Lung Injury in Neonatal Rats Exposed to Hyperoxia

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Keywords
Rac1 · Inflammasome · Hyperoxia · Neonatal lung injury · Bronchopulmonary dysplasia

Abstract
Background: Inflammatory injury, particularly the production of active interleukin (IL)-1β plays a major role in the pathogenesis of bronchopulmonary dysplasia (BPD) in preterm infants. The release of active IL-1β is controlled by post-transcriptional modifications of its proform (pro-IL-1β) through the inflammasome. Rac1 is a member of the Rho family of GTPases that regulate the inflammatory process. ***Objective:*** This study tested the hypothesis that Rac1 signaling increases inflammasome activation that results in damaging inflammation, and that the inhibition of Rac1 signaling prevents lung injury, by inhibiting inflammasome activation in a newborn rat model of BPD induced by hyperoxia. ***Methods:*** Newborn rat pups were exposed to room air or hyperoxia (85% O_2) and received daily intraperitoneal injections of placebo (normal saline) or NSC23766, a specific Rac1 inhibitor, for 10 days. The effects on lung inflammation, alveolarization, vascular development, vascular remodeling, right ventricular systolic pressure, and right ventricular hypertrophy (RVH) were then assessed. ***Results:*** Hyperoxia exposure upregulated Rac1 and increased the production of active IL-1β, which was accompanied by increasing expression of the inflammasome. In addition, hyperoxia induced the pathological hallmarks of BPD. However, treatment with NSC23766 significantly decreased inflammasome activation and macrophage infiltration, improved alveolar and vascular development, and reduced pulmonary vascular remodeling and RVH. ***Conclusion:*** These results indicate that Rac1 signaling regulates the expression of the inflammasome and plays a pivotal role in the pathogenesis of hyperoxia-induced neonatal lung injury. Therefore, targeting Rac1 signaling may provide a novel strategy to prevent and treat BPD in preterm infants.

Introduction

Bronchopulmonary dysplasia (BPD) remains the most common pulmonary complication of premature infants [1]. Inflammatory injury triggered by antenatal infection,

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mechanical ventilation, or oxygen toxicity is recognized as central to the pathogenesis of BPD [2–4], and there is increasing recognition that interleukin (IL)-1β plays a role here [5–7]. The cleavage of pro-IL-1β into active IL-1β is controlled by a multiprotein complex called the inflammasome that is comprised of the inflammasome proteins, NOD-like receptor (NLRP), the adaptor protein apoptosis-associated speck-like protein containing a caspase activation recruitment domain (ASC), and caspase-1 [8]. Active caspase-1 cleaves pro-IL-1β into active IL-1β. A recent study has demonstrated that the NLRP3-IL-1β axis is critically involved in the development of BPD [9].

Rac1 is a member of the Rho family of small GTPases and acts as a molecular switch to mediate multiple cellular mechanisms including inflammasome activation [10, 11]. Rac1 acts as a central regulator of cell migration through cytoskeletal arrangements that mediate inflammatory cell recruitment into lung tissue during the inflammatory process [12, 13]. Recent studies showed that Rac1 controls the NLRP3 inflammasome-mediated processing of pro-IL-1β in *Chlamydophila pneumoniae*-infected mononuclear cells [11]. In fact, treatment with NSC23766, a specific Rac1 inhibitor, decreases leukocyte infiltration and lung injury in animal models [14, 15]. Rac1 also regulates the expression of connective tissue growth factor (CTGF), which is a key factor in the pathogenesis of pulmonary vascular remodeling and fibrosis [16].

The hyperoxia-induced neonatal lung injury model is widely used as an experimental model for BPD [17]. Chronic exposure of neonatal rodents to high concentrations of oxygen induces lung inflammation and results in lung injury with the pathological hallmarks of BPD, including disrupted alveolar and vascular development, and increased vascular remodeling and pulmonary hypertension (PH). Thus, in this study we investigated whether the lung inflammation and subsequent lung injury associated with hyperoxia-exposed neonatal rats could be diminished by treatment with the Rac1 inhibitor, NSC23766, as has previously been reported for bacterially induced inflammation. Our data indicate that administration of NSC23766 did indeed decrease lung inflammation in hyperoxia-exposed neonatal rats with the resultant improved alveolarization and angiogenesis, and attenuated right ventricular hypertrophy (RVH). Moreover, NSC23766 actions appear to be inflammasome-mediated, as we also observed decreased inflammasome activation and decreased IL-1β processing. Thus, Rac1 may play a critical role in hyperoxia-induced inflammation and subsequent BPD-like pathology via inflammasome activation, suggesting that targeting Rac1 signaling may provide a novel strategy to prevent BPD in preterm infants.

Materials and Methods

Detailed descriptions of the materials and methods used are provided in the online supplement (for all online suppl. material, see www.karger.com/doi/10.1159/000450918).

Animal Model and Experimental Protocol

The study protocol was approved by the University of Miami Institutional Animal Care and Use Committee. Newborn Sprague-Dawley rats were randomized on postnatal day 2 (P2) into 4 groups (n = 10/group): the room air (21% O_2) + placebo (normal saline) group; the room air + Rac1 inhibitor NSC23766 (5 mg/kg) group; and the hyperoxia (85% O_2) + placebo and hyperoxia + NSC23766 group. NSC23766 (5 mg/kg) or placebo (equal volume) were administered by daily intraperitoneal injection for 10 days during continuous exposure to room air or hyperoxia. Exposure to 85% oxygen was used to produce severe lung injury and BPD. On P12, the animals were sacrificed.

Measurement of the Expression of Inflammasome Proteins, the Production of Active IL-1β, and Macrophage Infiltration

Expression of inflammasome proteins and the production of active IL-1β were determined by Western blot analysis. Macrophage infiltration was assessed by immunostaining for the macrophage marker Mac3; the numbers of Mac3-positive cells in the alveolar airspaces were then counted on 10 random images on each lung section to quantitate macrophage infiltration [17]. Immunostaining, double immunofluorescence staining, and Western blot were performed as previously described [17].

Lung Histology and Morphometry

Hematoxylin-and-eosin-stained tissue sections were used to measure mean linear intercept (MLI), as previously described [17]. Briefly, 10 random images were taken at ×20 magnification on each tissue section. The images were viewed under a field of equally spaced horizontal lines, and MLI was assessed as the average of total length of lines divided by the total intercepts of the alveolar septa from each lung.

Assessment of Pulmonary Vascular Morphometry

To determine vascular density, immunofluorescence staining for von Willebrand factor (vWF), an endothelial marker, was performed. Ten random images were taken at ×20 magnification on each lung section, and the average number of vWF-stained vessels (<50 μm in diameter) was calculated [17].

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 μm in diameter) that were positive for vWF and stained with α-SMA for >50% of their circumference was determined from 10 random ×20 magnification images of each lung section [17].

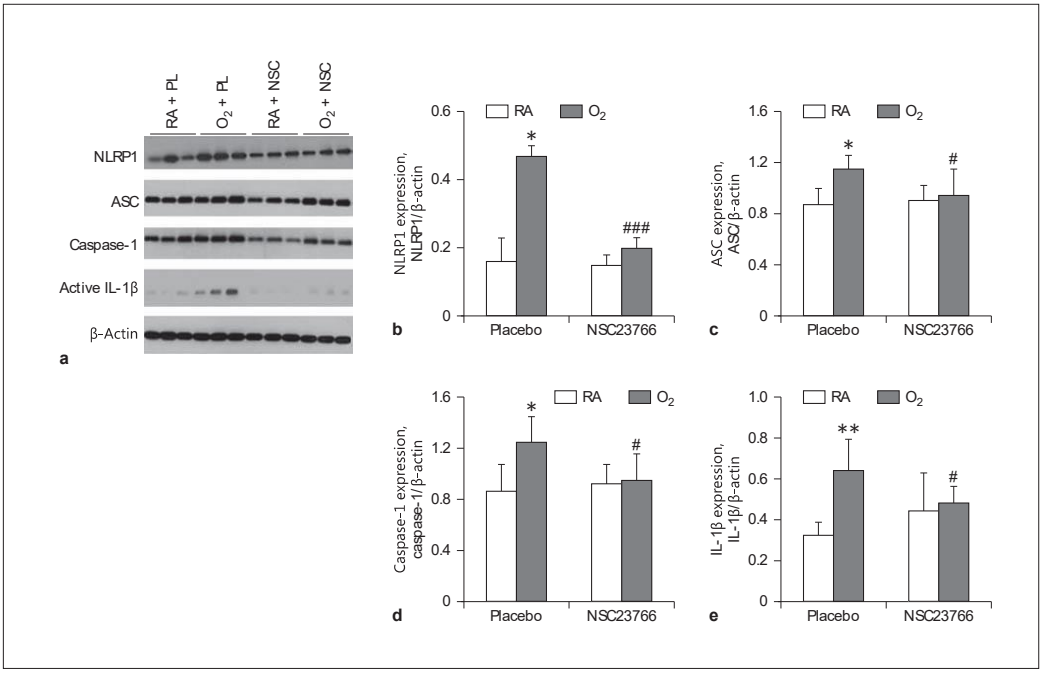

Fig. 1. Treatment with NSC23766 downregulates inflammasome protein expression and production of active IL-1β. **a** Representative photographic images of Western blots for inflammasome proteins. RA, room air; PL, placebo; O_2, hyperoxia; NSC, NSC23766. The relative expression levels of NLRP1 (**b**), ASC (**c**), active caspase-1 (**d**), and active IL-1β (**e**), analyzed by densitometry and normalized to β-actin, were increased by hyperoxia in the placebo group, and they were downregulated by treatment with NSC23766. $n = 5$/group. * $p < 0.05$ and ** $p < 0.01$ compared to room air + placebo; $^{\#}$ $p < 0.05$ and $^{\#\#\#}$ $p < 0.001$ compared to hyperoxia + placebo.

Assessment of PH and RVH

Right ventricular systolic pressure (RVSP) and the weight ratio, RV/left ventricle plus septum (RV:LV + S), were determined as indices for PH [17]. For RVSP measurement, a 25-gauge needle fitted to a pressure transducer was inserted after thoracotomy into the RV. Pressure levels were quantified and continuously recorded on a Gould polygraph (model TA-400; Gould Instruments, Cleveland, OH, USA). Thereafter, the hearts were dissected for measurement of the weight ratio, RV:LV + S [17].

Data Management and Statistical Analysis

Data were expressed as means (± SD), and comparisons were performed by ANOVA followed by the Student-Newman Keuls test. $p < 0.05$ was considered significant.

Results

Administration of NSC23766 Suppresses the Expression of Inflammasome Proteins and the Production of Active IL-1β

As demonstrated in Figure 1, hyperoxia increased the expression of all 3 inflammasome proteins in the placebo-treated animals, but this was significantly decreased in the NSC23766-treated rats (Fig. 1a–d). Importantly, the inhibition of Rac1 drastically decreased the production of active IL-1β (Fig. 1a, e). These data highlight the critical role of Rac1 signaling in mediating the hyperoxia-induced inflammasome protein expression and production of IL-1β in neonatal lungs.

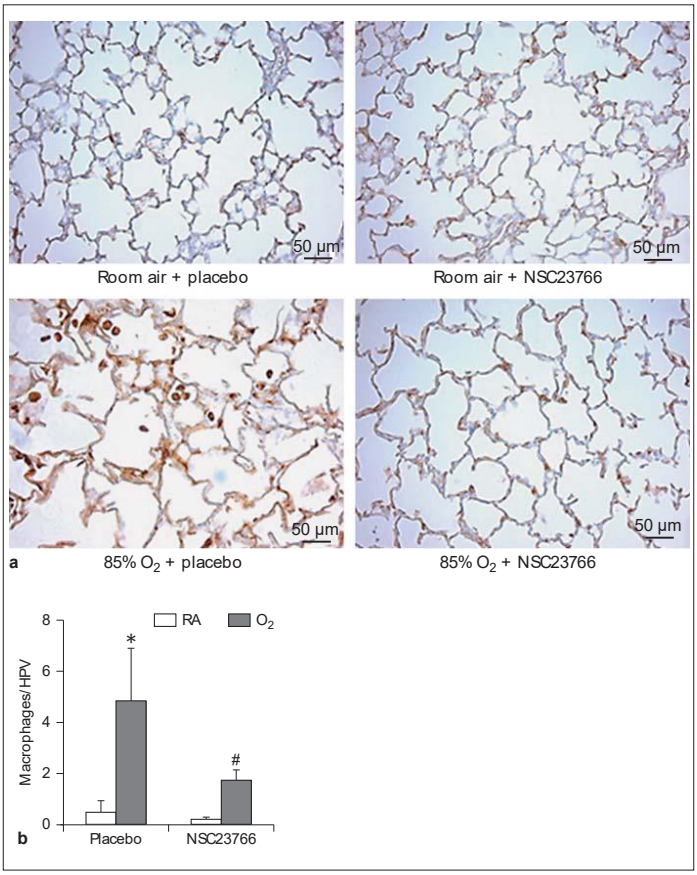

Fig. 2. Inhibition of Rac1 alleviates lung inflammation. **a** Immunostaining for Mac3 was performed. The average numbers of macrophages (brown) in the alveolar airspaces were counted from 10 random images on each lung section. ×20. **b** Hyperoxia exposure in the presence of the placebo significantly increased the number of macrophages compared with the room air group. However, administration of NSC23766 significantly decreased macrophage infiltration during hyperoxia. n = 5/group. * $p < 0.001$ compared to room air; # $p < 0.001$ compared to hyperoxia + placebo. HPV, high power view; RA, room air; O_2, hyperoxia.

Rac1 Inhibition Decreases Macrophage Infiltration

The macrophage counts were significantly increased in the hyperoxia + placebo-treated group compared to the room air + placebo group (Fig. 2a, b). However, the administration of NSC23766 significantly decreased the macrophage count during hyperoxia exposure (Fig. 2a, b).

Rac1 Inhibition Improves Alveolar Development

Upon histological examination, the lungs from the hyperoxia-exposed and the placebo-treated rats displayed larger and simplified alveoli (Fig. 3a). Treatment with NSC23766 modestly improved the alveolar structure in the hyperoxia-exposed animals. Morphometric analysis showed an increase in MLI in the lungs of the hyperoxia + placebo group compared to those of the room air + placebo group (Fig. 3b). Conversely, treatment with NSC23766 during hyperoxia exposure reduced MLI compared to the hyperoxia + placebo group (Fig. 3b). Thus, e inhibition of Rac1 signaling attenuates hyperoxia-induced impairment of alveolar structure.

Rac1 Inhibition Improves Vascular Development

The hyperoxia + placebo group showed a 50% decrease in vascular density compared to the room air + placebo group (Fig. 4a, b). However, treatment with NSC23766

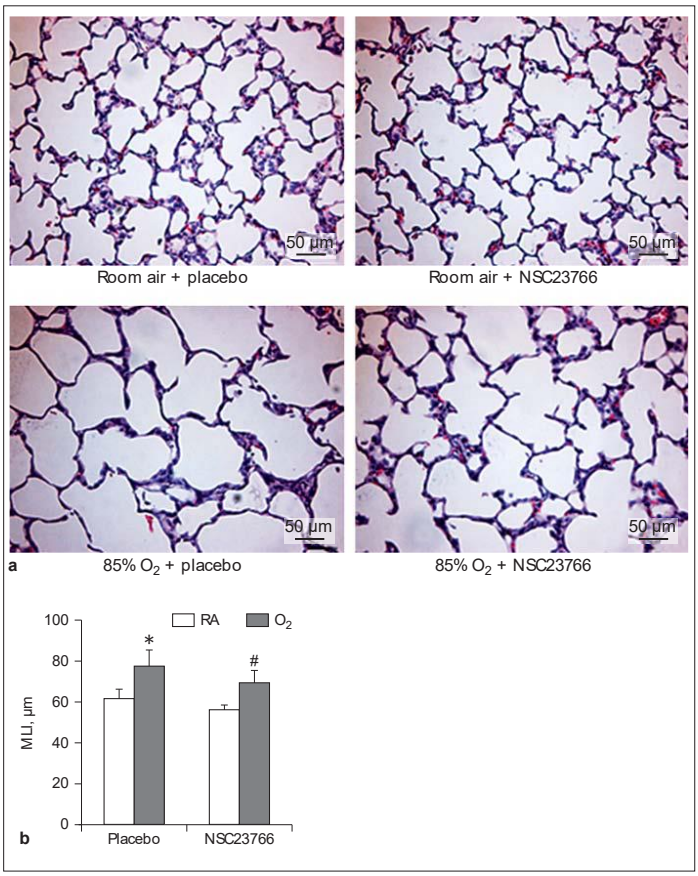

Fig. 3. Inhibition of Rac1 improves alveolarization. **a** Histological examination of the lung sections displayed larger and simplified alveoli in hyperoxia + placebo lungs, compared with hyperoxia + NSC23766 lungs which showed more and smaller alveoli. **b** Hyperoxia exposure significantly increased mean linear intercept (MLI) in placebo-treated rats; however, administration of NSC23766 significantly decreased MLI. $n = 5$/group. * $p < 0.001$ compared to room air; # $p < 0.05$ compared to hyperoxia + placebo. RA, room air; O_2, hyperoxia.

significantly increased the vascular density during hyperoxia compared to in the hyperoxia + placebo group (Fig. 4a, b). These results indicate that the inhibition of Rac1 signaling improves angiogenesis during hyperoxia exposure.

Inhibition of Rac1 Decreases Pulmonary Vascular Muscularization

The vessels with >50% muscularization were significantly increased in the hyperoxia + placebo lungs, and this was significantly decreased by treatment with NSC23766 (Fig. 5a, b). Correlating with decreased muscularization, the expression of CTGF, a critical factor causing vascular remodeling, was significantly reduced by the administration of NSC23766 (Fig. 5c, d). Thus, the inhibition of Rac1 signaling decreases hyperoxia-induced pulmonary vascular remodeling.

Rac1 Inhibition Decreases Hyperoxia-Induced RVH

PH with RVH is a sign of severe BPD. We evaluated the effects of Rac1 inhibition on PH and RVH by measuring the RVSP and RV:LV + Sweight ratios. Accordingly, there was a trend of slightly increased RVSP but significantly increased RVH in the hyperoxia + placebo group (Fig. 6a, b). The administration of NSC23766 during hyperoxia significantly reduced RVH (Fig. 6b). These data

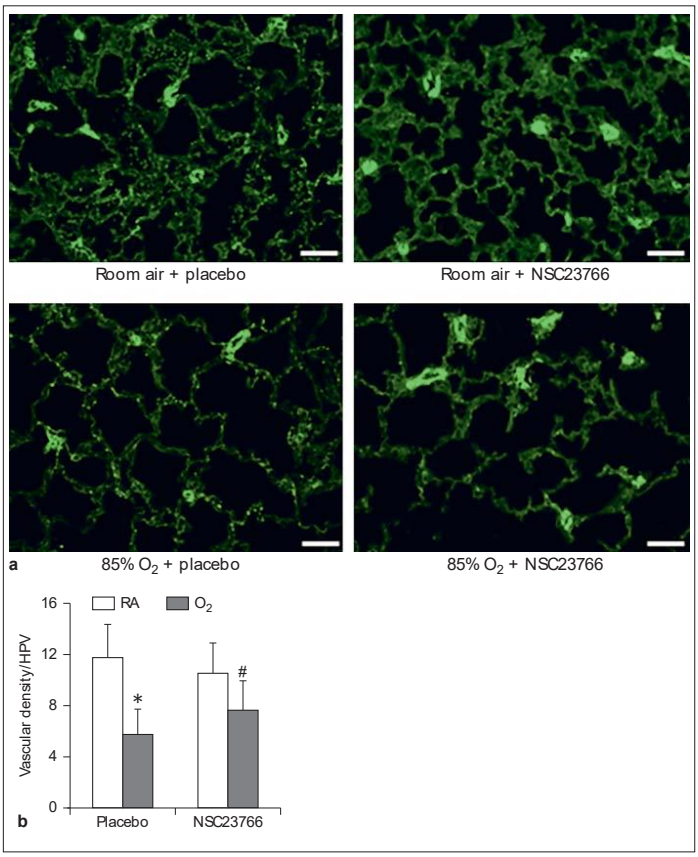

Fig. 4. Inhibition of Rac1 improves vascular development. **a** Immunofluorescence staining for vWF (green). Scale bar, 50 µm. **b** Vascular density was determined by counting vWF-positive vessels (<50 µm) on 10 random images from each lung section. Hyperoxia exposure in the presence of placebo significantly decreased vascular density compared with room air exposure, but the administration of NSC23766 significantly increased vascular density during hyperoxia. n = 5/group. * $p < 0.001$ compared to normoxic lungs; # $p < 0.001$ compared to hyperoxia + placebo lungs. HPV, high power view; RA, room air; O_2, hyperoxia.

indicate that the inhibition of Rac1 signaling protects against hyperoxia-induced RVH.

Discussion

This study demonstrates that hyperoxia activated the inflammasome-IL-1β axis and induced the pathological hallmarks of BPD in neonatal rats. Importantly, we showed that treatment with NSC23766, a specific pharmacological inhibitor of Rac1, downregulated the inflammasome-IL-1β axis, attenuated lung inflammation, improved alveolarization and pulmonary vascular development, and decreased pulmonary vascular remodeling. The inhibition of Rac1 signaling also decreased RVH. These data illustrate the critical role of a novel signaling pathway, the Rac1-inflammasome-IL-1β axis, in the pathogenesis of BPD.

Our results demonstrate that hyperoxia, in addition to inducing the pathological hallmarks of BPD, upregulates the expression of the inflammasome-related proteins NLRP1, ASC, and cleaved (active) caspase-1 in the lungs. Moreover, the induced inflammasome proteins appear to be activated, as we also found there was increased expression of active IL-1β. Our study also suggests that activation of the inflammasome-IL-1β axis is, at least in part, controlled by Rac1 signaling in hyperoxia-induced neonatal lung injury, because we found that treatment with NSC23766, a specific Rac1 inhibitor, significantly downregulated the expression of the inflammasome proteins

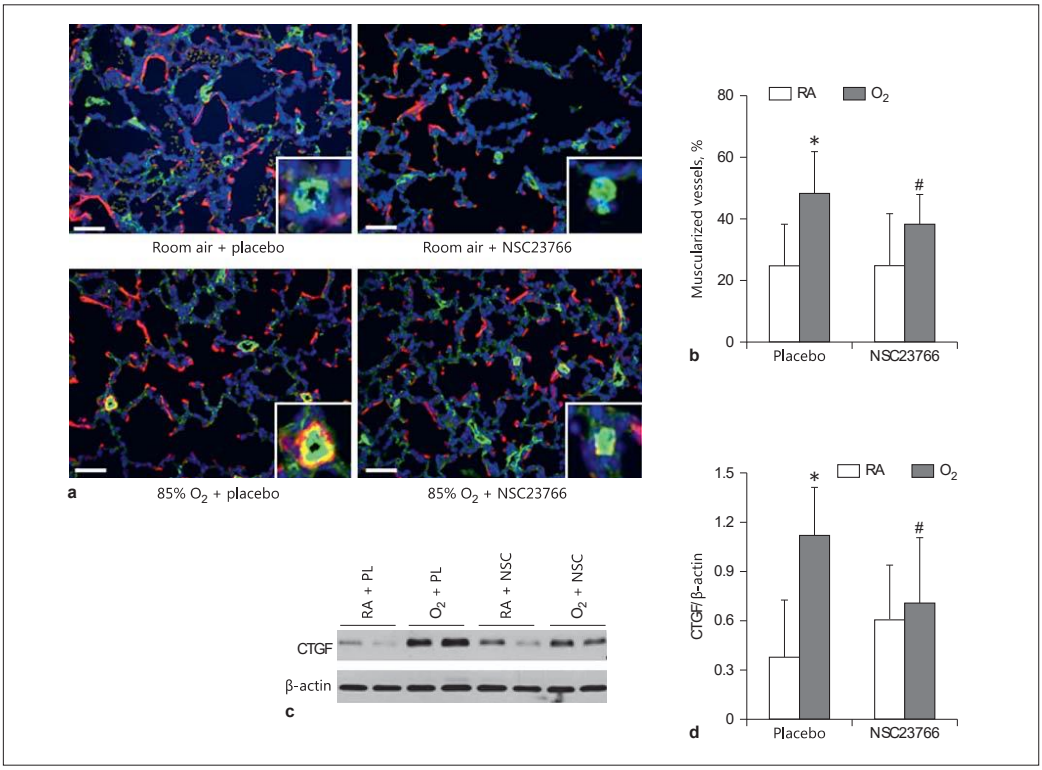

Fig. 5. Administration of NSC23766 decreases vascular remodeling. **a** Double immunofluorescence staining. vWF (green). α-SMA (red). DAPI nuclear stain (blue). Scale bar, 50 μm. **b** The percentage of muscularized peripheral vessels (<50 μm) was increased in hyperoxia + placebo lungs, and this was decreased by NSC23766. $n = 5$/group. * $p < 0.001$ compared to room air; # $p < 0.001$ compared to hyperoxia + placebo. **c** Representative Western blot images for CTGF and β-actin. RA, room air; PL, placebo; O_2, hyperoxia; NSC, NSC23766. **d** Relative CTGF expression level analyzed by densitometry and normalized to β-actin was increased by hyperoxia with placebo, and this was downregulated by treatment with NSC23766. $n = 5$/group. * $p < 0.001$ compared to room air; # $p < 0.001$ compared to hyperoxia + placebo. RA, room air; O_2, hyperoxia.

and the activation of IL-1β in hyperoxia-exposed lungs. Previous studies using other models of inflammatory lung injury appear to support our findings, as Rac1 inhibition with NSC23766 has been reported to decrease the macrophage/neutrophil infiltration and lung injury induced by lipopolysaccharide, streptococcal M1 protein, or sepsis, and also the expression of NLRP3 and active IL-1β in *C. pneumonia*-infected human mononuclear cells [11, 14, 15]. Thus, our study has revealed a novel mechanism by which Rac1 signaling regulates the hyperoxia-induced inflammatory response in the neonatal lung.

Clinical and animal studies have demonstrated that there are increased neutrophils and macrophages in tracheal aspirates in BPD [2, 17, 18]. We showed here that the inhibiton of Rac1 signaling decreased macrophage infiltration into the alveolar air spaces in hyperoxia-exposed lungs. It is known that Rac1 can act as a central regulator of cell migration through cytoskeletal arrangements, which mediates neutrophil recruitment into lung tissues during the inflammatory process [12, 13]. Therefore, our results support the findings that Rac1 signaling controls leukocyte infiltration in hyperoxia-induced neonatal lung injury.

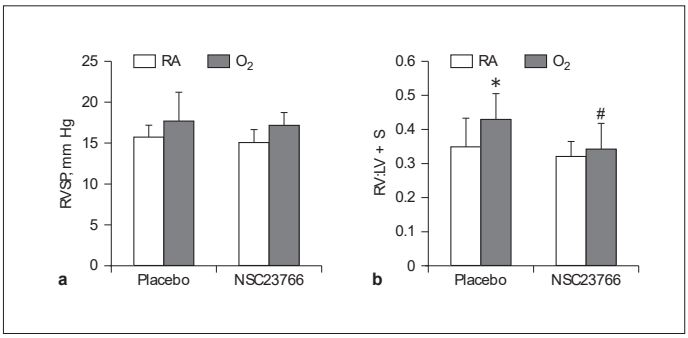

Fig. 6. Rac1 inhibition decreased right ventricular hypertrophy (RVH). **a** RVSP was slightly but not significantly increased in the hyperoxia + placebo group. **b** RVH, determined by the weight ratio RV:LV + S was increased in the hyperoxia + placebo group. Treatment with NSC23766 decreased RVH during hyperoxia exposure. $n = 5$ /group. * $p < 0.01$ compared to normoxic lungs; # $p < 0.01$ compared to hyperoxia + placebo lungs. RA, room air; O_2, hyperoxia.

Impaired alveolarization and disrupted angiogenesis are the main pathological characteristics of BPD [1, 19]. In this study, treatment with NSC23766 was not only effective in preventing hyperoxia-induced lung inflammation, it was also beneficial in preventing hyperoxia-induced alveolar structural damage. The inhibition of Rac1 signaling improved alveolarization during hyperoxia exposure. Alveolar development and angiogenesis in the lung go hand in hand, and impairment of alveolarization has a negative effect on angiogenesis, and vice versa [1, 4]. Here, we detected improved vascular density along with alveolarization in the NSC23766-treated hyperoxic rats. We hypothesize that the protective effects of NSC23766 in alveolar and vascular development are secondary to its anti-inflammatory properties. Our previous studies have shown that treatment with leukoadherin-1, a novel agonist of leukocyte surface integrins, decreased lung inflammation and increased alveolar and vascular development in hyperoxia-exposed neonatal rats [20]. Nold et al. [7] have recently shown that an IL-1 receptor antagonist increased alveolar numbers, decreased alveolar size, and increased the alveolar surface-area-to-volume ratio in perinatal inflammation and in postnatal hyperoxia-induced murine BPD. Their findings, along with our novel findings on Rac1 inhibition, support the notion that targeting the inflammatory response is key in preserving alveolar and vascular development in the immature lung.

Severe BPD is often complicated by PH, which significantly increases morbidity and mortality [4]. The development of PH in the immature lung is hallmarked by incomplete vascularization, impaired gas exchange, abnormal vasoreactivity, and vascular remodeling [4]. In this study, the inhibition of Rac1 signaling decreased pulmonary vascular remodeling and RVH in hyperoxic animals. We further demonstrated that treatment with NSC23766 drastically suppresses the hyperoxia-induced expression of CTGF. CTGF is a matricellular protein that plays an important role in tissue remodeling [21, 22]. It has increasingly been recognized as playing an important role in the pathogenesis of BPD and PH. The lungs of preterm infants who succumb to BPD have increased expression of CTGF [21]. Transgenic overexpression of CTGF in alveolar type II epithelial cells results in the pathological hallmarks of severe BPD with PH [22]. Furthermore, hyperoxia upregulates CTGF expression in the lungs of neonatal rats, and the inhibition of CTGF activity with a CTGF antibody attenuates BPD-like pathology and PH in these animals [21].

It is known that Rac1 signaling regulates CTGF expression [16, 23]. Lin et al. [16] showed that CXCL-12 activates Rac1 signaling which induces the expression of CTGF in human lung fibroblasts. CTGF is the downstream mediator of TGFβ-induced adventitial remodeling in carotid angioplasty [24]. Furthermore, CTGF stimulates vascular smooth muscle cell proliferation, migration, and the production of extracellular matrix in tissue culture [25]. We speculate that the downregulation of CTGF expression by NSC23766 is, at least in part, responsible for the decreased pulmonary vascular remodeling and RVH seen in this study. We also observed an elevation of RVSP in the hyperoxia-exposed rats that did not reach statistical significance, possibly because of the short length of oxygen exposure (10 days), as our previous study using 14 days of hyperoxia resulted in a significant elevation of RVSP [17, 21]. Nevertheless, the decreased pulmonary vascular remodeling and RVH highlight the importance of Rac-1-CTGF signaling in the development of experimental BPD with PH.

In conclusion, this study demonstrates that Rac1 inhibition has multiple beneficial effects against hyperoxia-induced neonatal rat lung injury, including decreasing the inflammasome activation/IL-1β processing, and lung inflammation, improving the alveolar and vascular growth, and reducing pulmonary vascular remodeling and RVH. Furthermore, we did not observe any negative effects of Rac1 inhibition on normal neonatal lung development. These observations suggest that additional experimental and clinical studies targeting Rac1 signaling could yield novel therapeutic approaches to the prevention and treatment of BPD in neonates.

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Author Contributions

Conception and design of the study: J.K.H., N.K., J.P.R.V., R.W.K., W.D.D., E.B., K.C.Y., and S.W. Acquisition, analysis, and interpretation of data: J.K.H., F.D., R.V., R.Z., S.L., S.C., and S.W.

Disclosure Statement

There were no conflicts of interest.

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Recombinant CCN1 prevents hyperoxia-induced lung injury in neonatal rats

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BACKGROUND: Cystein-rich protein 61 (Cyr61/CCN1) is a member of the CCN family of matricellular proteins that has 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 (O_2) for 7 or 14 days, and we assessed the expression of CCN1. In experiment 2, rat pups were exposed to RA or O_2 and received placebo or recombinant CCN1 by daily intraperitoneal injection for 10 days. The effects of CCN1 on hyperoxia-induced lung inflammation, alveolar and vascular development, vascular remodeling, and right ventricular hypertrophy (RVH) were observed.

RESULTS: In experiment 1, hyperoxia downregulated CCN1 expression. In experiment 2, treatment with recombinant CCN1 significantly decreased macrophage and neutrophil infiltration, reduced inflammasome activation, increased alveolar and vascular development, and reduced vascular remodeling and RVH in the hyperoxic animals.

CONCLUSION: These results demonstrate that hyperoxia-induced lung injury is associated with downregulated basal CCN1 expression, and treatment with CCN1 can largely reverse hyperoxic injury.

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, increase in 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, and nov) proteins belong to an important family of matricellular regulatory factors involved in internal and external cell signaling and have 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 the 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 exposure to hyperoxia induced CCN2 overexpression in lungs of neonatal rat (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, antifibrotic, 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 antifibrotic 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 has an important downregulatory role in the early inflammatory phase of wound-healing by stimulating the clearance of neutrophils via the process of efferocytosis (16). In addition,

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CCN1 promotes angiogenesis by increasing vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) expression and by enhancing endothelial cell adhesion, migration, and survival (18,22). However, the role of CCN1 in BPD pathogenesis is unknown.

We hypothesized that CCN1 should have 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 interleukin (IL)-1β are key components of the inflammasome cascade. Our results demonstrate that hyperoxia downregulated 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, and 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.

METHODS

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% O_2 for 7 or 14 days, and animals were killed 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 three groups: RA+placebo (PL, normal saline), O_2+PL, RA+CCN1, and O_2+CCN1. Recombinant CCN1 (1 mg/kg) or PL (equal volume) was administered by intraperitoneal injection on days 1, 4, 7, and 9 during continuous RA or exposure to hyperoxia. 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 killed 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-1β was determined by western blot analysis.

Lung Histology and Morphometry

Lungs were infused with 4% paraformaldehyde via a tracheal catheter at 20 cm H_2O pressure for 5 min, 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 μm in diameter) was counted from five 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 and vWF to assess the extent of muscularization. The percentage of peripheral vessels (<50 μm in diameter) that were stained with α-smooth muscle actin (>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 an α-smooth muscle actin antibody was performed. The percentage of vessels with at least one positive Ki67 nuclei was determined.

Assessment of Right Ventricular Hypertrophy

Right ventricular hypertrophy (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 (13,14) was determined.

Data Management and Statistical Analysis

Data were expressed as means ± 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.

Detailed descriptions of the Materials and methods are provided in **Supplementary methods** online.

RESULTS

Hyperoxia Downregulates CCN1 Expression in Neonatal Lungs

We evaluated the expression of CCN1 in lungs using western blot analysis on days 7 and 14 after continuous exposure to hyperoxia. As demonstrated in **Figure 1**, quantitative densitometry analysis demonstrated that hyperoxia exposure resulted in significant suppression of CCN1 expression on both day 7 (1.73 ± 0.33 vs. 0.4 ± 0.45, $P<0.001$, RA vs. O_2) and day 14 (2.25 ± 0.64 vs. 0.79 ± 0.28, $P<0.01$, RA vs. O_2; **Figure 1a,b**). These data suggest that CCN1 may have 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 with the RA+PL group (8.0 ± 4.63 vs. 1.6 ± 0.47, $P<0.001$, O_2+PL vs. RA+PL; **Figure 2a,b**). Similarly, neutrophil counts were also significantly elevated with hyperoxia exposure compared with RA exposure

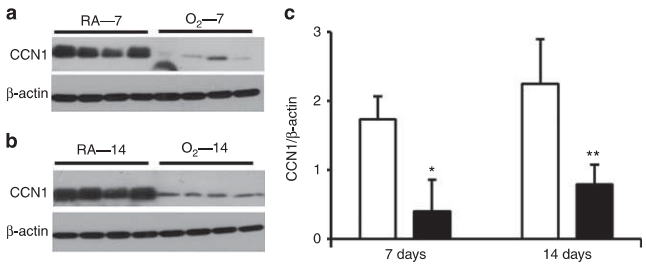


Figure 1. Hyperoxia downregulates CCN1 expression. Newborn rats were exposed to room air (RA, open bar) or to hyperoxia (85% O_2, solid bar) for 7 (**a**) or 14 (**b**), days and CCN1 expression in lung extracts was quantitated by western blot densitometry analysis after normalization to housekeeping gene β-actin. Representative western blot photo images are shown. (**c**) Hyperoxia exposure downregulated CCN1 expression at both 7 days (*$P<0.001$) and 14 days (**$P<0.01$) as compared with RA. Open bar: RA. Solid bar: hyperoxia.

(7.4 ± 4.11 vs. 1.0 ± 0.35, $P<0.001$, O_2+PL vs. RA+PL; Figure 2c,d). 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+CCN1 vs. O_2+PL; Figure 2a, b; neutrophil: 2.6 ± 1.33 vs. 7.4 ± 4.11, $P<0.001$, O_2+CCN1 vs. O_2+PL; Figure 2c,d).

We further evaluated the effects of recombinant CCN1 on lung inflammation by measuring expression of inflammasome component proteins and active IL-1β production. The lungs of the animals belonging to the hyperoxia+PL group had significantly increased expression of NLRP1, ASC, and active caspase-1 compared with those belonging to the RA group (Figure 2e–i). Hyperoxia-induced inflammasome protein expression appeared to be accompanied by inflammasome activation as we observed a significant elevation of active IL-1β in hyperoxia-exposed lungs compared with lungs belonging to animals in the RA group (1.27 ± 0.37 vs. 0.46 ± 0.21, RA+PL vs. O_2+PL, $P<0.001$, Figure 2e,i). Treatment with recombinant CCN1 resulted in significant reductions in all three elevated inflammasome proteins during hyperoxia (Figure 2e–h). In addition, similarly CCN1 treatment resulted in a significant decrease in active IL-1β expression in hyperoxia-exposed lungs (0.52 ± 0.11 vs. 1.27 ± 0.37, $P<0.001$, O_2+CCN1 vs. O_2+PL, Figure 2e,i). These results suggest a crucial role of CCN1 in protecting neonatal lungs against the hyperoxia-induced inflammatory response by downregulation of the inflammasome–IL-1β cascade.

Treatment with CCN1 Improves Hyperoxia-Suppressed Alveolar Development

We next evaluated the effects of CCN1 on alveolar development by measuring RAC. Compared with RA-exposed rats, the lungs from hyperoxia and PL-exposed rats had significantly reduced RAC, suggesting poor alveolar development (6.06 ± 0.4 vs. 8.93 ± 1.03, $P<0.001$, O_2+PL vs. RA+PL, Figure 3a,b). 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+PL vs. O_2+CCN1, Figure 3a,b). 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 μm in diameter) in lung tissue sections. As seen in Figure 4, hyperoxia exposure resulted in a significant reduction in vascular density compared with RA (5.30 ± 1.03 vs. 11.10 ± 2.87, $P<0.001$, O_2+PL vs. RA+PL, Figure 4a,b). 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+CCN1 vs. O_2+PL, Figure 4a,b). These results suggest that CCN1 improves vascular development in hyperoxia-exposed neonatal rat lungs.

Administration of CCN1 Reduces Hyperoxia-Induced Pulmonary Vascular Muscularization

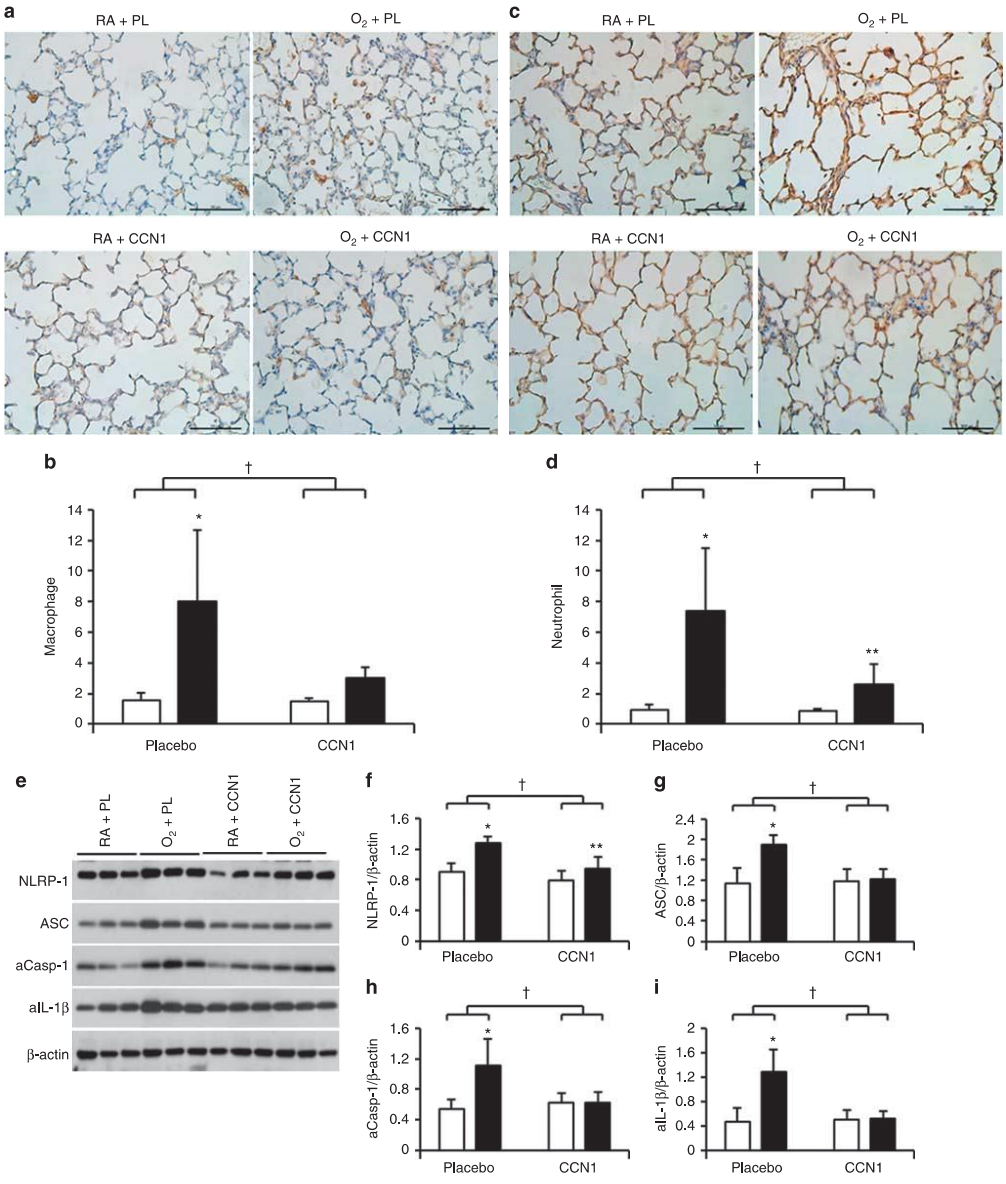
To assess whether CCN1 affects pulmonary vascular remodeling during hyperoxia, we measured the extent of muscularization of peripheral pulmonary vessels that are less than 50 μm in diameter and with more than 50% muscularization using double immunofluorescent vWF and α-smooth muscle actin staining of lung sections. The percentage of muscularized vessels was significantly increased in the hyperoxia group compared with that in the RA group (56% vs. 18%, $P<0.001$, O_2+PL vs. RA+PL, Figure 5a,b). Moreover, the percentage of muscularized vessel was significantly decreased by treatment with CCN1 during hyperoxia (56% vs. 33%, $P<0.01$, O_2+PL vs. O_2+CCN1, Figure 5a,b). 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%, $P<0.001$, O_2+PL vs.

RA+PL, Figures 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%, $P<0.001$, O_2+PL vs. O_2+CCN1, Figure 5c,d).

Treatment with CCN1 Decreases Hyperoxia-Induced RVH

To evaluate the degree of PH, we measured RVH (Fulton index). Hyperoxia exposure resulted in a significant increase in RVH compared with RA exposure (0.41 ± 0.04 vs. 0.31 ± 0.02, $P<0.001$, O_2+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, $P < 0.001$, O_2+CCN1 vs. O_2+PL, **Figure 6**). These results suggest that CCN1 can prevent hyperoxia-induced RVH in neonatal rats.

DISCUSSION

In this study, we report that hyperoxia downregulates CCN1 in newborn rat lungs. Moreover, we found evidence for a protective role of CCN1 in hyperoxia-induced neonatal lung injury by demonstrating that treatment with recombinant CCN1 decreases lung inflammation, improves alveolarization and vascular development, and decreases 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, if future studies show that CCN1 is also downregulated 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 are expressed during normal neonatal rat lung development and that hyperoxia downregulates 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 is upregulated by hyperoxia (14). These results suggest that CCN1 and CCN2 may have 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.* (25) 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. Further, Jin *et al.* (26) demonstrated that suppressing CCN1 expres-

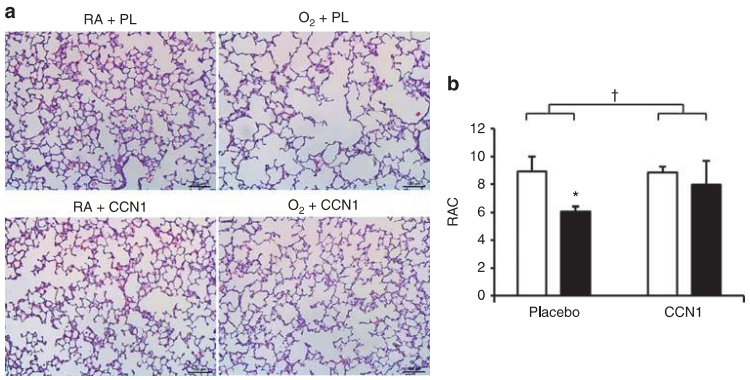


Figure 3. Treatment with CCN1 improves hyperoxia-suppressed alveolarization. (**a**) Histological examination of O_2+PL lung sections revealed larger and simplified alveoli in comparison with RA+PL lungs, which showed more numerous and smaller alveoli. CCN1 treatment reversed the effects of hypoxia as O_2+CNN1 lungs showed more alveolarization. Bar = 50 µm. (**b**) Morphometric analysis demonstrated that exposure to hyperoxia decreased radial alveolar count (RAC) in PL-treated rats, which was significantly reversed by administration of CCN1 (*$P < 0.001$: RA+PL vs. O_2+PL; †$P < 0.01$: O_2+PL vs. O_2+CCN1). $n = 6$/group. Open bar, room air; solid bar, hyperoxia. PL, placebo; RA, 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 HPV (×200) on each lung section (**b**). The O_2+PL lungs showed increased macrophage counts compared with RA+PL lungs, which were decreased by administration of CCN1 (*$P < 0.001$: RA+PL vs. O_2+PL; †$P < 0.01$: O_2+PL vs. O_2+CCN1). $n = 6$/group. Bar = 100 µm. Immunostaining with an anti-neutrophil elastase antibody was performed on lung tissue sections (**c**) and the average numbers of neutrophils in alveolar airspaces were counted from 10 random images, taken under the HPV on each lung section (**d**). Exposure to hyperoxia in the presence of the PL increased neutrophil infiltration into the alveolar airspaces, whereas treatment with CCN1 significantly decreased neutrophil infiltration during hyperoxia (*$P < 0.001$: RA+PL vs. O_2+PL; †$P < 0.001$: O_2+PL vs. O_2+CCN1; **$P < 0.05$: RA+CCN1 vs. O_2+CCN1). $n = 6$/group. Bar = 100 µm. (**e**) Representative western blot images for NLRP1, ASC, active caspase-1 (aCasp-1), active IL-1β (aIL-1β), and β-actin. The relative expression levels of NLRP1 (**f**), ASC (**g**), active caspase 1 (**h**), and active IL-1β (**i**) were analyzed using densitometry and were normalized to β-actin. All three inflammasome proteins and active IL-1β were increased by hyperoxia in the PL group as compared with the RA group (*$P < 0.001$ (NLRP-1); *$P < 0.001$ (ASC); *$P < 0.001$ (Caspase-1); *$P < 0.001$ (IL-1β)). However, treatment with CCN1 during hyperoxia decreased the expression of all three inflammasome proteins and active IL-1β as compared with the hyperoxia plus PL group (†$P < 0.001$ (NLRP-1); †$P < 0.001$ (ASC); †$P < 0.001$ (Caspase-1); †$P < 0.001$ (IL-β)). **$P < 0.05$: O_2+CCN1 vs. RA+CCN1. $n = 6$/group. Open bar, RA; solid bar, hyperoxia. HPV, high-power view; IL, interleukin; PL, placebo; RA, room air.

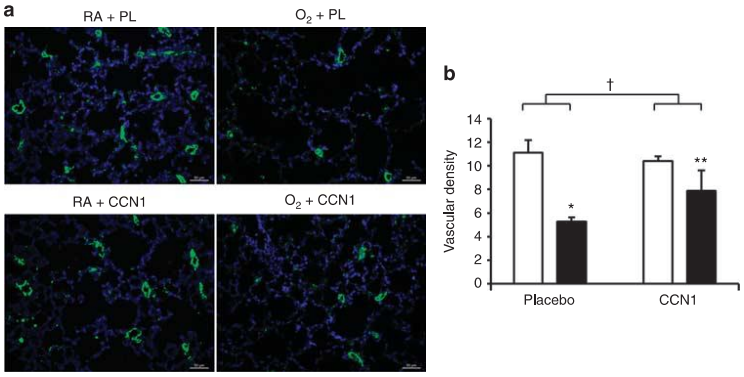


Figure 4. CCN1 administration improves hyperoxia-suppressed vascular development. (**a**) Immunofluorescence staining with an anti-vWF antibody (green signal) and 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining (blue signal) were performed on lung tissue sections. Bar = 50 µm. (**b**) Vascular density was determined by counting vWF-positive vessels (<50 µm) on five random images from each lung section. The vascular density was significantly decreased in O_2+PL lungs compared with normoxic lungs (*P<0.001: RA+PL vs. O_2+PL). Treatment with CCN1 significantly increased vascular density in hyperoxia-exposed animals (†P<0.05: O_2+PL vs. O_2+CCN1). **P<0.05: RA+CCN1 vs. O_2+CCN1. n = 6/group. Open bar, RA; solid bar, hyperoxia. PL, placebo; RA, room air; vWF, von Willebrand factor.

sion by small interfering RNA-accelerated lung-epithelial cell death after hyperoxia, and conversely that overexpressing CCN1, conferred increased resistance to hyperoxia-induced cell death. Although 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.* (27) conflicts with our finding that hyperoxia decreases lung CCN1 expression as they report hyperoxia-increased lung CCN1 mRNA and protein expression. However, it is of note that the models used were significantly different from our model. They used adult mice and exposed them to >95% O_2 for short period of time (24–48 h) to induce acute lung injury. On the contrary, we used a neonatal rat model, using 85% O_2 hyperoxia exposure for a longer period of 7–14 days to simulate chronic lung disease. Similarly, other studies suggesting that 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 physiological function of CCN1 in cell survival/death are dependent on cell and organ types, types of cellular stimuli, and the duration of inflammation.

Our results indicate that CCN1 therapy significantly reduced the neutrophil and macrophage counts in hyperoxia-exposed rats' lungs. Moreover, this may be partially related to the ability of CCN1 to increase efferocytosis of neutrophils, as has been described for wound tissue (16). However, 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-1β 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 lungs of hyperoxia-exposed neonatal mouse have increased caspase-1 and IL-1β activation (24). Our recent studies have demonstrated that hyperoxia activates NLRP1 inflammasome and inhibition of Rac1 signaling downregulates 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 upregulated expression of NLRP1, ASC, and active caspase-1, and production of active IL-1β, and that recombinant CCN1 treatment resulted in a significant downregulation of all four hyperoxia-elevated proteins, suggesting that 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 markedly 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 prevents 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 has 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

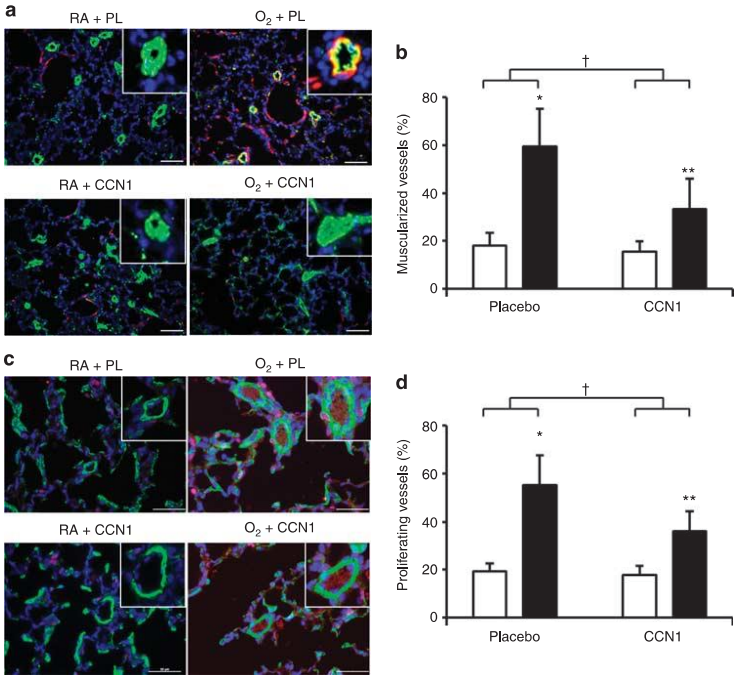


Figure 5. Treatment with CCN1 decreases hyperoxia-induced pulmonary vascular remodeling. (**a**) Double-immunofluorescence staining for vWF (green signal) and α-SMA (red signal) and DAPI nuclear staining (blue signal). Bar = 50 μm. (**b**) The percentage of <50-μm-diameter muscularized peripheral pulmonary vessels (≥50% of circumference α-SMA-positive) was significantly increased in lungs from the O_2+PL group. Administration of CCN1 significantly decreased vascular muscularization in hyperoxia-exposed animals (*$P < 0.001$: RA+PL vs. O_2+PL; **$P < 0.01$: RA+CCN1 vs. O_2+CCN1; †$P < 0.01$: O_2+PL vs. O_2+CCN1). $n = 6$/group. (**c**) Double immunofluorescence staining with Ki67 (red signal) and α-SMA (green signal) and DAPI nuclear staining (blue signal) were performed to assess vascular smooth muscle cell proliferation. Pink signals indicate Ki67-positive nuclei. Bar = 50 μm. (**d**) The percentage of vessels (<50 μm in diameter) with at least one Ki67-positive nuclei on each vessel was determined. O_2+PL lungs had increased proliferating vessels compared with RA lungs. Treatment with CCN1 decreased vascular proliferation (*$P < 0.001$: RA+PL vs. O_2+PL; **$P < 0.001$: RA+CCN1 vs. O_2+CCN1; †$P < 0.001$: O_2+PL vs. O2+CCN1). $n = 6$/group. Open bar, RA; solid bar, hyperoxia. α-SMA, α-smooth muscle actin; PL, placebo; RA, room air; vWF, von Willebrand factor.

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 have 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 have a critical role in vascular development in hyperoxia-induced neonatal lung injury.

We have shown 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 with the group exposed to hyperoxia+PL. Although 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 that 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

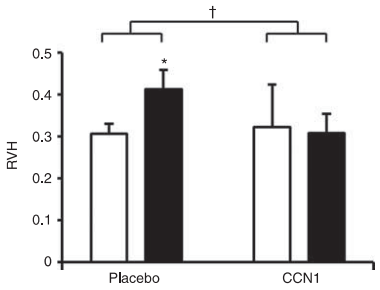


Figure 6. Effects of CCN1 on hyperoxia-induced RVH. Exposure to hyperoxia in the presence of the PL resulted in an increase in Fulton index (RV/LV+S), indicating RVH and PH. Administration of CCN1 significantly decreased RVH during hyperoxia (*$P < 0.001$: RA+PL vs. O_2+PL; †$P < 0.001$: O_2+PL vs. O_2+CCN1). $n = 6$/group. Open bar, RA; solid bar, hyperoxia. LV, left ventricle; PH, pulmonary hypertension; PL, placebo; RA, room air; RHV, right ventricular hypertrophy; S, septum.

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.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

STATEMENT OF FINANCIAL SUPPORT

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